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Towards more efficient biowaste utilization with black soldier fly larvae to produce more sustainable animal feed

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MORITZ GOLD

Dipl.-Ing. Environmental Engineering, University of Stuttgart

born 28.09.1986

citizen of Germany

accepted on the recommendation of

Prof. Dr.-Ing. Alexander Mathys, examiner

Prof. Dr. Christian Zurbrügg, co-examiner

Dr. Guido Bosch, co-examiner

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The future will be green, or not at all. – *Jonathon Porritt*

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Summary

Today's food production has enormous negative impacts on the environment and does not provide nutritious food in sufficient quantities for all people. Protein, largely provided through animal source foods, is a key nutrient associated with these shortcomings. Environmental impacts from animal source foods stem among others from the use of unsustainable feed ingredients (e.g. soybean and fish meal). At the same time, poor people often do not have access to sufficient quantities of affordable quality feed. This affects their livestock production, which is crucial for income, livelihood, nutrition, food security and resilience. In light of these shortcomings and the predicted increase in global food demand, the production of livestock feeds and animal source foods must be dramatically improved.

Larvae of the black soldier fly (BSFL), *Hermetia illucens* L. (Diptera: Stratiomyidae), promise benefits for the affordability, availability, quality, and sustainability of monogastric livestock feeds (i.e. poultry, fish, pigs, pets). BSFL are the natural food of some livestock and can replace and possibly outperform current feed ingredients. Producing BSFL that are more sustainable than current feed ingredients requires use of biowastes for growth. Thereby, BSFL rearing also has the potential to improve often inadequate waste management. However, unreliable and low rearing performance prohibits the use of many biowastes for efficient BSFL rearing. This thesis focuses on the development of solutions to improve BSFL rearing on biowastes by providing nutrients in ideal amounts and addition of microbial inoculants to the rearing substrate.

Formulation of biowaste mixtures based on protein and non-fibre carbohydrates is a promising approach for more efficient and predictable BSFL rearing using a range of biowastes. In comparison to individual wastes, biowaste formulations resulted in a higher rearing performance and lower variability. Biowaste formulation is especially beneficial since it integrates wastes with an individually low performance (e.g animal manures or slaughterhouse waste) into efficient BSFL substrates. In comparison to previous trial-and-error testing of biowaste mixtures, the proposed approach is systematic and considers the existing and growing knowledge on BSFL digestion. The open-access web application ensures formulation of biowaste mixtures when reliable data on the composition of biowaste is not available.

The rearing variability that remains despite formulation could be reduced by keeping other nutrients within narrower limits and by providing more accurate estimates of biowaste digestibility and larval nutrient and energy requirements. Development of the first *in vitro* simulation of BSFL digestion delivers such information. In addition, digestion of substrates in the *in vitro* model can indicate *in vivo* outcomes. Thereby *in vitro* simulation can be used for screening of different biowastes based on rearing performance. In order to improve the model accuracy, it is essential to promote an understanding of the complex digestion processes. This thesis showed that BSFL adjust the digestive residence time based on the substrate nutrient content, suggesting post-ingestive mechanisms and compensatory feeding by BSFL.

The addition of microbial inoculants did not result in performance improvements. Inoculants were obtained from the residues of a previous rearing cycle, which were typically rich in bacteria considered beneficial for substrate decomposition (i.e. *Providencia*, *Dysgonomonas*, *Morganella*, and *Proteus*). The absence of these bacteria from two agri-food waste residues highlighted that the complex microbial

processes involved in BSFL rearing are still poorly understood and further research is needed to reliably improve rearing performance. This is despite the growing knowledge for BSFL and five other fly species, reviewed in this thesis.

This thesis sets another cornerstone for efficient BSFL rearing on biowaste that vary in nutrients and microbiota. Major global improvements in the sustainability of animal source foods through the use BSFL-based feeds require an increase in the supply of BSFL substrates through improvements in waste management. In parallel, it is essential to develop solutions managing food safety risks associated with microbial and chemical contaminants in biowaste.

Zusammenfassung

Die heutige Nahrungsmittelproduktion hat enorme negative Auswirkungen auf die Umwelt und liefert nicht für alle Menschen Lebensmittel in ausreichender Menge und Qualität. Eiweiß, das größtenteils aus Lebensmitteln tierischer Herkunft stammt, wird oft mit diesen Problemen assoziiert. Ein grosser Teil der Umweltauswirkungen von Nahrungsmitteln tierischer Herkunft stammt aus der Verwendung nicht nachhaltigen Tierfutters (z. B. Sojabohnen und Fischmehl). Gleichzeitig haben Menschen mit geringem Einkommen oft keinen Zugang zu ausreichenden Mengen an hochwertigem Tierfutter. Dies wirkt sich auf ihre Tierzucht aus, die für Lebensunterhalt, Ernährung, Ernährungssicherheit und Resilienz von entscheidender Bedeutung ist. Angesichts dieser Mängel und des prognostizierten Anstiegs der weltweiten Nahrungsmittelnachfrage muss die Produktion von Tierfutter und Nahrungsmitteln tierischer Herkunft dramatisch verbessert werden.

Die Larven der Schwarzen Waffenfliege (engl.: black soldier fly larvae, BSFL), *Hermetia illucens* L. (Diptera: Stratiomyidae), könnten die Verfügbarkeit von kostengünstigem, hochwertigem und nachhaltigem Tierfutter ermöglichen. BSFL sind die natürliche Nahrung einiger Nutztiere und können die derzeitigen Futtermittelzutaten ersetzen und möglicherweise in ihrer Qualität sogar übertreffen. BSFL, die nachhaltiger als die derzeitigen Futtermittelzutaten sind, müssen allerdings auf Bioabfällen gezüchtet werden, die nicht bereits als Lebensmittel oder Tierfutter Verwendung finden. Dadurch kann die Aufzucht von BSFL auch zur Entsorgung und Wiederwendung von Bioabfall beitragen. Langsames und unbeständiges Wachstum auf Bioabfällen verhindert allerdings derzeit oft den effizienten Einsatz. Deshalb entwickelt diese Arbeit Lösungen für die Verbesserung des Larvenwachstums durch die optimale Bereitstellung von Nährstoffen und der Zugabe von mikrobiellen Inokula zum Aufzuchtsubstrat.

Die Zubereitung von Bioabfallmischungen basierend auf dem Protein- und Kohlenhydratgehalt ist ein vielversprechender Ansatz, um das Wachstum bei der Verwendung von verschiedenen Abfällen zuverlässig zu verbessern. Die Abfallrezeptur führte zu einem besseren und gleichmässigeren Wachstum im Vergleich zu den einzelnen Abfällen. Die Herstellung einer Mischung verschiedener Bioabfälle ist besonders vorteilhaft, weil Abfälle mit üblicherweise schlechter Qualität (z. B. Tierdung oder Schlachtabfälle) in effiziente Aufzuchtsubstrate integriert werden können. Im Vergleich zu früheren Arbeiten, welche Bioabfälle wahllos prozentual zusammenmischten, ist der in dieser Arbeit erarbeitete Ansatz systematisch und berücksichtig das derzeitige Wissen über die Verdauungsprozesse. Die entwickelte Webanwendung ermöglicht das Erstellen von Abfallmischungen, wenn keine zuverlässigen Nährstoffanalysen aus erster Hand zur Verfügung stehen.

Schwankungen im Larvenwachstum die trotz ähnlichem Protein- und Kohlenhydratgehalt bestehen, könnten verringert werden, indem der Gehalt der anderen Nährstoffe besser ausgeglichen wird oder Information über die Verdaulichkeit von Bioabfällen vorliegen. Die entwickelte *in vitro* Methode zur Simulation der Verdauung von BSFL liefert solche Informationen. Darüber hinaus kann die Verdauung von Substraten im *in vitro* Modell auf *in vivo* Ergebnisse hinweisen. Um die Genauigkeit des Modells zu verbessern, ist es wichtig, das Verständnis der komplexen Verdauungsprozesse weiter zu verbessern. Diese Arbeit zeigte beispielsweise, dass BSFL die Nahrungsaufnahme und die Verdauungsverweilzeit in Abhängigkeit vom Nährstoffgehalt des Substrats anpassen.

Die Zugabe von mikrobiellen Inokula führte nicht zu Verbesserungen im Larvenwachstum. Die Inokula wurden aus dem Rückstand eines früheren Aufzuchtzyklus gewonnen, die typischerweise reich an Bakterien sind, die für BSFL als vorteilhaft angesehen werden (z.B. *Providencia*, *Dysgonomonas*, *Morganella* und *Proteus*). Dass Nichtvorhandensein dieser Bakterien in den Rückständen von zwei Agrar- und Lebensmittelabfällen macht deutlich, dass die komplexen mikrobiellen Prozesse, die bei der Aufzucht der BSFL beteiligt sind, noch zu wenig verstand sind. Weitere Forschung ist erforderlich, um das Wachstum durch die Zugabe von Inokula zuverlässig zu verbessern.

Diese Arbeit legt einen weiteren Grundstein für die effiziente Verwertung von heterogenen Bioabfällen mit BSFL. Weitreichende Verbesserungen in der Nachhaltigkeit von Lebensmitteln tierischer Herkunft durch die Nutzung von Tierfutter basierend auf BSFL bedürfen der Verbesserung des Abfallmanagements. Darüber hinaus ist es unerlässlich Lösungen zu entwickeln, welche die Lebensmittelsicherheit trotz krankheitserregender Keime und chemischer Inhaltstoffe in Bioabfallen gewährleisten.

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Introduction

Today, most human health metrics such as undernourishment, life expectancy, infant and child mortality rates, and global poverty levels are superior to any other time in history (Whitmee et al., 2015). However, 690 million people still lack sufficient amounts of food (FAO et al. 2019), over 2 billion are micronutrient deficient (WHO, 2006) and meeting today's food demands has tremendous negative effects on the environment. In fact, global food production is the largest cause of climate change, ocean acidification, land degradation, water scarcity, overexploitation of fish, and biodiversity loss (Whitmee et al., 2015).

Protein is a key nutrient associated with inadequate supply and negative environmental impacts (Aiking, 2011). A large proportion of protein is provided through animal source foods (e.g., meat, fish) commonly having a higher environmental impact than plant foods (e.g., vegetables, pulses). These environmental impacts stem among others from the use of unsustainable feed ingredients (e.g., grains, fish meal, soybean meal). For example, around 750 million tonnes of cereals (Mottet et al., 2017), 200 million tonnes of soybean meal (Mottet et al., 2017) and 5 million tonnes of fish meal (FAO, 2020) are estimated to be used annually for feeding of livestock. These feeds partially compete with the production of human food and contribute to biodiversity loss and overfishing (Willett et al., 2019). Poor people frequently do not have access to global feed value chains. A lack of access to sufficient amounts of affordable quality feed affects sutaining livestock production crucial for income, livelihood, nutrition, food security and resilience (Kenis et al., 2014; Pechal et al., 2019). Overall, feed prices have been increasing over the last 20 years, especially for fish meal, from around 0.4 USD in 1990 to around 1.5 USD today (per kg).

Beyond these challenges, global demand of animal source foods and associated shortcomings of current practices such as unstainable feed ingredients are predicted to increase, especially in low- and middle-income countries in Sub-Saharan Africa and Asia, due to population growth (FAO, 2009; UN, 2019) and dietary shifts due to economic development (Sans and Combris, 2015). These trends will not solely be compensated by strategies to reduce the consumption and environmental impact of animal source foods such as replacement of ruminant with monogastric products or shifting to vegan diets (Willett et al., 2019). Animal source foods have a high nutritious value, and limited amounts (e.g., dairy, poultry, fish) have been recommended for benefits on consumer health (USDA, 2015). In addition, many people will not shift to diets low or absent of animal source foods, due to taste, texture, culture, ethics and wealth (Macdiarmid et al., 2016). In order to meet current and future demands while preventing further environmental degradation, the production of animal source foods must be changed dramatically.

Larvae of the black soldier fly (BSFL, Figure 1), *Hermetia illucens* L. (Diptera: Stratiomyidae), hold promise to be beneficial for the affordability, availability, quality, and sustainability of monogastric livestock feeds (i.e. poultry, fish, pigs, pets) (FAO, 2013). BSFL are the natural diet of livestock (e.g., poultry) and can substitute current feed ingredients (e.g. soybean and fish meal) while maintaining livestock production efficiency and meat quality (Altmann et al., 2020). BSFL are not only a source of bulk protein but can also be superior to conventional feed ingredients considering animal health and behaviour (Gasco et al., 2018). In Kenya, Abro et al. (2020) concluded that benefits of the introduction

of insect-based feeds also include increased employment, availability of fish and maize for human consumption, and reduced foreign currency spending.

Producing BSFL that are more sustainable than current feed ingredients requires use of substrates for growth that are not used as food (e.g., sorghum) and feed (e.g., mill byproducts, brewery side streams) (Bosch et al., 2019). Examples include municipal organic solid waste, animal and human manures and agri-food wastes (e.g., vegetable and fruit pomace/peels). BSFL grow on these substrates and convert substrate nutrients into their own body mass. By using BSFL as feed ingredient, resources in these substrates (e.g., water, nutrients, energy) are recycled within the food system according to circular economy principles. Consequently, BSFL grown on these substrates can have lower environmental impacts than current feed ingredients, especially associated with reduced greenhouse gas emissions and land use (Smetana et al., 2016; Bosch et al., 2019).

In addition to the potential to produce cost-effective and sustainable feeds, using biowastes and organic side streams for larval growth can contribute to waste management. For example, globally, around 1.3 billion tons of food are lost or wasted (Gustavsson et al., 2011). Especially in low- and middle-income countries, poor waste management such as indiscriminate dumping, unmanaged landfilling or open burning jeopardizes public and environmental health, contributes to climate change and is associated with a significant global economic and health loss (Hoornweg and Bhada-Tata, 2012). These challenges are expected to especially increase in low- and lower-middle income countries with the predicted population growth and development. Since feeds typically have a higher value than other biowaste treatment products such as compost and energy (e.g., biogas, electricity), revenues from BSFL can increase revenue to offset waste treatment costs. This financial incentive has contributed to the emergence of municipalities (e.g. Surabaya, Indonesia) and companies (e.g. Sanergy, Kenya) using BSFL for waste treatment.

Today, BSFL-based feed production it in its infancy considering feed demands. Globally, a few industrial-scale facilities exist producing around 2,600 tons of dry larval biomass per year (assumptions: 200 tonnes waste per day, 260 working days, 20% bioconversion rate, 75% waste moisture content) (Gold et al., 2020a). This means that in the order of 1,250 facilities would be needed to replace the current annual fish meal production that does not orginate from side streams from fish processing (3.25 million tonnes per year) (FAO, 2020). Working towards meeting these enormous demands requires development of solutions increasing the performance and reducing the variability of rearing BSFL on different biowastes. Today, typically unreliable and low performance prohibits the use of many biowastes for effcient BSFL rearing. Larval growth, bioconversion and waste reduction, as well as product composition (e.g., protein and lipid content) varies both when using the same type of substrate (e.g. different vegetable wastes) and when treating different waste types (e.g. vegetable compared to municipal solid waste) (Gold et al., 2018a). This impacts day-to-day operation and performance metrics crucial for the sustainability of facilities. More frequently, homogenous high-value substrates such as mill byproducts and brewery side streams are used today as substrates due to their consistent high rearing performance. However, these side streams may already be part of existing value chains, or used as feed ingredient, and diverting them to livestock production may trigger increased demand in conventional feed ingredients (e.g., soybean meal), jeopardizing environmental benefits of BSFL production (Smetana et al., 2019). Use of biowastes over side streams also means that more microbial

(e.g. bacterial and viral pathogens) and chemical contaminants need to be considered and managed to ensure food and feet safety (Van der Fels-Klerx et al., 2018).

As in livestock production, one approach to increase BSFL rearing performance is to provide nutrients in ideal amounts. It is established that organic matter, protein and non-fibre carbohydrates tend to increase, and lignin tends to decrease larval growth (Barragán-Fonseca, 2018a; Liu et al., 2018; Beniers and Graham, 2019). However, this knowledge is still frequently ignored and larval diet formulation follows a trial-and-error approach. Barragán-Fonseca et al. (2018b) was the first to provide substrates with similar amounts of protein and non-fibre carbohydrates, but this has still resulted in significantly different larval weights among substrates. This could be due to different nutrient qualities among substrates (e.g. type of non-fibre carbohydrates and fibres), inaccurate measures of nutrient composition (e.g. non-fibre carbohydrate estimation by difference) and larval nutrient requirements, and poor consideration of the larval digestion processes. BSFL nutrient requirements were previously always determined by monitoring growth and waste reduction in feeding experiments (Lalander et al., 2019). For other livestock, mass balance calculations between nutrients in feed and animal faeces, or *in vitro* digestions, to determine digestibility measurements, have resulted in efficiency gains (Cheli et al., 2012), but these methods have been so far absent for BSFL.

Inoculation of the substrate with pure-culture bacteria or bacteria mixtures isolated from the fly, soil or manure (e.g. *Bacillus natto*, *Bacillus subtilis*, *Lactobacillus buchneri*, and *Kocuria marina*) is also a promising approach to increase larval growth (Yu et al., 2011; Rehman et al., 2019; Somroo et al., 2019). However, this has been so far accomplished with few substrates, and performance improvements were sometimes absent or minimal (Callegari et al., 2020; Mazza et al., 2020).

For these innovations to translate into benefits for final food production and waste management, performance gains from controlled laboratory experiments have to be tested in realistic field conditions. So far, nutrient requirements were typically determined using high-value substrates (e.g., cellulose, protein powder, sunflower oil, pet food) (Barragán-Fonseca et al., 2018b; Beniers and Graham, 2019; Lalander et al., 2019) and it is yet unclear how relevant these findings are in practice. The same applies for microbial inoculants, since cultivation of pure-culture bacteria alongside BSFL may not always be practical for small-scale facilities or due to the required laboratory capacities.

This thesis focuses on the development of solutions to improve BSFL rearing performance on biowastes. Chapter 1 provides the background for this thesis by summarizing important processes involved in the conversion of biowastes into insect biomass by BSFL, based on existing research for five well-studied fly species phylogenetically similar to the black soldier fly. Chapter 2 assesses whether biowaste mixtures formulated based on substrate protein and digestible carbohydrate content, increases growth performance and reduces process variability in comparison to the individual wastes. Chapter 3 aims to provide pratical guidance for the selection and formulation of efficient BSFL substates. Chapter 4 and Chapter 5 deal with the increase of BSFL production efficiency by larval associated microbes. These chapters map bacterial dynamics in the residue and assesses whether the addition of residue-derived inoculates increases larval growth. To increase accuracy of future nutritional and microbial studies, Chapter 6 and Chapter 7 describe new research methods for BSFL, such as the first *in vitro* model of the BSFL midgut.

1. Chapter – Decomposition of biowaste macronutrients, microbes, and chemicals in black soldier fly larval treatment: A review

Moritz Gold[1,2], Jeffery K. Tomberlin[3], Stefan Diener[4], Christian Zurbrügg[2], Alexander Mathys[1]

[1]Swiss Federal Institute of Technology (ETH) Zurich, Institute of Food, Nutrition and Health, Sustainable Food Processing Laboratory, Schmelzbergstrasse 9, 8092 Zurich, Switzerland
[2]Eawag: Swiss Federal Institute of Aquatic Science and Technology, Sandec: Department Sanitation, Water and Solid Water for Development, Überlandstrasse 133, 8600 Dübendorf, Switzerland
[3]Texas A&M University, Department of Entomology, 370 Olsen Boulevard, College Station, TX 77843, USA
[4]Biovision Foundation, Heinrichstrasse 147, 8005 Zurich, Switzerland

1.2 Abstract

Processing of biowaste with larvae of the black soldier fly, *Hermetia illucens* L. (Diptera: Stratiomyidae), is an emerging waste treatment technology. Larvae grown on biowaste can be a relevant raw material for animal feed production and can therefore provide revenues for financially viable waste management systems. In addition, when produced on biowaste, insect-based feeds can be more sustainable than conventional feeds. Among others, the scalability of the technology will depend on the availability of large amounts of biowaste with a high process performance (e.g., bioconversion of organic matter to protein and fat) and microbial and chemical product safety. Currently, in contrast to other waste treatment technologies, such as composting or anaerobic digestion, the process performance is variable and the processes driving the decomposition of biowaste macromolecules, inactivation of microbes and fate of chemicals is poorly understood. This review presents the first summary of the most important processes involved in black soldier fly larvae (BSFL) treatment, based on the available knowledge concerning five well-studied fly species. This is a starting point to increase understanding regarding the processes of this technology, with the potential to increase its efficiency and uptake, and support the development of appropriate regulations. Based on this review, formulating different types of biowaste, e.g. to produce a diet with a similar protein content, a balanced amino acid profile and/or pre- and co-treatment of biowaste with beneficial microbes, has the potential to increase process performance. Following harvest, larvae require heat or other treatments for microbial inactivation and safety.

1.3 Black Soldier Fly (BSF) Biowaste Processing

Especially in low- and middle-income countries, indiscriminate disposal, poor-quality treatment, and uncontrolled landfilling of biowaste contributes to negative environmental and public health outcomes (Hoornweg and Bhada-Tata, 2012; Komakech et al., 2014). Poor biowaste management practices also waste energy and nutrients, that could be used to meet the increasing global resource demands (Alexander et al., 2017a; Diener et al., 2014). Black soldier fly (BSF) biowaste processing is a relatively new treatment technology that has received increased attention over the last decades (Čičková et al., 2015; De Smet et al., 2018; Zurbrügg et al., 2018; Makkar et al., 2014). The BSF, *Hermetia illucens* L. (Diptera: Stratiomyidae), can today be found in many countries with year-round warm, tropical or sub-tropical climates (Dortmans et al., 2017; Rozkosny, 1983; Üstüner et al., 2003). The natural diet of BSF larvae (BSFL) include animal manures, human excreta, fruit and vegetable wastes, and carrion (Rozkosny, 1983; Schremmer, 1986). BSFL consume this biowaste, convert it into larval biomass, and leave behind a compost-like residue, with characteristics similar to immature compost (Zurbrügg et al., 2018; Xiao et al., 2018). A typical BSF biowaste processing facility consists of waste pre- processing (e.g. particle size reduction, dewatering, removal of inorganics), biowaste treatment by BSFL, separation of BSFL from process residue, and lastly, refinement of the larvae and residue into marketable products (Dortmans et al., 2017). Refinement of the larvae may include killing, cleaning, sterilisation, drying and fractionation (i.e. separation of proteins, lipids and chitin), and of the residue, (vermi-)composting or anaerobic digestion (Zurbrügg et al., 2018). In addition, a nursery maintaining healthy adult and larval BSF ensures a reliable and consistent supply of offspring for biowaste treatment (Dortmans et al., 2017).

1.4 Potential of BSF Biowaste Processing for Waste Management

The interest in BSFL biowaste processing has been largely attributed to the production of larval biomass for animal feeds with a high market value. BSFL biomass contains 32-58% protein and 15-39% lipids (dry weight, Table 1.1 and Table 1.2), which are valuable for the production of animal feeds for livestock (e.g. poultry, swine, and fish) (Barragán-Fonseca et al., 2017; Makkar et al., 2014; Sánchez-Muros et al., 2014; Wang and Shelomi, 2017), pets (Bosch et al., 2014) and biofuels (Leong et al., 2016; Manzano-Agugliaro et al., 2012; Surendra et al., 2016; Zheng et al., 2012). De-fatted BSFL can have a protein content of 55-65% (dry weight) (Schiavone et al., 2017; Surendra et al., 2016) and an amino acid profile similar to that of feed components, with promising results in animal feeding experiments (Sánchez-Muros et al., 2014). Lauric acid, antimicrobial peptides, and chitin in BSFL have the potential to make larval biomass an even superior feed component (Gasco et al., 2018).

Due to the high market prices for conventional feed components (soya bean meal 44% protein ~400 USD/tonne, fish meal 64-65% protein ~1600 USD/tonne, dry weight, based on data cited by FAO (2016) for 2011-15), revenues from BSF biowaste processing products could be much higher than for other treatment technologies. For example, compost from biowaste commonly has a low market value (6-16 USD/tonne) (Danso et al., 2006; Diener et al., 2014). These revenues could contribute to the implementation and sustainable operation of biowaste management, especially in low and middle-income countries, where financial resources for implementation and operation are frequently scarce (Guerrero et al., 2013).

1.5 Current Challenges for BSF Biowaste Processing

Even though the scientific output on the topic has been increasing in the past decade (35 publications 2009-2013, 173 publications 2014-September 2018) (Scopus, 2018), BSF biowaste processing is a relatively new treatment technology (see Lohri et al. (2017) for the evolution of research articles published for different biowaste treatment technologies). Current, challenges for the efficient and sustainable implementation and operation of this emerging technology include:

- Precise, reliable, and efficient operation of the BSF nursery (e.g. egg yield per female fly, hatching rate) to maximize young larvae production.
- Low technology readiness levels/economy of scale of technologies and facilities. This influences their financial their financial viability and the possibility for the product to enter into markets. New partnerships between insect companies and technology providers are being established to deliver BSF biowaste processing facilities at scale (AgriProtein, 2017; BITS, 2017).
- Missing benchmarks for products: Even though research and companies start demonstrating the benefits of products (e.g. insects-based feeds) from BSFL, these benefits are still waiting to penetrate into the associated industries and result in a large product demand. This is also related to the low scale of facilities discussed above.
- Incomplete or restrictive regulations: Several countries (e.g., EU, USA, Canada, Mexico, Australia, China, South Africa, Kenya, Uganda) have started allowing the use of BSFL for production of feeds under certain conditions (e.g. registration, processing, animal specific) (EC, 2017; KEBS, 2017; Lähteenmäki-Uutela et al., 2017; UNBS, 2017).

Challenges that will be specifically discussed in this review include the variable BSFL treatment process performance and larvae and residue product safety.

Table 1.1 Description of biowaste and side streams used in BSFL treatment. This review defines wastes as discarded by products or side streams of urban activities that are typically heterogeneous in their characteristics and have a low economic value. In contrast, side streams are more homogenous and therefore commonly have a higher value. Poultry feed is also included as it is frequently used as an indicator for process performance.

Biowaste/side-stream types	Description	References
Human manures	Human faeces from source separation toilets. Faecal sludge from onsite sanitation technologies.	(Banks et al., 2014; Diener et al., 2011b; Lalander et al., 2013; Nyakeri et al., 2017b)
Animal manures	Excreta of poultry, cows or swine. Depending on the management practices, this can include bedding material (e.g. straw, hay) and animal feed.	(Liu et al., 2008; Moon et al., 2001; Myers et al., 2008; Newton et al., 2005; Nguyen et al., 2013; Nyakeri et al., 2017a; Oonincx et al., 2015b; Rehman et al., 2017; Sheppard et al., 1994; Zhou et al., 2013)
Fruit wastes	Discarded fruits (e.g. apples, pears, oranges or coconut endosperm). Typically produced by food companies or fruit markets.	(Jucker et al., 2017; Mohd-Noor et al., 2017)
Vegetable wastes	Discarded vegetables, for example sugar beet pulp, banana peels, cowpea, soya bean curd residue, lettuce, beans, cabbage. Typically produced by food companies or vegetable markets.	(Jucker et al., 2017; Nyakeri et al., 2017a; Oonincx et al., 2015a; Rehman et al., 2017; Tinder et al., 2017)
Municipal organic solid wastes	Mixed waste of discarded fruits, vegetables, and food scraps. Produced by households, restaurants, markets, malls, companies, and public institutions.	(Diener et al., 2011b; Nguyen et al., 2013; Nyakeri et al., 2017b; Oonincx et al., 2015a; Spranghers et al., 2017)
Millings and brewery side streams	Side streams typically produced by the milling and brewery industry: Sorghum, dried distiller grains with solubles, wheat, bran, spilled grains and grinding dust.	(Nyakeri et al., 2017b, 2017a; Tinder et al., 2017; Tschirner and Simon, 2015)
Poultry feed	Feed used for poultry. Frequently used as a control feed in BSFL feeding experiments.	(Diener et al., 2009; Gobbi et al., 2013; Liu et al., 2017; Nguyen et al., 2013; Oonincx et al., 2015a)

Variability in BSFL treatment performance

Table 1.1 summarizes biowaste previously used in research on BSFL treatment and introduces the classification into different biowaste types used in this review. Values in Table 1.2 demonstrate that one current challenge of BSF biowaste processing is the precise, reliable, and efficient operation of BSFL treatment. Considering typical process and product parameters, BSFL process performance (e.g. bioconversion of organic matter to protein and fat) is currently variable for the same biowaste, and between different biowaste types. Even though scalability of data generated from these bench-scale experiments could also be an issue, as it is unknown if these results truly translate to industrial production levels, challenges with variable process performance are also being reported from BSF biowaste processing operators. This impacts day-to-day operation (e.g. operation over or under the treatment capacity) and the sustainability and scalability of this technology.

Influence of BSFL treatment performance on sustainability

By providing BSFL biomass to global food and feed systems, BSF biowaste processing has the potential to contribute to meeting the increasing nutritional demands of the growing global human population (FAO, 2009). But, feeds produced from BSFL do not necessarily have a lower environmental impact than conventional feeds such as soya bean and fish meal, which are currently frequently deemed unsustainable (Alexander et al., 2017a; Salomone et al., 2017; Smetana et al., 2016; Van Huis and Oonincx, 2017). Biowaste that is not already used as animal feed or for other resource recovery options has the largest potential for sustainable products. New methodologies to quantify the sustainability of waste management and feed production systems based on BSF biowaste processing are being developed (Chaudhary et al., 2018; Smetana et al., 2016).

Influence of BSFL treatment performance on technology scalability

Existing BSF biowaste processing facilities indicate that they are financially sustainable when processing several tonnes to several hundred tonnes of biowaste per day with a high process performance (AgriProtein, 2018; Diener et al., 2015a; Protix, 2018). As access to large and continuous amounts of biowaste is typically already subject to fierce competition, BSF biowaste processing facilities will rely on a mixture of biowaste for their long-term operation. Scale is also required for this technology to have an impact on global food systems, hundreds of thousands of tonnes of BSFL would need to be produced per year, considering around 145 million tonnes soya bean (in 2007) (Hardy, 2010) and 15.8 million tonnes fish meal (in 2014) (FAO, 2016) is being produced per year.

Reasons for the variable process performance

The biowaste macronutrients, proteins, carbohydrates, fibres and lipids are frequently thought to have the largest influence on process performance (Nguyen et al., 2013; Oonincx et al., 2015; Tinder et al., 2017). As shown in Figure 1.1, biowaste and organic side streams previously used in BSFL treatment have a variable composition in these nutrients which could explain the variability in process performance. Based on the median of all macronutrient results included in this review, human and animal manures, milling and brewery side streams and municipal organic solid wastes are higher in proteins in comparison to fruit and vegetable wastes which are higher in carbohydrates. Human and animal manures and fruit and vegetable wastes have a higher median fibre and ash content. Municipal organic solid wastes are highest in lipids.

Moisture content and pH that are not specifically considered in this review, also have a large influence on process performance (Cammack and Tomberlin, 2017; Ma et al., 2018). As shown in Figure 1.1, biowaste moisture content is typically 70-80%, suitable for BSFL treatment (Dortmans et al., 2017). A moisture content in this range is important for larval development and BSFL and residue separation (Cheng et al., 2017).

Other reasons for the variability include the lack of standard operating procedures for conducting BSFL feeding experiments. Parameters including, but not limited to, different operating parameters such as biowaste descriptions, genetics (Zhou et al., 2013), feeding rate (Diener et al., 2009; Myers et al., 2008), feeding intervals (Banks et al., 2014), larval density, temperature (Harnden and Tomberlin, 2016; Tomberlin et al., 2009) and time of harvest (Liu et al., 2017) can vary significantly across studies.

Table 1.2. Previous results that were produced with poor biowaste preparation, feeding rates or larval densities, and experimental methods based on the current BSFL treatment process understanding were excluded from this table as they underestimate process performance (Oonincx et al. (2015b), Tschirner et al. (2015), Banks et al. (2014) and Diener et al. (2009)). Larval dry mass is typical 60-70% (Diener et al., 2009; Spranghers et al., 2017).

Biowaste		Prepupa larval weight	Development time	Waste reduction		Bioconversion rate	Protein	Lipids
		mg wet wt.	days	% wet wt.	% dry wt.	% wet wt.	% dry wt.	% dry wt.
Human and animal manure	Poultry	150-255[a,b]	-	50[b]	32-62[a,c]	3.7[b]	34-35[a]	-
	Swine	113-218[a,d]	34[e]	39[f]	29-53[a,c]	4.0[f]	32-43[a,g]	33[g]
	Cow	74-147[a,d]	24-31[d,h,i]	63[h]	26-58[a,c,d,h,i]	6.3[h]	34-35[a]	-
	Human	70-299[j,w]	27[j]	46-55[k]	55[j]	-	45[w]	18[w]
Fruit waste		55-174[l,w]	15-52[l,p,w]	-	32-36[p]	-	35-58[p]	15-38[p,w]
Vegetable waste		101-184[l,j]	16-48[h,l,m,w]	74[h]	72[h]	9.7[h]	44[m]	-
Fruit/vegetable waste		123-154[e,l]	29-37[e,l]	-	-	-	39[v]	33[v]
Municipal organic solid waste		101-220[e,q]	16-37[e,o,q,r,w]	-	60-68[j]	11.8[q]	36-46[o,r,w]	25-39[o,r,w]
Millings and brewery side streams		78-290[m,n,w]	16-39[m,s,w]	38-59[n]	-	-	37-45[m,n,w]	27-39[m,n,w]
Poultry feed		99-184[e,t]	15-24[e,o,r,t,u]	-	42[t]	-	33-39[o,t]	34[o]

[a](Zhou et al., 2013) [b](Sheppard et al., 1994) [c](Oonincx et al., 2015b) [d](Myers et al., 2008) [e](Nguyen et al., 2013) [f](Newton et al., 2005) [g](St-Hilaire et al., 2007) [h](Rehman et al., 2017) [i](Li et al., 2011) [j](Diener et al., 2011b) [k](Banks et al., 2014) [l](Jucker et al., 2017) [m](Tinder et al., 2017) [n](Tschirner and Simon, 2015) [o](Spranghers et al., 2017) [p](Mohd-Noor et al., 2017) [q](Diener et al., 2011a) [r](Oonincx et al., 2015a) [s](Tomberlin et al., 2009) [t](Diener et al., 2009) [u](Gobbi et al., 2013) [v](Nyakeri et al., 2017a) [w](Nyakeri et al., 2017b)

Figure 1.1 Macronutrient (in % of dry mass) and moisture (in % as received) in biowastes typically used in BSFL treatment. Bars are the range of all reported values. The vertical line within the bar is the median of all reported values as a metric for their variability. Carbohydrates were calculated by subtracting the sum of protein, fibre, lipids and ash from 100%. See Table 1.1 for the descriptions of the biowaste types. (Diener et al., 2009; Jucker et al., 2017; Li et al., 2011; Mohd-Noor et al., 2017; Nguyen et al., 2013; Rose et al., 2015; Sanergy, 2018; Spranghers et al., 2017; Tinder et al., 2017; Tschirner and Simon, 2015).

BSF biowaste processing product safety

Biowaste include a high number and diversity of microbes. In addition, human and animal manures may contain pharmaceuticals, poorly stored milling and brewery side stream mycotoxins, fruit and vegetable waste pesticides, and municipal organic solid waste heavy metals and other toxins such as dioxins, polychlorinated biphenyls (PCBs) and polyaromatic hydrocarbons (PAHs). Thus, transfer and accumulation of these contaminants to BSFL in the feed and food chain are of concern to this new industry (Van der Fels-Klerx et al., 2018).

Previous research identified that BSFL treatment can reduce numbers of microbes (Lalander et al., 2013). In addition, dioxins, PCBs and PAHs, and selected pesticides, pharmaceuticals and mycotoxin did not accumulate in BSFL in the few existing studies (Bosch et al., 2017; Charlton et al., 2015; Lalander et al., 2016; Purschke et al., 2017). In contrast, cadmium, lead, mercury, zinc, and arsenic are taken up by BSFL from biowaste and can exceed the maximum permissible levels of animal feed regulations (Biancarosa et al., 2017; Diener et al., 2015b; EC, 2002; Gao et al., 2017; Purschke et al., 2017; van Der Fels-Klerx et al., 2017). Due to the volume of biowaste reduced in BSFL treatment, heavy metals concentrate in the residue and could limit its application as compost. Whereas microbes

in and on the larvae can be a risk for animal feed safety, and in the residue for public and environmental health (Lalander et al., 2013), several studies have shown that inoculation of biowaste with microbes can increase the BSFL process performance (Xiao et al., 2018; Yu et al., 2011; Zheng et al., 2012).

A lack of understanding about the fate of potential contaminants (including parasites and viruses) in BSF biowaste processing currently limits the use of biowaste with the highest potential to produce sustainable animal feeds. For example, in the EU, use of biowaste (except for vegetable and fruit wastes) are currently not permitted for BSFL production as animal feed (EC, 2017; EFSA, 2015; Makkar et al., 2014).

1.6 Purpose of this Review

In contrast to other biowaste treatment technologies, process mechanisms in BSFL treatment are poorly understood. BSFL have a similar ecological niche and phylogenetic order (relative to well-studied insects from other orders) to larvae of well-studied fly species, this suggests that their digestion developed in a similar way from the same original digestive process (Terra and Ferreira, 2012, 1994; Terra and Regel, 1995). These include housefly larvae (HFL), *Musca domestica* (Diptera: Muscidae), green bottle fly larvae (GBFL), *Lucillia sericata* (Diptera: Calliphoridae), stable fly larvae (SFL), *Stomoxys calcitrans* (Diptera: Muscidae), and common fruit fly larvae (FFL) (also called vinegar fly), *Drosophila melanogaster* (Diptera: Drosophilidae).

Using available research on larvae of these well-studied fly species, this review summarizes the current knowledge on the decomposition of biowaste macromolecules and chemicals and the inactivation of microbes by fly larvae and in BSFL treatment. It then uses this knowledge to discuss the influence of biowaste macromolecules, microbes, and chemicals on BSFL treatment process performance, and highlights future research directions.

The goal of this review is to increase the understanding of BSFL treatment to lead to the enhancement of its performance and product safety, for uptake of heterogeneous biowaste. This could result in improved biowaste management, especially in low- and middle-income countries.

1.7 Black Soldier Fly Larvae (BSFL) Biowaste Treatment

BSFL treatment usually takes place in boxes, bins or containers. For example, 40-80 cm x 60-200 cm x 17-30 cm used by Diener et al. (2011a) and Zurbrügg et al. (2018). At the beginning of BSFL treatment, young BSFL with an age of typically 4-9 days and a weight of approximately 1-2 mg, reared on a standard diet (often poultry feed), are placed on a defined amount of biowaste (Jucker et al., 2017; Spranghers et al., 2017; Tschirner and Simon, 2015). Diener et al. (2009) proposed feeding rates of 100-125 mg per larva, per day (based on poultry feed, with a 60% moisture content). Biowaste is either provided once, or through periodic feeding (Banks et al., 2014; Dortmans et al., 2017).

During treatment, BSFL pass through six life stages between which, larvae moult (i.e. process of shedding their exoskeleton, allowing larvae to grow) (May, 1961). As summarized in Table 1.2, the last BSFL instar (prepupa) is reached after 15-52 days. At this life stage, BSFL are 95-299 mg (Table 1.2) and 6-20 mm (Nguyen et al., 2013; Rozkosny, 1983; Tinder et al., 2017). Full-scale operations tend to reduce the number of feedings to reduce operational costs and select their harvest time to maximize larval production and product quality (Liu et al., 2017).

1.8   System Description of BSFL Treatment in this Review

As shown in Figure 1.2, conceptually, the container in which BSFL treatment takes place can be divided into two reactors. The fly larva as a reactor which includes all processes inside the BSFL, and the biowaste as a reactor which includes all processes in the biowaste outside BSFL. Processes in both reactors are influenced by operational parameters such as container dimensions, temperature, larval density, humidity, feeding rate, and feeding interval. As will be reviewed in the following sections, fly larvae and waste reactors are connected through ingestion, secretions, and excretions. In addition, both reactors consume and produce gases (Beskin et al., 2018; Oonincx et al., 2010). The overall system includes multiple fly larva reactors that operate in parallel within the biowaste reactor. For example, Dortmans et al. (2017) use four larvae per cm^2 of the biowaste reactor surface area.

1.9   Description of Processes in the Fly Larva Reactor

Well-studied fly larvae

Previous BSFL research focused on measuring the influence of biowaste macronutrients, microbes, and chemicals in controlled feeding experiments, but few did study the underlying physiological, microbial, and biochemical processes. Fortunately, such research is available for other fly larvae species.

Adult house flies are a recognized pest of humans, pets, and livestock; HFL typically feed on animal manures. Extensive research exists on digestion mechanisms (e.g. physiology, enzymes, nutrient absorption) of HFL (Espinoza-Fuentes and Terra, 1987; Lemos et al., 1993; Lemos and Terra, 1991b; Pimentel et al., 2018; Terra and Jordão, 1989; Terra and Regel, 1995; Zhang et al., 2017). GBFL have mostly been studied for their importance in maggot therapy, with research focusing on antimicrobial processes (Cazander et al., 2009; Lerch et al., 2003; Luther, 1951; Mumcuoglu et al., 2001; Sanei-Dehkordi et al., 2016). GBFL typically feed on living animal or human flesh in some instances, depending on the species; however, they consume decaying animals, and manures. After their medical importance, GBFL have also been studied for their use in forensic entomology (Tarone et al., 2011). The stable fly has been studied as it is an important animal pest. SFL typically feed on animal manures (Albuquerque and Zurek, 2014; Scully et al., 2017) mixed with urine and straw, or other cellulose-based materials. It has mainly been studied for interactions between SFL and microbes (Rochon et al., 2004; Romero et al., 2006). FFL typically feed on decaying fruits. They are of importance as a model organism for genetics and more recently immunology, host-microbe interactions and metabolism (Jeon et al., 2011; Lee and Brey, 2013; Mirth and Piper, 2017; Rodrigues et al., 2015; Shin et al., 2011; Storelli et al., 2011; Wong et al., 2016; Yamada et al., 2015). The common fruit fly is one of the most well studied organisms in the world with decades of knowledge of its genomics and molecular biology which allows for unique research on metabolic pathways.

HFL were previously also studied for treatment of poultry, swine, and cow manure (Barnard et al., 1998; Calvert et al., 1970; Hussein et al., 2017; Koné et al., 2017; Nordentoft et al., 2017; Schuster et al., 2013; Wang et al., 2017; Wang et al., 2016; Wu et al., 2017; Yang et al., 2015; Zhang et al., 2014, 2012) and GBFL for treatment of poultry slaughterhouse waste, fish waste and swine manure (Nuov et al., 1995; Yehuda et al., 2011). In contrast to the BSF, all of these fly species feed as adults which makes them vectors for the transmission of pathogens (Čičková et al., 2015).

General fly larvae digestion and gut physiology

Similar to other insects and mammals, fly larvae feed to obtain nutrients for their metabolic requirements (Cohen, 2005). The monomer of carbohydrates, glucose, is used by fly larvae as a building block for tissues and as fuel. Fly larvae are also surrounded by chitin which is made of proteins and carbohydrates (Cohen, 2005). Amino acids, the building blocks of proteins are important molecules for the production of fly larvae tissue, hormones, and transport proteins (Cohen, 2005). Arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine are considered essential amino acids for insects (Cohen, 2005). Lipids serve as energy storage, provide structural components for cell membranes and organelles, and are important for the production of hormones (Carvalho et al., 2012; Cohen, 2005). Sterols, vitamins, and minerals are also indispensable for larval development, but due to the scare information on their decomposition and importance for larval development they are not considered in this review (Cohen, 2005).

Fly larvae feed through mouthparts located at the anterior end of their elongate-oval shape. Little research exists on fly larvae mouthparts. The existing research concludes that fly larvae ingest liquids and solids without specifying the maximum particle size (Brookes and Fraenkel, 1958; Oliveira et al., 2015). Following this, the food passes through the food pipe (oesophagus) (Mumcuoglu et al., 2001), the proventriculus (a valve with a potentially grinding function), into the midgut (Chapman, 2013). The midgut, shown in Figure 1.2, is the longest and most important part of the larva digestive tract for diet sensing, decomposition and nutrient absorption, that doubles back and forth upon itself between the anterior and posterior end of the fly larvae (Chapman, 2013; De Smet et al., 2018; Luther, 1951). Kim et al. (2011b) extracted enzymes from the BSFL gut and salivary glands and the latter included only 10% of the total enzymatic activity. Along the midgut, through the combined action of the gut environment, enzymes, and microbes, the diet is broken down into smaller molecules for absorption through the gut cell into the haemolymph (Chapman, 2013). The haemolymph is analogous to the blood in vertebrates and transports nutrients within the larval body (Chapman, 2013). Nutrients are stored in the larval fat body, which is important for the accumulation of lipids and controlling the larval metabolism (Chapman, 2013). From the midgut, the diet passes to the hindgut and malpighian tubules. The malpighian tubules, located at the mid- and hind-gut junction are connected to the haemolymph and are important for maintaining a balance among nutrients, water, and ions within the larvae. They exchange nutrients, ions, nitrogenous substances (e.g., uric acid) and other metabolic wastes with the haemolymph and hindgut, that exceed the larval demands (Chapman, 2013; Murakami and Shiotsuki, 2001). These molecules and non digestible diet components, enzymes, metabolites, microbes, antimicrobial proteins, or diet components not absorbed are then excreted from the hindgut (Chapman, 2013; Engel and Moran, 2013; Espinoza-Fuentes and Terra, 1987). Fly larvae are continuous feeders and the diet has a residence time in the larvae of around 60-180 minutes (Dadd, 1970; Espinoza-Fuentes and Terra, 1987; Mumcuoglu et al., 2001; Terra and Ferreira, 2012).

Gut environment

Oxygen and gut pH are important properties of the gut environment as they influence the decomposition of diet components, the activity of enzymes and shape the number and diversity of gut microbes.

The physiology of fly larvae suggests that the midgut has both aerobic and anaerobic sections. Openings at the front and end of BSFL connect to their respiratory system and could supply oxygen (Rozkosny,

1983). Active transport mechanisms or diffusion through the small diameter of the midgut could allow for an oxygen supply into the gut (Chapman, 2013). This has also been indicated by the cultivation of aerobic, facultative aerobic and anaerobic bacteria from guts of all reviewed fly species (Jeon et al., 2011; Scully et al., 2017; H. Wang et al., 2017; Zurek et al., 2000).

Based on research for HFL and FFL, the fly larvae midgut pH is buffered longitudinally in several sections: a neutral anterior midgut (pH 7), an acidic mid midgut (pH 2), and a neutral to alkaline posterior midgut (pH 6.3-9.3) (Espinoza-Fuentes and Terra, 1987; Overend et al., 2016; Shanbhag and Tripathi, 2009). The variability in the posterior midgut is due to a higher alkalinity reported for FFL.

Gut enzymes

Enzymes are the most important driver for diet decomposition. They hydrolyse macromolecules into smaller molecules to be absorbed through the gut cells for the fly larva metabolism (Chapman, 2013). The decomposition of one macromolecule is likely due to the action of dozens of enzymes that sequentially reduce molecule size. In this way, carbohydrates are reduced to simple sugars, proteins into amino acids and lipids into glycerides and fatty acids (Carvalho et al., 2012; Kim et al., 2011b). For example, in the adult common fruit fly, around 350 enzymes are estimated to catalyse the decomposition of carbohydrates, proteins and lipids (Lemaitre and Miguel-Aliaga, 2013).

In HFL, most digestive enzymes are found in the posterior midgut, which suggests it is where diet decomposition and nutrient absorption primarily take place. Except during moults, enzymes are continuously released into the midgut through the release of secretory vessels from the gut cells (Espinoza-Fuentes and Terra, 1987). Excreted enzymes vary based on the diet, gut environment (e.g. pH) and along the gut and its compartments (Espinoza-Fuentes and Terra, 1987). Important enzymes in fly larvae that hydrolyse glycoside links in carbohydrates include amylase, maltase, aminopeptidase and glucosidase, peptide bonds in protein pepsin, trypsin and serine, and ester bonds in lipids triacylglycerol lipase, phospholipase and phosphatases (Blahovec et al., 2006; Greenberg and Paretsky, 1955; Kim et al., 2011a, 2011b; McDonald et al., 2011; Zhang et al., 2017). Fly larvae also have a number of lysozymes, chitinases and glucanases that may participate in the decomposition of microbes (Fujita, 2004; Lemaitre and Miguel-Aliaga, 2013). Cytochrome P450 and glutathione-S-transferase enzymes are important for the decomposition of mycotoxins or insecticides (Cochrane and Leblanc, 1986; Fusetto et al., 2017).

As shown in Figure 1.2, the fly larva midgut is lined by a structure attached to the anterior midgut, known as the peritrophic matrix. The peritrophic matrix can be found in most insects and compartmentalizes the midgut into sections for the different phases of enzymatic hydrolysis of diet molecules (Bolognesi et al., 2008; Terra, 2001). Espinoza-Fuentes and Terra (1987) reported that diet molecules and enzymes are transported forward by water fluxes from the anterior to posterior midgut, and within the posterior midgut.

Decomposition of macromolecules

Even though fly larvae may excrete enzymes such as amylase or maltase from the salivary glands through the mouth onto the diet, as reported for HFL, most diet decomposition takes place in the midgut (Terra et al., 1988). Following ingestion, the diet enters the endoperitrophic space (i.e., outside of the peritrophic matrix) where it has contact with enzymes catalysing the initial decomposition of

macromolecules such as amylase and maltase (carbohydrates), pepsin and trypsin (protein) and triacylglycerol lipase and phospholipase (lipids) (Pimentel et al., 2018; Terra and Ferreira, 2012). With the flux of diet and water, macromolecules pass along the gut until their size has been sufficiently reduced by the enzymes to enable them to penetrate together with the enzymes through the peritrophic matrix into the ectoperitrophic space (i.e. inside of the peritrophic matrix). With the water flux in the posterior midgut, enzymes and diet molecules are then returned to the anterior section of the posterior midgut where decomposition takes place by enzymes such as maltase (carbohydrates) and aminopeptidase (proteins) (Espinoza-Fuentes and Terra, 1987). Enzymes are then returned through the peritrophic matrix into the endoperitrophic space to locate new diet macromolecules for hydrolysis (Espinoza-Fuentes and Terra, 1987). As shown in Figure 1.2, in HFL, glucose produced by amylase from diet carbohydrates is already absorbed in the mid-midgut. In the posterior midgut, residual diet carbohydrates and carbohydrates originating from the decomposition of microbes are thought to be decomposed and absorbed (Pimentel et al., 2018). Results for HFL and FFL suggest that fly larvae do not have enzymes for the initial decomposition of celluloses and hemicelluloses, but microbes in the larval gut may decompose them into a form that can be metabolized by fly larvae (Espinoza-Fuentes and Terra, 1987; Lemaitre and Miguel-Aliaga, 2013; Terra and Ferreira, 2012). Through diffusion or active transport, monomers are then transported into the haemolymph.

Metabolic pathways and regulation

Based on studies of FFL, ingestion, decomposition and absorption of diet constituents, and larval development in fly larvae, is controlled by complex hormonal regulations. Different processes adjust larval development and the decomposition of diet constituents to the current nutritional demands, which change over time as larvae grow (Almeida de Carvalho and Mirth, 2017; Colombani et al., 2003; Lajeunesse et al., 2010; Lemaitre and Miguel-Aliaga, 2013).

Nutrient sensing appears to start before ingestion. Shen and Cai (2001) demonstrated that feeding rate is regulated by a hormonal pathway and increases with sugars in the diet. However, Almeida de Carvalho and Mirth (2017) conclude that FFL regulate food intake to maintain protein intake at the cost of consuming too little or too much carbohydrates. As FFL aim to keep the development time as short as possible (Rodrigues et al., 2015), protein excess bears a metabolic cost due to the production of toxic nitrogenous wastes (e.g. ammonia, uric acid) and protein deficiency prolongs development time (Almeida de Carvalho and Mirth, 2017).

Following ingestion, the larva digestive tract appears to balance nutrient decomposition by regulating the amount of enzymes secreted into the gut, secreting less enzymes for nutrients in excess and more enzymes for nutrients in deficit (Clissold et al., 2010; Rodrigues et al., 2015). For example, for carbohydrates, Benkel and Hickey (1986) showed that the presence of glucose reduced amylase production (Brown et al., 1999; Wu et al., 2003). For lipids, Zinke et al. (2002) and Handke et al. (2013) showed an upregulation in genes for lipid synthesis in the larval body, on low-protein diets, which was also confirmed by Pimentel et al. (2017) for BSFL.

Ultimately, demonstrated by the extensive research on FFL, larval development (e.g. larval size and composition, growth rate, development time) appears to be regulated by the larval fat body in response to nutrition (Chapman, 2013; Koyama and Mirth, 2018). In FFL, illustrated in Figure 1.2, in the presence of amino acids, the fat body secretes signals that lead to the release of insulin like hormones

from the brain that initiates complex metabolic processes that ultimately lead to larval growth (Arquier et al., 2008; Colombani et al., 2003; Géminard et al., 2009; Okamoto and Yamanaka, 2015). These processes called insulin/insulin-like growth factor signalling (IIS) pathway and the Target of rapamycin (TOR) pathway are suppressed during starvation or on low-protein diets and reduce growth rate and development time (Koyama and Mirth, 2018; Shin et al., 2011; Storelli et al., 2018, 2011).

Gut microbes

Fly larvae feed on diets with a diverse composition and number of microbes. Microbes in the fly larvae gut have multiple functions that are important for larvae development. (Douglas, 2010; Lemaitre and Miguel-Aliaga, 2013).

Several processes enable fly larvae to use microbes, such as bacteria and fungi as food (Brookes and Fraenkel, 1958; Chang and Wang, 1958; Cohen, 2005; Lam et al., 2009a; Schmidtmann and Martin, 1992). For example, decomposition of carbohydrates such as starch in the anterior midgut, and absorbed in the mid-midgut, reduce the nutrients available for microbial metabolism (Espinoza-Fuentes and Terra, 1987). In addition, fly larvae excrete pepsin and lysozyme into the midgut and are exposed to acidic pH levels that decompose microbial cells. Also, fly larvae excrete proteins with antimicrobial properties (e.g., antimicrobial peptides, reactive oxygen generating enzymes) (Choi et al., 2012; Elhag et al., 2017; Espinoza-Fuentes and Terra, 1987; Lemaitre and Miguel-Aliaga, 2013; Lemos et al., 1993; Lemos and Terra, 1991a; Nayduch and Joyner, 2013; Pöppel et al., 2015; Vogel et al., 2018). Lysis of bacteria and fungi in the midgut thereby releases nutrients that can be decomposed and absorbed in the posterior midgut.

Such decomposition of microbes via gut-based mechanisms including pH, enzymes and antimicrobial proteins explain the selective inactivation of microbes, as reported for fly larvae. Through the use fluorescent bacteria, it was identified that there was complete inactivation of *Escherichia coli* and *Bacillus subtilis* through the gut passage of GBFL (Lerch et al., 2003; Mumcuoglu et al., 2001; Valachova et al., 2014). *E. coli* and *Salmonella enteritidis* number were also reduced in the larva of HF and S. enterica and ΦX174 virus in BSFL (Lalander et al., 2014; Nordentoft et al., 2017). Other species, for example, *Enterococcus faecalis* in BSFL and *E. coli* for SFL had low reductions in the larvae and residue (Lalander et al., 2013; Rochon et al., 2004). This demonstrates that results vary among fly larvae and inactivation is sometimes contradictive for the same fly species (Lalander et al., 2013; Rochon et al., 2004; Valachova et al., 2014). Variable results observed for GBFL in their medical applications, suggest that microbe inactivation by fly larvae depends on the specific microbe and strains, and varies with microbial dose, nutrient availability and the duration that larvae feed on the diet (Barnes et al., 2010). Similar mechanism likely apply to fungi. When exceeding a certain size and density, due to their inactivation mechanisms, fly larvae are able to "control" fungi in the diet for which they compete for nutrients (Lam et al., 2009b; Rohlfs et al., 2005), with certain fungi surviving the gut passage (Coluccio et al., 2008).
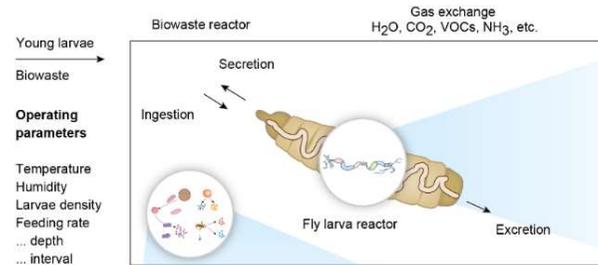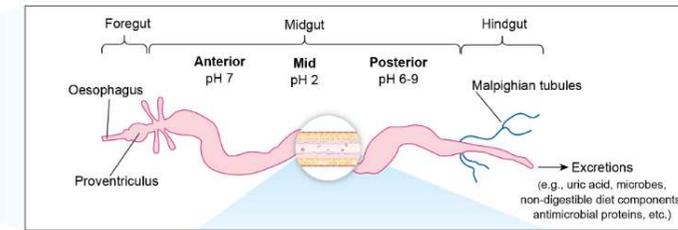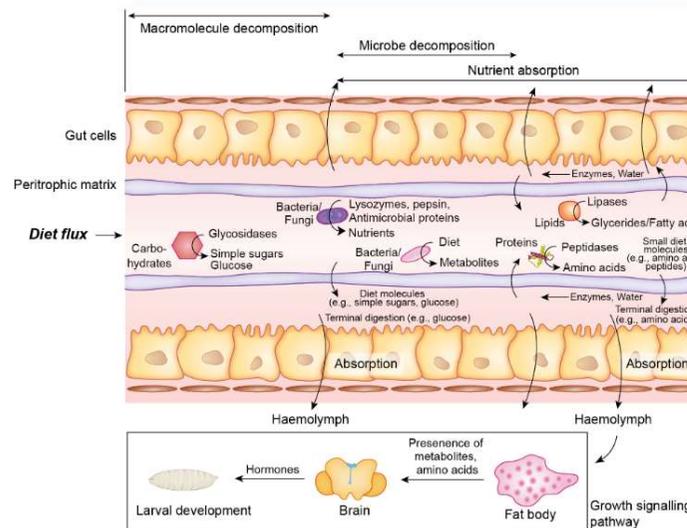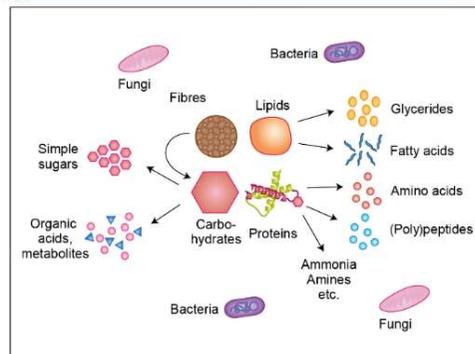
Figure 1.2 Schematic summary of important processes in BSFL treatment. (a) BSFL treatment conceptually divided into a biowaste and larva reactor. (b) The larva midgut is most important for nutrient sensing, decomposition and absorption. The midgut is compartmentalized longitudinally considering pH and enzymes, and lined by the peritrophic matrix for the different phases of enzymatic hydrolysis of diet molecules. Size of nutrient macromolecules is reduced along the midut, with nutrient absorption mostly taking place in the posterior midgut. Some enzymes are returned via water fluxes. The fat body signals larva development in the presence of amino acids via the IIS/TOR pathway. (c) Enzymatic and microbial decomposition of biowaste macromolecules in the biowaste reactor. Adapted from: (Beskin et al., 2018; De Smet et al., 2018; Espinoza-Fuentes and Terra, 1987; Lee and Brey, 2013; Lemaitre and Miguel-Aliaga, 2013; Wong et al., 2016).

Microbes that survive the gut passage are candidates for microbes that contribute more to larval development, besides just being a nutrient source. This appears to be especially important in low-protein diets, or diets that lack other essential nutrients (e.g. methionine) (Rhinesmith-Carranza et al., 2018). That fly larvae cohabit in some form of symbiosis (Douglas, 2010) with microbes is indicated by several studies that were not able to grow FFL, HFL or SFL on diets with an absence of bacteria (Lysyk et al., 1999; Romero et al., 2006; Watson et al., 1993), whereas the addition of certain bacteria promoted their development. In contrast, it appears that fly larvae maintain their own gut microbes originating by transfer from the adult fly, larval diet and the environment (Jeon et al., 2011; Scully et al., 2017; Zurek and Nayduch, 2016).

In all reviewed species, gut bacteria are dominated by the phyla *Firmicutes*, *Proteobacteria*, *Bacteroidetes* and *Actionbacteria* (Boccazzi et al., 2017; Scully et al., 2017; Shin et al., 2011; Singh et al., 2014; Storelli et al., 2011; Su et al., 2010; H. Wang et al., 2017; Wong et al., 2014; Zhao et al., 2017; Zheng et al., 2013; Zurek et al., 2000). Dominating genera have a large variability between fly larvae and studies, likely due to the variability in diets. In BSFL, *Bacterioides*, *Dysgonomonas*, *Phascolarctobacterium* were abundant genera of bacteria (Zheng et al., 2013) and *Pichia*, *Trichosporon*, *Rhodotorula* and *Geotrichum* of fungi (Boccazzi et al., 2017).

The role of fly larval gut microbes on larval development is poorly understood. One key function of gut microbes includes the provision of metabolic products through the decomposition of macromolecules. These are relevant for the larval metabolism and protection from pathogens (Lemaitre and Miguel-Aliaga, 2013; Wong et al., 2016). As fly larvae feed on diets high in carbohydrates (Figure 1.1), gut microbes can metabolize starch, sugars and fibres into organic acids such as short chain fatty acids and/or simple alcohols (Figure 1.2) (Cohen, 2005; Romero et al., 2006; Wong et al., 2016). Jeon et al. (2011) and Lee et al. (2014) identified that bacteria in the BSFL gut possess enzymes that can hydrolyse starch, cellulose, proteins and lipids, and thus contribute to biowaste decomposition. Zhao et al. (2017) analysed gut microbes in SFL and concluded that they likely contribute to the decomposition of fibres. In low-nutrient diets, Shin et al. (2011) identified that carbohydrate decomposition by *Acetobacter pomorum* in FFL produces metabolites that affected larval development by influencing growth signalling pathways. Storelli et al. (2011) reported that in FFL, *Lactobacillus plantarum* increases amino acids extracted from low-protein diets which increases larval development. Erkosar et al. (2015) attributed this to the activation of larval enzymes that decompose proteins. Other bacteria and fungi may likely still be discovered which produce metabolites that are involved in other pathways influencing larval development (Lee and Brey, 2013).

Decomposition of chemicals

In nature, fly larvae grow on diets with potential contaminants such as secondary plant metabolites, insecticides or mycotoxins. Thus, they have strategies that allow them to nevertheless thrive in the presence of contaminants. Pharmaceutics, pesticides and mycotoxins used in feeding experiments so far have not had a detrimental effect on BSFL development (Lalander et al., 2016; Purschke et al., 2017). Similar to macronutrients, the decomposition of chemicals involves a number of complex metabolic processes, mostly taking place in the larval midgut, malpighian tubules and fat body. In general, detoxification of chemicals involves cytochrome P450 monooxygenases and glutathione-S-transferase enzymes, but also relies on the action of gut microbes (Chung et al., 2009; Cochrane and Leblanc, 1986; Fusetto et al., 2017). The decomposition of the insecticide imidacloprid in FFL has been

well studied. The chemical is metabolized by larval enzymes into oxidative metabolites and by gut microbes into nitro-reduced metabolites, of which some are toxic and are excreted by the larvae (Hoi et al., 2014). Such detoxification pathways likely also apply for BSFL (Bosch et al., 2017; Charlton et al., 2015; Lalander et al., 2016; Purschke et al., 2017). However, results by Bosch et al. (2017) for the decomposition of the mycotoxin aflatoxin B1 by BSFL suggest that the exact decomposition pathways for chemicals can vary between fly species (Foerster et al., 1983).

Fate of heavy metals

Heavy metals such as copper, zinc or iron are essential for metabolic processes in fly larvae (Balamurugan et al., 2007). However, at high concentrations they have been shown to be detrimental to larval development (i.e. reduced larval biomass and an increased development time) (Diener et al., 2015b; Purschke et al., 2017). They are sequestered from the diet to cells or vesicles in the epithelium of the midgut, malpighian tubules or fat body (Sohal, 1974; Sohal et al., 1977). Heavy metals that are toxic in lower quantities such as cadmium, lead and mercury are also integrated into these structures by proteins called metallothioneins that bind cadmium and copper and store them within cell organelles called lysosomes (Maroni et al., 1986; Maroni and Watson, 1985). These pathways could also apply for BSFL which accumulate heavy metals. Uptake of cadmium in insects is also mediated through calcium channels in the gut cells. As BSFL have more than ten times the level of calcium than HFL, they may sequester more cadmium than HFL, as reported by Wang et al. (2017) (Braeckman et al., 1999; Finke, 2013).

1.10 Description of Processes in the Biowaste Reactor

Several studies with BSFL and HFL operated biowaste treatments with fly larvae next to biowaste treatments without fly larvae. The results suggest that treatments without fly larvae had a considerable waste reduction (30% dry weight reduction), as well as some inactivation of pathogens (e.g. <2 log10 *S. enterica*), pharmaceuticals and pesticides (Lalander et al., 2013). However these were lower than when biowaste was treated with fly larvae (Lalander et al., 2016, 2013; Nordentoft et al., 2017; Wang et al., 2016). As biowaste often has a high number and diversity of microbes, such waste reduction and inactivation without fly larvae indicate a significant contribution of microbial processes.

Microbial decomposition of macromolecules

Microbial decomposition of biowaste constituents involves the metabolism of a diverse group of microbes that use products from each other's metabolism. Hydrolytic enzymes produced by certain microbes are similar in function to those found in fly larvae and can decompose carbohydrates, proteins, and lipids, but also have the potential to use a large number of other biowaste molecules or larval excretions (e.g. uric acid). More importantly, facultative and obligate anaerobic microbes found in biowaste, have cellulase enzymes that hydrolyse fibres (i.e. cellulose, hemicellulose, and lignin) that are typically difficult to decompose by fly larvae and most insects (Terra and Ferreira, 2012). For example, in swine manure treatment with HFL, Zhang et al. (2012) identified glucosidase, cellulose, protease and phosphatase enzyme activities in the residue which indicated that enzymes are present that can hydrolyse glycoside links in fibres.

Reduction of fibres in BSFL treatment varies between studies. BSFL treatment of fermented corncobs did not reduce cellulose and hemicellulose, and reduced lignin by 2% (Li et al., 2015). Treatment of cow manure reduced cellulose by 17% and hemicellulose by 5% in Li et al. (2011) and around 50% for both fibres in Rehman et al. (2017). These large differences in fibre reduction could be due to the variable chemical composition of different fibres, analyses methods, different BSFL treatment operations, and different compositions and quantities of microbes, nutrients, and environmental parameters influencing microbial ecology and metabolism. Temperature, oxygen and the presence of glucose and other readily metabolizable sugars can supress the microbial fibre decomposition (Gikes et al., 1991; Linden and Shiang, 1991). Results by Zheng et al. (2012) for BSFL treatment of rice straw and restaurant waste, exemplify that the presence of sufficient amounts of bacteria can contribute to the hydrolysis of fibres. The inoculation of biowaste with an unknown mix of microbes (Rid-X®) increased reduction of cellulose by 37% and hemicellulose by 23%.

These results suggest that microbial number and diversity in different biowaste types have a large influence on the microbial contribution to the decomposition of biowaste constituents. Different types of biowaste such as human excreta, different types of animal manures, municipal organic solid waste and fruit waste have very different microbial compositions and quantities. Ryckeboer et al. (2003) summarized microbial communities in biowaste and concluded that species of the phyla *Firmicutes*, *Bacteroidetes*, *Actinobacteria* and *Proteobacteria* dominate. Even for the same type of biowaste, such as for different animal manures, different microbial compositions can be expected due to factors such as animal species, diet and manure storage duration (Albuquerque and Zurek, 2014).

Products of the microbial hydrolysis of biowaste (e.g. organic acids, sugars) are then available for microbial and/or larval metabolism. Larvae likely benefit from the action of microbes as they provide molecules for their metabolism, that without their action would not have been available (Chapman, 2013; Douglas, 2010). In addition, microbes can convert non protein molecules into microbial biomass that can then be decomposed by the larva and thereby increase the overall pool of amino acids (Raubenheimer and Simpson, 1999). However, microbes may also compete with fly larvae for biowaste constituents. Albuquerque et al. (2014) reported that SFL development on horse manure was shorter for manure stored for one week compared to fresh manure, but development decreased on manure with further storage. This could be attributed to a complete decomposition of biowaste constituents by microbes, or conversion into a form that has a low value for fly larvae development (Karigar and Rao, 2011).

Microbial dynamics

Microbial number and diversity are different between biowaste types and change during fly larvae treatment (Scully et al., 2017). Results for HFL and FFL suggest that the overall diversity of bacteria and fungi decreases. Zhang et al. (2012) and (2014) concluded that the decomposition of microbes in the fly larvae gut, aeration of the biowaste by fly larvae, reduction in nutrient availability and increase in temperature and pH reduces the microbial diversity in full-scale treatment of swine manure with HFL and shifted it from anaerobic to aerobic microbes (Beskin et al., 2018; Wang et al., 2016). Stamps et al. (2012) reported that FFL reduce the fungal diversity. In addition, fly larvae transfer specific bacteria from their gut to the biowaste reactor. Zhao et al. (2017) concluded that HFL transfer bacteria of the phyla *Actinobacteria*, *Proteobacteria*, *Bacteroidetes* and *Firmicutes* from the gut, to biowaste. Thereby, fly larvae may promote microbes in biowaste that assist their development (Storelli et al., 2018; Vogel et al., 2018).

Microbial decomposition of chemicals

The microbial metabolism and the change in the physical and chemical environment in the waste reactor also decompose mycotoxins, pharmaceuticals and pesticides (Lalander et al., 2016; Li et al., 2013; Zhang et al., 2014). Lalander et al. (2016) observed reductions between 30-90% for three pharmaceuticals (carbamazepine, roxithromycin, trimethoprim) and >99% for two pesticides (azoxystrobin, propiconazole).

The processes leading to these reductions in biowaste are poorly understood, but likely similar to those of other biowaste treatment technologies, such as composting. Here, depending on the compound, reductions in the order of 50% to > 99.99% were reported for composting of animal manures (Ho et al., 2013; Ramaswamy et al., 2010; Selvam et al., 2012). In composting, processing conditions (e.g. temperatures >75°C) and the microbial metabolism and associated enzymes are thought to contribute to their decomposition (Karigar and Rao, 2011; Mondini et al., 2004). For example, bacteria and fungi typically found in biowaste of the genera *Lactobacillus*, *Bacillus*, *Pseudomonas*, *Flavobacterium* and *Aspergillus* have been reported to be involved in the decomposition of mycotoxins (Dalié et al., 2010; Ji et al., 2016).

1.11 Influence of Biowaste Composition on Process Performance

Figure 1.2 summarizes the most relevant processes in BSFL treatment, driven by the microbial and larval metabolism that decompose biowaste macronutrients and chemicals, and inactive microbes. This conceptual understanding can be used to understand the influence of macronutrients on process performance (i.e. larval development time, weight and composition) and the influence and fate of microbes and chemicals. This understanding can then be used to develop solutions to reduce the variability in the process performance of heterogeneous biowaste and ensure product safety.

Carbohydrates

Biowaste is mostly comprised of carbohydrates (Figure 1.1). In BSFL treatment, carbohydrates are decomposed by various enzymatic processes in the larvae and biowaste. As shown in Figure 1.2, carbohydrates are ingested by fly larvae, or following hydrolysis by microbes resulting in the production of simple sugars, organic acids, and other metabolites. In the midgut, these compounds and certain microbes are decomposed into monomers, used by microbes, or absorbed by the gut cells for use in the larval metabolism.

Results of this review suggest that biowaste carbohydrates influence the BSFL lipid content. On low-protein and high-carbohydrate diets, carbohydrates are converted by larvae into lipids and stored in the fat body (Handke et al., 2013; Pimentel et al., 2017; 2002). Thus, BSFL produced on low protein and high-carbohydrate diets are typically higher in lipids in comparison to BSFL produced on diets more balanced in carbohydrates and protein (Barragán-Fonseca, 2018a). For example, Spranghers et al. (2017) produced BSFL prepupae with 37% lipids on vegetable waste with 45% carbohydrates in comparison to 21% lipids for digestate with 7% carbohydrates. Jucker et al. (2017) reported a higher BSFL lipid content for fruit waste than vegetable waste, which is lower in carbohydrates (Figure 1.1). Tinder et al. (2017) reported a higher energy content for BSFL prepupae produced on biowaste higher in carbohydrates and lower in protein. As lipids are higher in energy than proteins, this can indicate an increase in BSFL lipids.

Fibres

It is likely that BSFL do not have enzymes for the decomposition of fibres. However, microbes in the larval gut and biowaste can hydrolyse them and make the nutrients available for larval development. As shown in Figure 1.2, carbohydrates, simple sugars and other metabolites result from the hydrolysis of fibres. As fibres are a large and chemically diverse group of biowaste constituents with variable decomposition processes and a continuum to carbohydrates, groups of fibres that can be decomposed by BSFL still have to be established. This area of research could be quite fruitful if methods were developed to either allow BSFL to consume cellulose degradation products after microbial decomposition, or if BSFL were found to contain cellulose degrading microbes that could be amplified to allow more efficient use of nutrients locked in the cellulose matrix.

Barragán-Fonseca (2018a) demonstrated that one key driver for high BSFL treatment process performance is the diet protein and carbohydrate content. Because fibres are indigestible, large amounts of fibres can reduce the process performance by reducing the overall nutrient density for BSFL development. This could be one contribution to the lower dry weight waste reduction and longer development time of biowaste high in fibres, such as animal manures (Table 1.2, Figure 1.1). Treatment of biowaste before BSFL treatment (i.e., pre-treatment) or inoculation at the beginning of BSFL treatment with microbes (i.e., co-conversion) that can degrade fibres, offer important future research possibilities to improve BSFL process performance (Zheng et al., 2012). In addition, future biowaste characterization should consider the function of different carbohydrates (e.g. starch, sugars, available carbohydrates) for BSFL (Barragán-Fonseca, 2018a).

Proteins

Protein has been identified as the most important biowaste macronutrient influencing BSFL process performance (Nguyen et al., 2013; Oonincx et al., 2015; Tinder et al., 2017). Proteins in biowaste deliver amino acids for larval development. As shown in Figure 1.2, amino acids are ingested as proteins, (poly)peptides or amino acids by fly larvae (Chapman, 2013). Proteolytic processes of microbes in the biowaste before or during BSFL treatment could also convert biowaste proteins into non-protein nitrogen, such as ammonia and thereby reduce the pool of amino acids. In the midgut, proteins and amino acids are decomposed by peptidases and absorbed by the gut cells. In the presence of sufficient amounts of amino acids, insulin-like hormones that trigger larval development are released. On low-protein diets, gut microbes can signal larval development by the production of metabolites from carbohydrates and increase the amount of amino acids that can be extracted from the diet. These results were produced with mono-associated FFL on low-protein diets and should be validated by future research with BSFL.

This understanding suggests that when operating with a variety of biowaste, the quantity of protein in the diet is a key parameter that drives larval development. Formulating diets with variable protein contents to a standard protein content could be a key driver for an efficient and reliable process performance (Barragán-Fonseca, 2018a). Previous research identified that in general, BSFL grown on biowaste higher in protein have a higher larval weight, bioconversion rate, feed conversion rate and larval protein, and a lower developmental time and lipid content (Nguyen et al., 2013; Oonincx et al., 2015; Tinder et al., 2017). BSFL grown on biowaste lower in protein have a higher developmental time, are smaller and have more lipids (if the biowaste includes high amounts of carbohydrates) (Jucker et al., 2017).

Barragán-Fonseca (2018a) and Cammack and Tomberlin (2017) worked with artificial diets and only varied the amount and ratio of protein and carbohydrates. This research identified that both the quality, quantity and ratio of protein to carbohydrate is important for process performance and that there this a trade-off between development time, and larval weight and composition. The authors concluded that development time was shorter on balanced or protein-biased diets with protein:carbohydrate ratios in the order of 1:1-4:1. In contrast, larval weight was higher and fat content lower on carbohydrate-biased diets with protein:carbohydrate ratios of 1:2-1:4 (Barragán-Fonseca, 2018a). Barragán-Fonseca (2018a) demonstrated that diets high in protein can also produce larvae with a high lipid content, and thereby reduce the larval protein content.

Current biowaste characterisation before BSFL treatment does not reflect the biological function protein has for BSFL. Biowaste protein is frequently estimated through determination of biowaste nitrogen and multiplication with a conversion factor. This method overestimates the protein content of biowaste that include significant amounts of non-protein, nitrogenous constituents such as ammonia, nitrate and nitrite (Chen et al., 2017). For example, based on complete amino acid analysis, Chen et al. (2017) determined a conversion factor of nitrogen to protein of 4.78-5.36 for different animal manures, compared to 6.25, which is the typically used factor. This could be one explanation for the lower process performance of animal manures in comparison to other biowaste (Table 1.2).

After the quantity of protein, protein quality (including amino acid composition and digestibility) could be a key parameter driving larval development (Barragán-Fonseca, 2018a). The comparative slaughter technique (i.e. comparison of the amino acid composition of the diet to the amino acid composition of the animal following slaughtering) previously used to determine energy requirements of livestock, but also proposed for insects, can provide preliminary information on how well the amino acid requirements of BSFL are met by biowaste (Cohen, 2005; McDonald et al., 2011). Figure 1.3 shows the composition of essential amino acids in biowaste protein, next to BSFL prepupae. It shows the quality of proteins of different biowaste types in comparison to the essential requirements of BSFL. It shows that fruit and vegetables are low in essential amino acids. In combination with their low protein content (Figure 1.1), this could contribute to a lower developmental time of BSFL grown on fruit and vegetable wastes (Table 1.2) (Jucker et al., 2017). Millings and brewery side streams, municipal organic wastes and animal manures have a more similar amino acid composition to BSFL. Interestingly, poultry feed which has the lowest developmental time for BSFL (Table 1.2) has the most similar amino acid composition to BSFL. Thus, formulations of biowaste with different amino acids that complement each other could increase process performance beyond simply the mean performance of the different biowaste types (McDonald et al., 2011). The comparative slaughter technique is an oversimplification for BSFL treatment, as microbes in the biowaste and larval gut can be additional consumers or producers of amino acids.

The digestibility of protein could be an additional future parameter to account for different protein qualities of biowaste. Protein quality in feeds for livestock is frequently determined *in vitro* using pepsin and hydrochloric acid (McDonald et al., 2011). However, corrective equations to account for shortcomings of this method have not yet been established for fly larvae. Digestibility of proteins can be both positively and negatively influenced by biowaste processing such as size reduction or heat treatment that also occur during biowaste production or management (Cohen, 2005; McDonald et al., 2011).

Amino acid composition results for BSFL in Figure 1.3 suggest that biowaste protein and amino acids have a smaller influence on the BSFL amino acid composition than biowaste fatty acids on BSFL fatty acids. Spranghers et al. (2017) and Liland et al. (2017) determined the amino acids profile of BSFL produced on different diets. The standard deviation of BSFL essential amino acids between diets ranged from 2-21%. This is much lower than the influence of the diet on the BSFL fatty acid composition which can exceed 100% (Liland et al., 2017; Oonincx et al., 2015; Spranghers et al., 2017).

Lipids

Lipids are typically a minor constituent of biowaste (Table 1.2). As shown in Figure 1.2, biowaste lipids are decomposed in the biowaste and/or larval gut into free fatty acids or mono- and diglycerides for absorption by the gut cells and use in the larval metabolism.

Research for BSFL suggest that lipids do not likely limit larval development, unless provided in excess. When changing both protein and lipids in different diets, Oonincx et al. (2015) reported that protein had a larger influence on larval development than lipids. In addition, BSFL on a low-lipid diet were higher in lipids, potentially as this diet was higher in carbohydrates. Excess lipids such as in restaurant waste have been suspected to decrease larval development (Nguyen et al., 2013).

In contrast to carbohydrates and proteins that are decomposed into monomers for use in completely new body molecules, the composition of biowaste lipids directly influences the BSFL fatty acid composition. Carvalho et al. (2012) demonstrated that the diet lipid composition directly influenced the FFL fatty acid composition. Plant-based diets that are high in long unsaturated fatty acids produced FFL phospholipids, that are longer and more unsaturated, in comparison to diets depleted in lipids. St-Hilaire et al. (2007) and Liland et al. (2017) confirmed this influence of diet lipid composition for BSFL. BSFL content of omega-3 fatty acids increased when fish offal and seaweed that are high in these fatty acids were formulated into the diet.

Microbes

Biowaste used in BSFL treatment typically have a high number and diversity of microbes. As presented in this review, and shown in Figure 1.2, microbes in the biowaste and larval gut have multiple functions that are important for the BSFL process performance. In the biowaste, microbes are important for the hydrolysis of biowaste macromolecules, especially fibres that typically cannot be decomposed by BSFL. Following ingestion, microbes are selectively inactivated by gut pH and by enzymes and antimicrobial proteins and used by larvae as food providing additional nutrients than those found in diet molecules. Microbes that survive these processes are potential candidates for gut microbes that can contribute to larval development. Gut microbes are also excreted together with antimicrobial proteins into the biowaste. Over time, BSFL change the microbial diversity in the biowaste.

BSFL are able to reduce microbes that can be feed or food pathogens. However, these antimicrobial processes in the larval gut and biowaste are yet poorly understood. They depend on several factors including the composition of microbes, nutrient availability and composition, and operating parameters such as temperature and pH. In BSFL, *E. coli* were reduced more at temperatures of 27°C and 32°C, than 23°C, and in poultry manure compared to hog manure (Erickson et al., 2004; Liu et al., 2008). De Smet et al. (2018) suggest the lack of reduction in hog manure is due to the reduced stability of antimicrobial proteins produced by BSFL, due to the lower pH compared to poultry manure. Vogel et al. (2018) suggest that BSFL adjust their antimicrobial proteins based on their diet. Similar to FFL, research for BSFL suggest that different mechanisms exist for gram-negative and gram-positive bacteria, which could explain the survival of E. faecalis in BSFL, a common gram-positive gut bacteria (Lalander et al., 2016; Lemaitre and Miguel-Aliaga, 2013; Zdybicka-Barabas et al., 2017). These results suggest complex and dynamic processes for inactivation of food and feed pathogens, including spores that likely also survive the gut passage (Coluccio et al., 2008). Thus, heat or other treatments for microbial inactivation after BSFL harvest, are crucial to ensure food and feed safety (Schlüter et al., 2017; Van der Fels-Klerx et al., 2018).
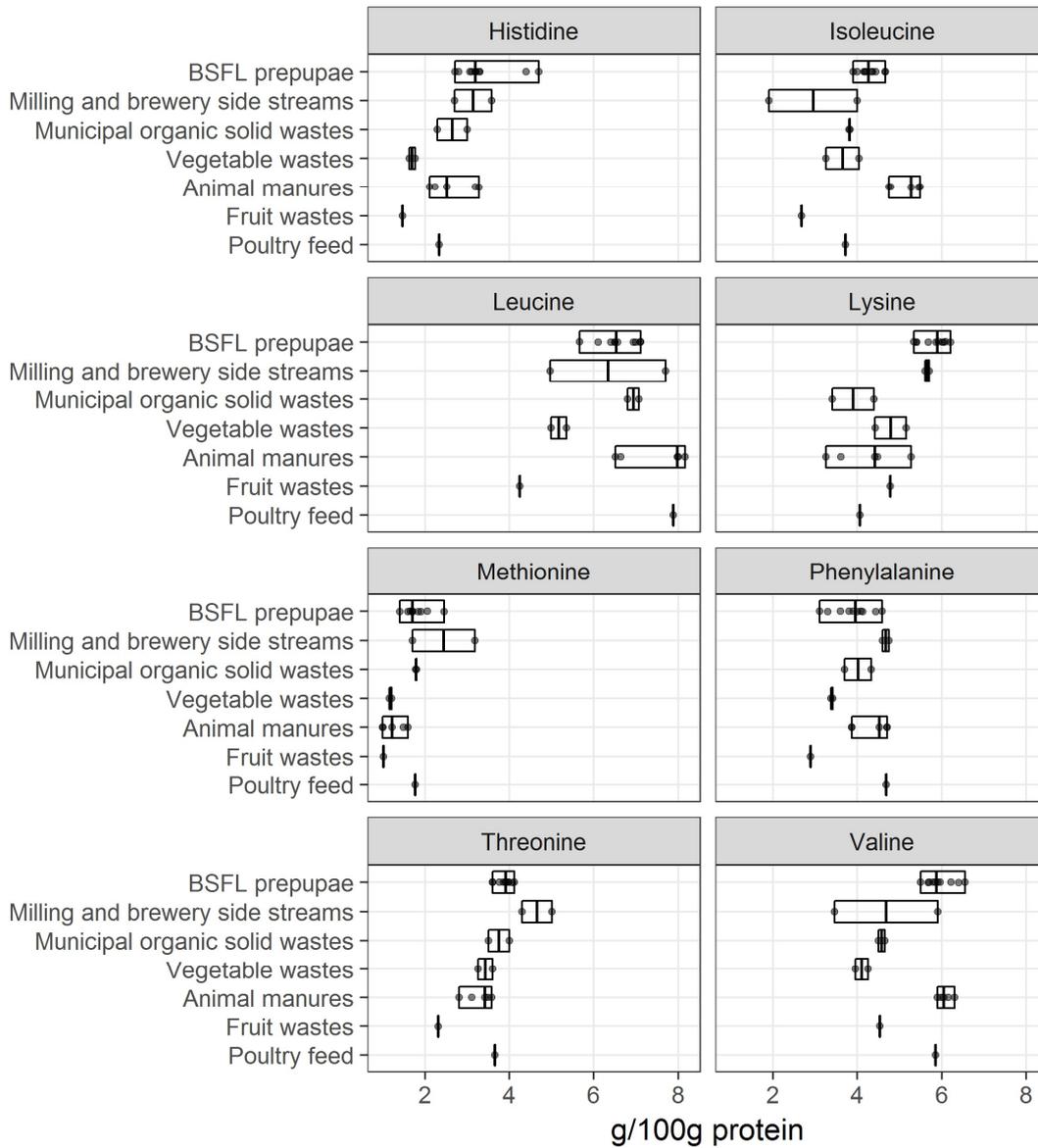
Figure 1.3 Essential amino acids (in g/100 g protein) in BSFL prepupae produced on different biowastes and in biowastes typically used in BSFL treatment. Results for fruit and vegetable waste were calculated based on Jucker et al. (2017) with data from the USDA Food and Nutrition Information Center (https://www.nal.usda.gov) (Attia and Abou-Gharbia, 2011; Bosch et al., 2014; Chen et al., 2017; De Marco et al., 2015; Liland et al., 2017; Myer et al., 2000; Spranghers et al., 2017; St-Hilaire et al., 2007; Tschirner and Simon, 2015).

Selected microbes in BSFL treatment have the potential to result in a large increase in process performance. Two studies with BSFL demonstrated that co-conversion of biowaste with *B. subtilis*, isolated from the larval gut, can increase the process performance. Addition of *B. subtilis* to poultry manure increased BSFL weight by 9-22%, bioconversion rate by 13% and waste reduction by 13% compared to BSFL without *B. subtilis* addition (Xiao et al., 2018; Yu et al., 2011). Authors of these studies concluded that *B. subtilis* decomposes biowaste molecules that cannot be decomposed by BSFL, such as fibres. On low protein diets, *A. pomorum* and *L. plantarum* in FFL guts promoted the development of larvae mono associated with these bacteria (Shin et al., 2011; Storelli et al., 2011). As these bacteria boosted larval development and are easy to cultivate, they are also two interesting candidates for BSFL treatment.

Chemicals

Based on the results of this review, BSFL have mechanisms for the decomposition of biowaste chemicals that are supported by microbes present in the biowaste and gut. Pharmaceuticals, pesticides and mycotoxins are decomposed into different forms by microbial action in the biowaste and enzymes in the larval gut. Reduction rates for these compounds are similar to those determined for composting. However, more research is required as pharmaceuticals and pesticides include enormous and heterogeneous groups of compounds with different properties influencing their decomposition. Also, the influence of potential degradation products of chemicals on animal health has not been investigated. In addition, whether antibiotics, nowadays commonly used for livestock (McDonald et al., 2011), alter microbial numbers and diversity (which is important for many processes in BSFL treatment, and thereby reduces the process performance, requires further research.

In contrast to pharmaceuticals, pesticides and mycotoxins, heavy metals are sequestered in the larval midgut from the biowaste, with transporters or proteins and stored in the larval body. Thus, protecting biowaste from high heavy metal contaminations is currently the only way to prevent heavy metals in BSFL and in the residue.

1.12 Summary and Outlook

BSFL biowaste processing is an emerging biowaste treatment technology which can produce marketable high-value products that can contribute towards sustainable and financially viable resource recovery-based waste management systems. One challenge of the technology includes an efficient operation of BSFL treatment and safe larval and residue production with several biowaste streams that have variable characteristics.

Research on the processes in BSFL treatment are in its infancy. Knowledge for different well studied fly larvae summarized in this review are consistent between fly species and to the current knowledge on BSFL. Thereby, this review is a starting point to increase the process understanding of this technology.

Numerous biowaste characteristics that are frequently intertwined and microbes in the biowaste and larval gut likely contribute to the process performance. This creates a challenge in predicting BSFL treatment process performance and product safety. Processes that drive process performance are dynamic, based on larval age/size, biowaste macromolecule composition and microbial numbers and diversity.

Especially protein and digestible carbohydrate quantity, quality and their ratio appear to be important for process performance. Future research should include biowaste characterization that reflects the biological function of nutrients in the larvae. Following, formulating biowaste with a similar protein/carbohydrate content, balanced in amino acids and different types of carbohydrates has the potential to increase the efficiency and reliability of the process performance.

Recent research started exploring the manifold roles that microbes play in BSFL treatment. Pre- and co-treatment of biowaste with beneficial microbes has a large potential to increase process performance, for example to reliably decompose fibres that can comprise a significant fraction of biowaste. However, BSFL treatment operators are yet hesitant, considering that microbes could also be pathogenic for animals and need to be inactivated to ensure microbial product safety.

Acknowledgments

2. Chapter – Biowaste treatment with black soldier fly larvae: increasing performance through the formulation of biowastes based on protein and carbohydrates

Moritz Gold[1,2], Cecille Marie Cassar[1,2,3], Christian Zurbrügg[2], Michael Kreuzer[d], Samy Boulos[5], Stefan Diener[6], Alexander Mathys[1]

[1]ETH Zurich: Swiss Federal Institute of Technology Zurich, Institute of Food, Nutrition and Health, Laboratory of Sustainable Food Processing, Schmelzbergstrasse 9, 8092 Zurich, Switzerland
[2]Eawag: Swiss Federal Institute of Aquatic Science and Technology, Sandec: Department Sanitation, Water and Solid Water for Development, Überlandstrasse 133, 8600 Dübendorf, Switzerland
[3]University of Stuttgart: Institute for Sanitary Engineering, Water Quality and Solid Waste Management, Chair of Waste Management and Emissions, Bandtäle 2, 70569 Stuttgart, Germany
[4]ETH Zurich: Swiss Federal Institute of Technology Zurich, Institute of Agricultural Sciences, Animal Nutrition, Universitätstrasse 2, 8092 Zurich, Switzerland
[5]ETH Zurich: Swiss Federal Institute of Technology Zurich, Institute of Food, Nutrition and Health, Laboratory of Food Biochemistry, Schmelzbergstrasse 9, 8092 Zurich, Switzerland
[6]Biovision Foundation, Heinrichstrasse 147, 8005 Zurich, Switzerland

2.1 Abstract

A key challenge for black soldier fly larvae (BSFL) treatment is its variable reliability and efficiency when applied to different biowastes. Similar to other biowaste treatment technologies, co-conversion could compensate for variability in the composition of biowastes. Using detailed nutrient analyses, this study assessed whether mixing biowastes to similar protein and non-fibre carbohydrate (NFC) contents increased the performance and reduced the variability of BSFL treatment in comparison to the treatment of individual wastes. The biowastes examined were mill by-products, human faeces, poultry slaughterhouse waste, cow manure, and canteen waste. Biowaste formulations had a protein-to-NFC ratio of 1:1, a protein content of 14-19%, and a NFC content of 13-15% (dry mass). Performance parameters that were assessed included survival and bioconversion rate, waste reduction, and protein conversion efficiency. In comparison to poultry feed, vegetable canteen waste showed the best performance and cow manure performed worst. Formulations showed significantly improved performance and lower variability in comparison to the individual wastes. However, variability in performance was higher than expected for the formulations. One reason for this variability could be different fibre and lipid contents, which correlated with the performance results of the formulations. Overall, this research provides baseline knowledge and guidance on how BSFL treatment facilities may systematically operate using biowastes of varying types and compositions.

2.2 Introduction

The treatment of biowaste by black soldier fly larvae (BSFL) is an emerging waste management technology (Čičková et al., 2015; Gold et al. 2018a; Zurbrügg et al., 2018). This process converts waste into larval biomass, reduces waste dry mass and generates the raw materials for the production of soil conditioner and fertilizer (Setti et al., 2019), lubricants and biodiesel (Leong et al., 2016; Li et al., 2011), pharmaceuticals (Vilcinskas, 2013) and animal feeds (Barragán-Fonseca et al., 2017; Makkar et al., 2014; Sánchez-Muros et al., 2014; Wang and Shelomi, 2017).

A key challenge for BSFL biowaste treatment is its variable reliability and efficiency. Currently, performance—as measured by bioconversion rate, larval weight, and larval biomass composition (e.g. protein and lipid content)—varies both when using the same type of biowaste (e.g. different vegetable wastes) and when treating different types (e.g. vegetable waste compared to mill by-products) (as summarised by Gold et al., 2018a). The sustainable operation of BSFL biowaste treatment facilities likely depends on the use of different waste types of varying quantity and composition. Homogenous or highly nutritious biowastes such as food industry by-products (e.g. bread and mill by-products) or canteen and restaurant wastes are often already used elsewhere (e.g. as animal feeds and for energy recovery). In addition, poor waste management practices, such as a lack of organic waste segregation, incentives for landfill disposal, and complex collection and transport logistics, often hinder access to high-quality wastes. Importantly, the use of different wastes from different sources will adversely affect the day-to-day operation (e.g. running over or under capacity) of BSFL treatment facilities with concurrent impacts on BSFL growth and waste treatment performance. This affects the sustainability (Mertenat et al., 2019; Smetana et al., 2019, 2016) and scalability of this technology and the down-stream application of products (e.g. live-feed for aquaculture) (Gold et al. 2018a).

Similar to other animal species used for production, the nutrient content of biowaste is hypothesised to have the largest influence on performance under similar operating conditions (e.g. feeding rate, larval

density and temperature) (Nguyen et al., 2013; Oonincx et al., 2015; Tinder et al., 2017). Factors determining the nutritional quality of biowaste include the density, ratio and type of nutrients it contains. Nutrients considered to be decisive include the sum of all macronutrients, organic matter, proteins, non-fibre carbohydrates (NFC), fibre and lipids (Barragán-Fonseca et al. 2018b, 2018c; Gold et al. 2018a; Lalander et al., 2019). For example, manures are typically low in organic matter and fibre, restaurant and canteen wastes are rich in NFC and lipids, and fruit and vegetable wastes are low in proteins (Gold et al. 2018a). In response to these different nutritional conditions, fly larvae adjust their growth rate and nutrient accretion, with the main goal of accumulating enough reserves to complete the non-feeding life-stages of metamorphosis and adulthood (Danielsen et al., 2013; Gold et al. 2018a). Similar to other animals, an insufficient amount or an unfavourable ratio of nutrients prolongs development, reduces growth and related biomass production, and limits the efficiency of waste reduction (Danielsen et al., 2013).

BSFL feeding experiments and assessments of the midgut (the main organ involved in digestion) suggest that protein, NFC and lipids are highly digestible by BSFL and, therefore, their supply enhances performance (Barragán-Fonseca et al. 2018c; Beniers and Graham, 2019; Bonelli et al., 2019; Lalander et al., 2019). In contrast, fibre including cellulose and lignin are less digestible and tend to decrease larval growth rates (Liu et al., 2018). Among these nutrients, several studies have concluded that the protein (and amino acid) content of biowastes is most important. For example, Lalander et al. (2018) concluded that protein has the greatest impact on the development time to prepupa. Beniers and Graham (2019) also observed that protein has greater importance for larval weight than NFC. As amino acids allow larvae to proceed to the next instar (Gold et al. 2018a) and BSFL accumulate lipids during later instars (as energy reserves for later life-stages) (Liu et al., 2017), waste with a greater protein content can also increase larval lipid content. Research on the common fruit fly larvae (*Drosophila melanogaster*) indicates that fly larvae control their feeding with respect to protein and may overfeed on other nutrients such as NFC (Almeida de Carvalho and Mirth, 2017). This further emphasises that protein is so essential for development. For BSFL, protein supply may influence larval weight and lipid content when receiving low-protein and high-carbohydrate feeds as carbohydrates may be converted into body lipids (Pimentel et al., 2017; Spranghers et al. 2017). By contrast, lipids in biowaste can impede or promote larval development. Nguyen et al. (2013) suspected that excess lipids in fish offal may decrease larval development; however, results from Oonincx et al. (2015a) for food industry by-products and from Nguyen et al. (2013) for liver and kitchen waste suggest that lipids can also increase performance as the energy density of the feed is increased (Brouwer, 1995). Oonincx et al. (2015a) also observed high feed-conversion efficiencies for feeds with a high lipid and protein content. The ash content of biowaste positively correlates with larval ash content (Spranghers et al. 2017) and negatively with bioconversion rates (Lalander et al., 2019).

A reliable high-performance BSFL treatment for biowaste requires strategies that build on existing knowledge of the influence of variable waste nutrient compositions on larval performance. Similar to other biowaste treatment options such as anaerobic digestion or composting, co-treatment, i.e. the treatment of a mixture of several biowastes, could increase performance and reduce variability (Li et al., 2009). Specifically, mixing multiple biowastes can provide a more nutritious and balanced feed for larval growth. Rehman et al. (2017) and Nyakeri et al. (2019) observed that mixing cow and human manure with food wastes and food production by-products (e.g. soybean curd residue and banana peels) increased larval weight compared to these individual wastes. Similar to composting (i.e. carbon-to-

nitrogen ratio) and anaerobic digestion (e.g. methane potential), a systematic approach to co-conversion based on biowaste nutrients could compensate for the variability in biowaste composition.

The formulation of appropriate biowaste mixtures based on nutrients requires the reliable determination of composition using parameters that are relevant for BSFL growth. Such an approach, and also incorporating cost considerations, is widely applied for feed formulation in commercial livestock production (McDonald et al., 2011). Barragán-Fonseca et al. (2018b) were the first to formulate feeds for BSFL with similar protein and NFC contents using combinations of food industry by-products; however, larvae still performed significantly different between these waste mixtures. These authors concluded that nutrient quality (e.g. amino acid content, type of NFC and fibre) must be considered to reduce this variability. Previous studies have not considered this sufficiently when determining biowaste composition. For example, biowaste has been characterised based on its carbon, nitrogen and protein content (using generic nitrogen-to-protein conversion factors) or the sum of other nutrients to estimate its NFC content (Barragán-Fonseca et al. 2018b; Lalander et al., 2019). Nitrogen may not, however, be an accurate measure as it may also include non-protein nitrogenous compounds of low nutritional value such as urea, ammonia, nitrate and nitrite (Chen et al., 2017). Similarly, carbon includes less digestible fibre such as cellulose and lignin. The importance of these compounds is difficult to assess as the extent to which non-protein nitrogen and fibre are used by the gut microbes of BSFL is not yet known. Thus, generic nitrogen-to-protein conversion factors may overestimate protein content. In addition, carbon may greatly overestimate NFC when the ash, lipid, protein and fibre content is subtracted from 100% rather than the sum of digestible carbohydrates such as glucose and starch.

This study aimed to assess the performance of BSFL treatment as applied to different waste formulations prepared from six types of biowaste following the determination of their respective nutritional composition. It was hypothesised that biowaste formulations with a similar protein and NFC content would increase performance and reduce variability in comparison to the individual wastes. Thereby, this research sought to generate knowledge and advice on how BSFL treatment facilities may best operate with biowaste of varying type and composition.

2.3 Materials and Methods

Biowastes used in the feeding experiments

Six different types of biowaste were used in feeding experiments, namely mill by-products, human faeces, poultry slaughterhouse waste, cow manure, and canteen and vegetable canteen waste. Two different batches of human faeces were used as BSFL treatment performance was unexpectedly high with the first batch. Mill by-products were obtained from a Swiss wheat-milling company. The human faeces were obtained from dry toilets separating urine and faeces at the Swiss Federal Institute of Aquatic Science and Technology (Eawag) in Dübendorf, Switzerland. The poultry slaughterhouse waste consisted of discarded body parts (feet, head, liver, stomach, and intestine) from a poultry slaughterhouse of Micarna, a leading meat processing company in Switzerland. The cow manure was obtained from a farm near Zurich, Switzerland. The vegetable waste was obtained from the Eawag canteen and consisted of a mixture of vegetables with and without salad dressing. The difference between the vegetable canteen waste and the canteen waste was that the latter had the addition of sausage and other meat offal.

Following their collection, the wastes were homogenised with a kitchen blender to mimic the pre-treatments used in BSFL treatment facilities (Dortmans et al., 2017), and moisture content was determined in duplicate with a halogen moisture analyser (BM-65, Phoenix instrument, Garbsen, Germany) The wastes were then portioned into plastic bags, frozen and stored at -20°C until the start of the feeding experiment (Diener et al., 2009; Lalander et al., 2019; Myers et al., 2008; Nguyen et al., 2015). The wastes were thawed at 4°C for 24 h and brought to the experimental temperature of 28°C prior to each feeding experiment.

Composition of the experimental biowastes

Oven-dried (105°C) wastes and poultry feed (used as a high-performance benchmark) were analysed for gross nutrient composition, moisture content and pH using standard procedures (AOAC 1997; Van Soest et al., 1991). The second batch of human faeces was only analysed for protein, lipid and organic matter content. Moisture and organic matter were determined in quintuplicate with an automatic thermogravimetric determinator (TGA-701, Leco, St. Joseph, MI, USA). Nitrogen content was determined in triplicate using a C/N analyser (Type TruMac CN, Leco Cooperation, St. Joseph, MI, USA). Fibre fractions including neutral (NDF) and acid detergent fibre (ADF) were assessed in duplicate using a fibrebag system (Fibretherm, Gerhardt Analytical Systems, Germany) according to methods 6.5.1 and 6.5.2 of the Association of German Agricultural Analytic and Research Institutes (Naumann et al., 2012). Lipids were analysed from ether extracts from freeze-dried samples by Eurofins Scientific, Schönenwerd, Switzerland, according to Regulation (EC) No 152/2009 (European Commission (EC) 2009). The extraction solvent used was petroleum ether at 40–60°C following hydrolysis with 3 M hydrochloric acid. pH was analysed with a portable meter and pH probe (HQ40d, Hach Lange GmbH, Switzerland).

Amino acids were analysed in triplicate in freeze-dried samples (Çevikkalp et al., 2016; Kwanyuen and Burton, 2010; White et al., 1986; Zhang et al., 2009). The samples were hydrolysed at 110°C for 16-24h with 5 M sodium hydroxide (tryptophan) or 6 M hydrochloric acid containing 0.1% phenol (for all other amino acids). For tryptophan, the hydrolysed samples were subsequently neutralised, diluted and analysed by RP-HPLC-FLD using an Agilent 1200 series LC-system including a fluorescent detector (FLD) (Agilent Technologies, Santa Clara, USA) and a C18 analytical Pico Tag amino acid analysis column (3.9 x 150mm) in combination with a Nova-Pak C18 guard column (3.9 x 20mm) (Waters AG, Baden, Switzerland). The fluorescence detector was operated at an excitation wavelength of 280 nm and an emission wavelength of 340 nm. For all other amino acids, the hydrolysed amino acids were transformed into their phenylthiocarbamyl derivatives with phenyl isocyanate and analysed by RP-HPLC using an Agilent 1100 series LC-system including a diode array detector (DAD) operated at 254 nm (Agilent Technologies, Santa Clara, USA) and the same column as above. α-methyl-DL-tryptophan and L-Norleucine was used as an internal standard. The HPLC results were corrected with the respective recovery rates of the internal standards. Only results with an internal standard recovery >70% were considered further. More details on amino acid analyses are included in the Supplementary Material.

Glucose and starch were determined in triplicate using freeze-dried samples with a commercial enzyme assay (Megazyme, 2019). In brief, glucose was removed from each sample with ethanol. Then, following centrifugation, the glucose concentration was determined in the supernatant and the pellet was used for starch analysis. Resistant starch was converted into maltodextrins in potassium hydroxide. Amylase

and amyloglucosidase were used to hydrolyse the remaining starch into glucose. Glucose was then quantified with a spectrometer (Genesys 10S, Thermo Fisher Scientific, USA) in comparison to a glucose standard.

Protein was calculated by multiplication of the nitrogen results with waste-specific conversion factors, namely 5.6 for poultry feed (based on results for maize and soybean meal) (Sriperm et al., 2011), 4.3 for cow manure (Chen et al., 2017), 5.4 for mill by-products (based on results for cereals) (Mariotti et al., 2008), 5.4 for canteen waste, 5.0 for vegetable canteen waste, and 5.0 for poultry slaughterhouse waste (based on results for meat, fish, cereals and vegetables) (Mariotti et al., 2008). No conversion factors were available for human faeces and so this was estimated as the ratio of the sum of all amino acids divided by the nitrogen content. Samples of human faeces (mixed with sawdust) (Nyakeri et al., 2019) and pit latrine sludge provided by Sanergy, Nairobi, Kenya, were also included in the analysis to cover the typical variability of human faeces and faecal sludge (Gold et al., 2017b, 2017a). Caloric content was estimated by multiplying the mean results for lipids, NFC and protein with their gross caloric content of 9.4, 5.4, and 4.1 kcal/g, respectively (Merrill, 1973; Wu, 2016). Hemicelluloses were determined as the difference between NDF and ADF. ADF was assumed to be a reliable estimate of cellulose and lignin content. The sum of glucose and starch was assumed to reflect the total NFC.

Formulation of the biowaste mixtures

For the feeding experiments, either the six individual wastes or six mixtures of the wastes (Table 2.1) were used. The mixtures were based on the composition of the biowastes and aimed to achieve a protein-to-NFC ratio of approximately 1:1 (DM) considering the low content of NFC (Barragán-Fonseca et al. 2018b; Cammack and Tomberlin, 2017). In contrast to Barragán-Fonseca et al. (2018b), no high-value ingredients such as sunflower oil or cellulose were added to balance the unavoidable variability in fibre and lipid content as this is not typically practicable for cost reasons.

Table 2.1 Dry mass proportion of individual wastes in the biowaste formulations (F1-F6).

Formulation	F1	F2	F3	F4	F5	F6
Mill by-products	23	37	51	60	33	65
Canteen waste	-	7	-	20	33	-
Human faeces	16	-	14	20	-	-
Poultry slaughterhouse waste	-	-	-	-	-	22
Cow manure	11	35	34	-	-	12
Vegetable canteen waste	50	21	-	-	33	-

The formulations were generated using Visual Basic for Applications in Microsoft Excel and were always based on mill by-products complemented with two to three other wastes. The formulations were prepared from thawed wastes on the day of feeding and were mixed thoroughly. Formulation 3 was prepared with two different batches of human faeces based on the nutrient composition of the first batch. These batches appeared to have a similar composition based on their lipid (20.9 and 19.3% DM), crude protein (20.5 and 21.8% DM) and ash (13.7 and 15.8% DM) content. In the following discussions, the two human faeces formulations are referred to as formulation F3 (1) and formulation F3 (2), respectively.

Table 2.2 shows the realised nutrient composition of the six formulations, calculated based on the proportions shown in Table 1 and the results of the analyses of the individual biowastes. The formulations contained between 14 and 19% DM of protein and between 13 and 15% DM of NFC.

Table 2.2 Mean dry mass nutrient contents of the different biowaste formulations (F1-F6) based on the percent dry mass proportion of individual wastes in the biowaste formulations (Table 2.1) and the composition of their constituent wastes (Table 2.3).

Formulation	Proteins	Non-fibre carbohydrates	Fibres	Lipids	Organic matter	Moisture content
F1	13.8	13.6	38.5	19.0	90.5	80.8
F2	14.0	13.0	48.7	11.2	88.9	81.5
F3*	14.0	12.7	50.1	5.9	88.4	79.9
F4	19.1	15.8	43.8	13.0	92.1	72.5
F5	19.6	15.4	39.8	22.3	93.1	76.9
F6	19.0	15.4	45.8	12.0	92.1	73.9
Mean	16.6 (2.9)	14.3(1.4)	44.5 (4.7)	13.9 (5.9)	90.7 (2.2)	77.6 (3.7)

in parentheses: standard deviation
*Formulation (3)

Feeding experiments

Feeding experiments were designed as outlined by Lalander et al. (2018) and Liu et al. (2018). Three individual sets of experiments with different batches of larvae were carried out. First with the individual wastes (experiment 1) and then with formulations 1 to 3 (experiment 2) and finally with formulations 4 to 6 (experiment 3). Larvae were obtained from the BSFL research colony at Eawag maintained according to Dortmans et al. (2017). The BSFL hatched within 24 h and were first fed *ad libitum* with poultry feed (UFA 625, UFA AG, Switzerland) for 12-14 d until they reached a mean individual weight of 3.8 ± 0.5 mg DM. The larvae had a similar content of carbon (55-56% DM), protein (36-38% DM) and ash (13-14% DM) across the experiments. From these populations, 4 to 5 × 80 randomly selected larvae per treatment were manually counted and placed in plastic containers (7.5 cm diameter, 11 cm height) with individual wastes or waste formulations, giving a larval density of approximately 2 larvae/cm². Larvae were also freeze-dried for the analysis of larval composition. The experimental containers were covered with paper towels or mosquito nets and randomly arranged in a climate chamber (HPP 260, Memmert GmbH, Germany) providing a steady microclimate of 28°C and 70% relative humidity. Feed was provided every 3 d. Considering the increase in the nutrient requirements of BSFL with growth (Nyakeri et al., 2019), the feeding rate was increased over the 9-day experiment from 15 to 25 and 40 mg DM/larva per day on days 0, 3 and 6, respectively. Due to the expected improved nutritional quality of the formulations, the feeding rate was lowered by 25% for each feeding in experiment two and three.

In contrast to previous studies, which have typically terminated experiments after the first appearance of prepupae (Bosch et al., 2019; Lalander et al., 2019), all experiments were terminated after 9 d, before the appearance of prepupae. Prepupae are richer in chitin and lipids and, therefore, not optimal for animal feed applications (Nyakeri et al., 2019). Larvae were manually separated from the residue, cleaned with tap water, and dried with paper towels. Subsequently, larvae were manually counted, weighed and freeze-dried. Residues were dried in a laboratory oven at 80°C. Both the dried larvae and the residues were then weighed and stored at 4°C.

Analysis of larval composition

The dried larvae were milled and treatment replicates were combined equally by mass. Samples were then analysed in triplicate for DM, carbon and nitrogen content using the same analyser as for the wastes. Larval protein content was calculated as the nitrogen content × 4.67 following Janssen et al. (2017). Carbon content was divided by the total amount of organic matter; as lipids typically contain more carbon than proteins and carbohydrates (Brouwer, 1995), the ratio of carbon-to-organic matter was used as an indicator of larval lipid content.

Determination of the performance of BSFL treatment

Larval counts, and residue and larvae dry weights, were used to calculate five BSFL performance parameters. First, larval survival rates were calculated using equation (1) as the ratio of larvae at the end (larvae$_{end}$) and the beginning (larvae$_{beg}$) of the experiments (Van Der Fels-Klerx et al., 2016).

$$\text{Survival rate (\%)} = \frac{\text{larvae}_{end}}{\text{larvae}_{beg}} \times 100 \tag{1}$$

Waste reduction was calculated using equation (2) as the ratio of residue dry mass (residue$_{mass}$) to the dry mass of total feed (feed$_{mass}$) provided (Diener et al., 2009):

$$\text{Waste reduction (\% DM)} = \left(1 - \frac{\text{residue}_{mass}\ (g)}{\text{feed}_{mass}\ (g)}\right) \times 100 \tag{2}$$

The bioconversion rate was calculated using equation (3), for which the larval dry weight gain (larval$_{gain}$) was calculated as the difference between the final larval dry weight and the initial larval dry weight multiplied by the number of larvae at the end of the experiment:

$$\text{Bioconversion rate (\% DM)} = \frac{\text{larvae}_{gain}\ (g)}{\text{feed}_{mass}\ (g)} \times 100 \tag{3}$$

Waste conversion efficiency (Liu et al., 2018), also called efficiency of conversion of ingested/digested food (Diener et al., 2009; Oonincx et al., 2015b), was calculated using equation (4):

$$\text{Waste conversion efficiency (\% DM)} = \frac{\text{larvae}_{gain}\ (g)}{\text{feed}_{mass}\ (g) - \text{residue}_{mass}\ (g)} \times 100 \tag{4}$$

Finally, the protein conversion efficiency was calculated using equation (5) as the ratio of the amount of larval protein accumulated (protein$_{gain}$) to feed provided (feed$_{mass}$). Larval protein accumulated was calculated as the difference between the amount of final larval protein and the initial larval protein multiplied by larvae$_{end}$. The amount of larval protein was calculated by multiplying the larval protein content with the larval weight:

$$\text{Protein conversion efficiency (\% DM)} = \frac{\text{protein}_{gain}(g)}{\text{feed}_{mass}\ (g)} \tag{5}$$

Performance benchmark

As in previous research, poultry feed (60% moisture content) was fed to larvae in parallel to the individual biowastes and biowaste formulations as a high-performance benchmark (Lalander et al., 2019). As shown in Figure 2.1, the results for poultry feed varied between experiments but no single

experiment stood out as being different across all of the performance parameters. Even though larvae had a similar weight and composition at the start of the experiment, variability between experiments could be due to differences in age, feeding rates or other confounding factors (e.g. differences in airflow in the climate chamber due to varying numbers of containers).
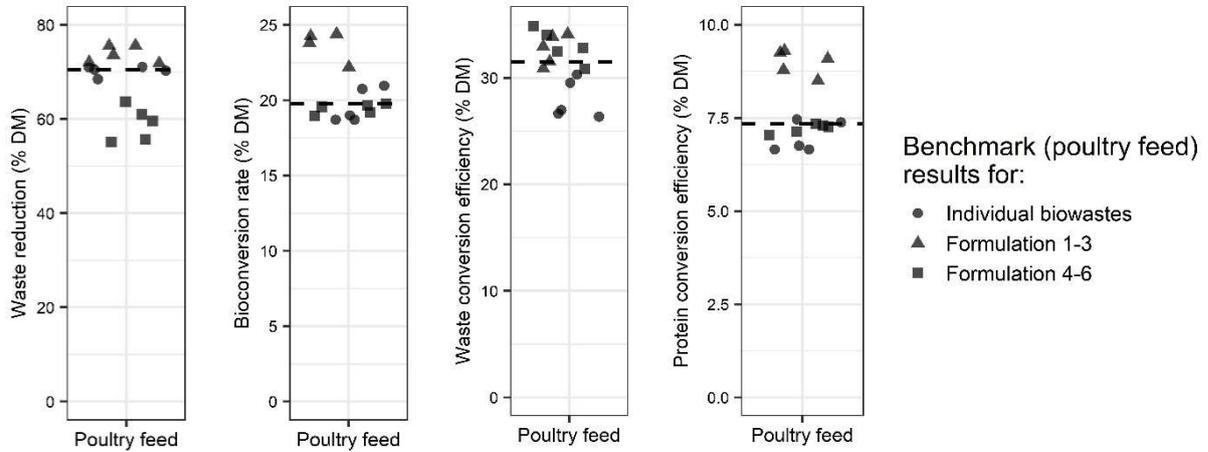
Figure 2.1 Performance of BSFL fed on poultry feed used a high-performance benchmark. Medians are shown as dashed lines.

To ensure a consistent basis for comparison between the three experiments, and between the individual wastes and waste formulations, performance parameters were also expressed as percentage differences (medians; cf. Figure 2.1) in comparison to the results for the poultry feed using equation (6). For this, the results for each performance parameter (Performance$_{treatment}$) were subtracted from the median result obtained using the poultry feed (performance$_{benchmarkl}$) over all three experiments:

$$\text{Performance in \% to benchmark} =$$

$$\frac{\text{performance}_{treatment} - \text{median performance}_{benchmark}}{\text{median performance}_{benchmark}} \times 100 \tag{6}$$

Even though all of the parameters were corrected by the mass of total feed provided, variations in feeding rate, which differed between experiments, could influence performance results. Therefore, poultry feed and mill by-products were also fed to larvae at two different feeding rates: 27 mg DM/larva per day as used in experiment 1 and 20 mg DM/larva per day as used in experiments 2 and 3. This comparison included in Supplementary Figure S1 did not reveal an influence of feeding rate on performance, thus justifying the comparisons across the experiments.

Data analyses and statistics

Data were analysed using R software and RStudio version 1.1.463 (RStudio Inc., Boston, MA, USA). The mean, median, standard deviation, and range (difference of maximum and minimum) of the biowaste composition and performance parameters were calculated. Significance of differences in mean nutrient composition and mean performance parameters between the individual wastes and the waste formulations were tested using analysis of variance (ANOVA) followed by pairwise Tukey post-hoc comparisons. Due to the small size sample size per group (n=3-5), normality and homogeneity of

variance were assessed visually by residuals versus fits and Q-Q plots (Ricci et al., 2019). These graphs are shown in Supplementary Material. A conservative p-value of < 0.01 was chosen to declare significance due to the small sample size per group, which could lead to misinterpretation of model assumptions. The Mann–Whitney U test ($p<0.05$) used to identify significant differences between the distributions of performance parameters of all the individual wastes (n=29) in comparison to all the waste formulations (n=29). The Levene and Shapiro-Wilk test ($p<0.05$) identified that the data in those two groups violated the assumptions for parametric tests. Following visual assessment of normality (Supplementary Material), Pearson correlation coefficients ($p<0.01$) were also calculated to identify linear dependencies between biowaste composition and feeding experiment results.

2.4   Results and Discussion

Biowaste composition

Nutrient composition varied significantly between the biowastes (Table 3). The results for amino acids are included in Supplementary Material. The biowastes showed large variability with respect to protein content, which was highest in poultry slaughterhouse waste, canteen waste and human faeces, and lowest in cow manure, vegetable canteen waste and mill by-products. Protein quality may also differ. In contrast to the other wastes, the protein in human faeces and cow manure was likely protein from gut microbial biomass (Rose et al., 2015).

This was the first study in which nitrogen-to-protein conversion factors were determined for human faeces and faecal sludge. Human faeces collected in Zurich and pit larine sludge collected in Nairobi had conversion factors of 3.9 and 3.8, respectively. These conversion factors are comparable to those for animal manures in the range of 2.8 to 4.3 (Chen et al., 2017). In comparison, human faeces collected in Nairobi had less non-protein nitrogen, with a conversion factor of 5.2. Potential reasons for this difference could be the differing diets between the residents of Zurich and Nairobi (Rose et al., 2015) or storage conditions (e.g. temperatures) leading to the volatilisation of nitrogen. Overall, these results confirm that multiplying nitrogen results with the generic factor of 6.25 (i.e. the inverse of the mean nitrogen content of protein) can greatly overestimate true protein (i.e. amino acid) content (Mariotti et al., 2008). This is well established but has not been implemented even in recent BSFL research (Lalander et al., 2019; Liu et al., 2018).

Wastes were low in glucose, starch and total NFC. NFC was highest in the mill by-products and vegetable canteen waste. The addition of meat to the vegetable canteen waste increased protein content from below 15 to over 30% DM but concurrently decreased NFC content by half. Cow manure, human faeces, and slaughterhouse waste had almost no NFC. This was expected as animal tissue contains only very small amounts of glycogen and most NFC is digested or fermented in the gut of humans and animals (Riesenfeld et al., 1980). The sum of glucose and starch was much lower than when NFC was calculated as the difference between DM and ash, protein, fibre, and lipids. For example, the calculated value for human faeces was 17% DM compared to 1.7% DM for glucose plus starch (Rose et al., 2015; Spranghers et al., 2017). This indicates that there are either large amounts of non-sugar-non-starch-non-fibre organic matter or that there is an accumulation of analytical error in the gross nutrient measurements, or both. Overall, in the present study, low NFC was the reason why the protein-to-carbohydrate ratios in the waste formulations did not exceed 1:1 and that the mean carbohydrate contents did not exceed 14% DM. The corresponding values described by Barragán-Fonseca et al. (2018b) and Cammack and Tomberlin (2017) were 1:1 to 1:2 and 21-30% DM, respectively.

Table 2.3 Mean nutrient composition of individual biowastes as percent dry mass, moisture content in percent, pH, and calorific content as kcal per 100 g dry biowaste.

Wastes	pH	Moisture content	Proteins	Non-fibre carbohydrates Total	Glucose	Starch	Fibre Total	Cellulose & lignin	Hemicellulose	Lipids	Organic matter	P:NFC* ratio	Caloric** content
Mill by-products	6.2 (0.1)	70.0	14.5[d] (0.3)	23.2[b] (0.2)	1.7[b] (0.0)	21.2[b] (0.6)	51.7 (0.9)	22.1 (1.0)	29.6 (1.9)	3.0	93.8[c] (1.3)	1:2	211
Canteen waste	4.3 (0.0)	74.0 (1.2)	32.2[b] (0.8)	7.5[d] (0.7)	3.5[a] (0.4)	4.0[d] (0.4)	36.2 (1.4)	22.8 (0.6)	13.4 (0.9)	34.9	93.0[c] (0.7)	4:1	501
Human faeces (1)	6.0 (0.0)	76.7 (0.9)	20.1[c] (0.9)	1.7[e] (0.1)	1.0[bc] (0.0)	0.7[e] (0.1)	27.9 (0.6)	19.5 (0.3)	8.4 (0.6)	20.9	86.4[b] (0.3)	12:1	288
Poultry slaughterhouse waste	5.7 (0.1)	66.7 (1.2)	37.3[a] (0.5)	0.3[e] (0.1)	0.2[d] (0.1)	0.1[e] (0.0)	20.8 (1.9)	9.3 (0.9)	11.5 (2.7)	42.9	94.0[c] (1.3)	152:1	557
Cow manure	7.2 (0.1)	87.0 (0.2)	11.1[e] (0.4)	1.8[e] (0.6)	0.7[cd] (0.3)	1.0[e] (0.4)	58.4 (0.4)	40.9 (1.7)	17.4 (1.2)	4.4	80.7[a] (0.5)	7:1	96
Vegetable canteen waste	3.8 (0.0)	82.7 (0.1)	12.1[e] (0.1)	15.5[c] (0.9)	3.7[a] (0.3)	11.6[c] (0.6)	31.5 (1.8)	24.0 (1.5)	7.5 (0.3)	28.9	92.4[c] (0.5)	1:1	404
Mean	**5.5 (1.3)**	**74.5 (10.0)**	**21.2 (10.2)**	**8.3 (9.2)**	**1.8 (1.5)**	**6.4 (8.4)**	**37.7 (14.5)**	**23.1 (10.2)**	**14.6 (8.2)**	**22.5 (16.3)**	**90.1 (5.4)**	**29:1**	**343 (177)**
Poultry feed (benchmark)	5.7 (0.0)	60.0	19.1[c] (0.7)	28.5[a] (0.8)	0.5[cd] (0.2)	27.5[a] (1.4)	22.0 (1.0)	8.6 (0.0)	13.5 (1.1)	4.8	98.2[ab] (4.0)	1:2	274

in parenthesis: standard deviation for samples where n ≥ 3 and differences between analyses where n=2
results with no shared letter are significantly different from each other
*P:NFC = ratio of protein to non-fibre carbohydrates (NFC), **gross caloric content of protein, NFC, and lipids

The content of lipids, fibre and ash also varied among the wastes. Poultry slaughterhouse waste had a high lipid content and low ash and fibre content; the opposite was true for cow manure and mill by-products. The addition of meat to the canteen waste markedly increased the lipid content, while both of the canteen wastes were low in ash. Also, both batches of human faeces were rich in lipids, with values exceeding 20% DM. These results are high considering values ranging from 2 to 21% DM have been reported in the literature. The lipid content of the human faeces and pit latrine sludge samples collected in Nairobi were 9.4 and 16.6% DM, respectively. This suggests that the amount of lipids in faecal sludge can vary depending on management practices (e.g. residence time in the containment and the addition of sawdust) (Gold et al., 2017b), the presence of unabsorbed lipids, endogenous lipid losses (e.g. bile) and microbial processes (Aylward and Wood, 1962; Rose et al., 2015). Overall, the data show that the cow manure, human faeces, and poultry slaughterhouse wastes had low amounts of digestible nutrients and protein-to-carbohydrate ratios, whereas both the canteen wastes were rich in digestible nutrients and had a high caloric content, however, it is unknown how much of this energy can be harnessed by the fly larvae. Similar to mill by-products, the vegetable canteen waste was balanced or slightly NFC biased with proteins and NFC ratios of 1:1 and 1:2, respectively. Thus, these wastes were expected to perform best in feeding experiments when offered alone (Barragán-Fonseca et al. 2018b; Cammack and Tomberlin, 2017). For the other biowastes, mixing those with complementary nutrient compositions was expected to be advantageous.

Treatment performance of individual biowastes

All individual biowastes supported the development of BSFL (Table 4). The mean survival rates were 90-99% and were not significantly different between the biowaste types. They were also comparable to those found in previous research, where survival rates were shown to be above 80% (summarised by Rehman et al., 2017). Lalander et al. (2018) reported survival rates for different biowastes in the range of 81 to 100%, except for wastewater sludge which supported survival rates of only 39 to 81%. These results suggest that experimental conditions applied in the present study were suitable and confirms that BSFL can develop on a wide variety of biowastes. That said, BSFL treatment performance varied widely between the different biowastes (Figure 2.2). Mean performances values were significantly different among most of the biowastes and those that performed best were not always the same for each performance parameter. Waste reduction and protein conversion efficiency were lower for all of the wastes in comparison to the poultry feed, and cow manure had the poorest performance in all of the performance parameters.

Using vegetable canteen waste and mill by-products resulted in the highest waste reduction even though values were still 17 to 20% lower than for the poultry feed. This could be due to the high NFC content of these two wastes, which are easily digested and absorbed into the haemolymph of fly larvae (Bonelli et al., 2019; Pimentel et al., 2018). However, comparison of the waste reduction and larval weight results of mill by-products and human faeces demonstrate that this higher waste reduction did not necessarily result in higher larval weight. The level of waste reduction in the mill by-products exceeded the sum of easily digestible nutrients which are assumed to be reflected by the sum or proteins, NFC and lipids (Table 3) based on the morphofunctional features of the BSFL midgut reported by Bonelli et al. (2019).

Table 2.4 Mean performance of BSFL treatment on the different biowastes and formulations.

	Survival rate %	Larval weight mg DM	Waste reduction % DM	Bioconversion rate % DM	Larval biomass composition		
					Protein % DM	Ash % DM	Carbon % OM*
Individual wastes							
Mill by-products	96.2 (1.5)	41.7 (0.9)	56.4 (1.2)	14.9 (0.3)	42.1 (0.4)	7.3	58.0 (0.7)
Canteen waste	92.3 (3.1)	44.2 (5.9)	37.9 (3.8)	15.3 (2.1)	36.1 (0.3)	5.2	62.8 (0.3)
Human faeces (1)	99.1 (0.6)	58.8 (1.7)	39.1 (1.5)	22.7 (0.6)	26.7 (0.4)	13.6	65.8 (0.1)
Human faeces (2)	96.2 (2.5)	50.2 (1.2)	48.6 (0.3)	18.8 (0.8)	27.1 (0.1)	13.1	65.1 (0.6)
Poultry slaughterhouse waste	90.7 (2.9)	39.4 (0.7)	30.7 (4.7)	13.4 (0.5)	31.5 (0.7)	4.1	64.6 (0.4)
Cow manure	89.8 (7.5)	14.3 (0.4)	12.7 (0.9)	3.8 (0.2)	36.2 (0.2)	23.1	56.1 (0.3)
Vegetable canteen waste	97.5 (2.7)	59.1 (2.6)	58.4 (1.4)	22.7 (1.1)	24.5 (0.2)	5.1	65.4 (0.1)
Poultry feed (benchmark)	97.9 (2.1)	55.6 (5.1)	67.7 (6.9)	21.0 (2.4)	36.3 (0.8)	12.2	60.4 (0.7)
Waste formulations							
F 1	99.8 (0.6)	64.2 (1.1)	64.1 (0.6)	31.8 (0.6)	25.2 (0.3)	8.0	65.8 (0.2)
F 2	97.8 (3.7)	39.1 (0.3)	51.1 (0.7)	20.9 (0.9)	33.9 (0.5)	11.4	60.9 (0.4)
F 3 (1)	100.0 (0.0)	29.7 (1.2)	45.3 (1.1)	16.4 (0.7)	38.7 (0.3)	16.1	56.9 (0.3)
F 3 (2)	99.7 (0.6)	29.2 (2.0)	49.2 (1.6)	14.5 (1.1)	39.0 (0.6)	15.9	56.9 (0.6)
F 4	98.0 (1.4)	48.9 (2.4)	58.3 (1.1)	22.9 (1.1)	36.9 (0.3)	8.3	61.7 (0.5)
F 5	97.0 (3.4)	62.8 (1.6)	65.2 (2.0)	30.9 (1.6)	28.6 (0.3)	4.9	65.3 (0.7)
F 6	99.0 (1.0)	39.8 (2.1)	56.6 (0.7)	19.8 (1.1)	38.1 (0.3)	8.1	61.0 (0.2)

in parenthesis: standard deviation for samples where $n \geq 3$

*OM=organic matter

This suggests that some fibre, likely hemicelluloses, were decomposed during BSFL treatment. Gold et al. (2018b) also observed some decomposition of hemicelluloses in BSFL treatment with artificial diets, but this happened to a much smaller extend than that reported by Rehman et al. (2017) with cow manure. Such differences in digestibility have not yet been considered in biowaste formulation and could lead to unexpected performance results when designing formulations based on the glucose and starch content of NFC alone.
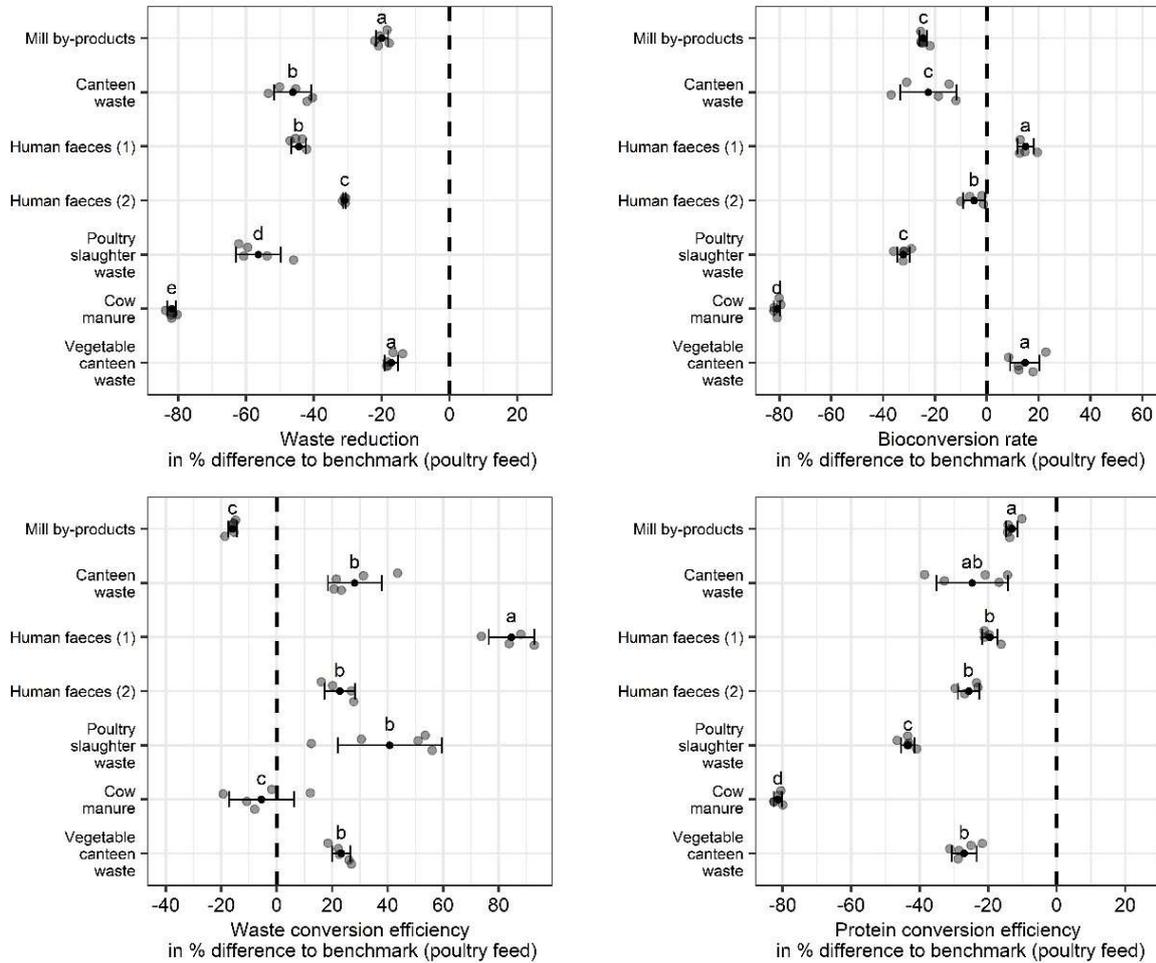
Figure 2.2 Effects of the different individual wastes on waste reduction (top, left), bioconversion rate (top, right), waste conversion efficiency (bottom, left) and protein conversion efficiency (bottom, right) in comparison to the benchmark poultry feed (dashed vertical line). Means, standard deviations and results per replicate are displayed. Performance results with no shared letter are significantly different from each other. All results are in dry mass.

Human faeces supported a bioconversion rate that was comparable to the poultry feed (and this was higher for human faeces (1) and lower for human faeces(2)), despite having a much lower waste reduction. This was due to an 85% higher waste conversion efficiency than the poultry feed. BSFL showed a significantly lower performance using poultry slaughterhouse waste than human faeces. The lowest performance was found for cow manure, which was low in protein, NFC, and lipids. In contrast, the human faeces and poultry slaughterhouse wastes were high in protein and lipids. These results thus suggest that NFC is less important for larval development than high overall nutrient content.

The protein conversion efficiencies were less variable and trends were different in comparison to the other performance parameters. Human faeces (1) and vegetable canteen waste had the highest bioconversion rate but not the highest protein conversion efficiency. This was due to varying larval composition arising from the different wastes and their associated larval weights. Larval protein content was notably higher when fed on mill by-products and canteen waste than on human faeces and vegetable canteen waste (Table 4). Larvae fed with human faeces and vegetable canteen waste likely incorporated more lipids, as indicated by a higher proportion of carbon in organic matter in comparison to the mill by-products and the canteen waste. Larva growing on human faeces contained the most ash.

Considering these findings, the most promising biowaste thus depends on the objective of the BSFL treatment. Mill by-products and vegetable canteen waste performed best with respect to waste treatment whereas human faeces and vegetable canteen waste were more favourable with respect to larval biomass production efficiency. The most protein per unit of biowaste was produced using the mill by-products and the canteen waste. Thus, for facilities targeting insect protein meal production, these wastes would be favourable. Poultry slaughterhouse waste and cow manure resulted in generally poor performance. However, not all wastes can be employed in BSFL treatment facilities for animal feed production given legal resolutions (Lähteenmäki-Uutela et al., 2017). For example, in the European Union, only mill by-products and pre-consumer wastes (in nutrient composition similar to canteen wastes) can currently be used (European Commission (EC), 2017).

Food, restaurant, and canteen wastes also resulted in the highest, and animal manures the lowest, BSFL treatment performance in previous studies (Lalander et al., 2019; Nyakeri et al., 2019; Oonincx et al., 2015b). Lalander et al. (2018) reported a bioconversion rate of 14% DM for food waste in comparison to 15–23% DM for canteen wastes in this study. The corresponding values for waste reduction were 55% DM in comparison to 38–58% DM. For human faeces, the bioconversion rate was 11% DM as reported by Lalander et al. (2018) compared with 19–23% DM in the present study, and waste reduction data were 48% DM in comparison to 39–49% DM, respectively. In contrast to the present study, Lalander et al. (2018) observed a higher BSFL performance using slaughterhouse waste compared to food waste and human faeces. Values for waste reduction in the literature for cow manure range from 29 to 58% DM (Miranda et al., 2019; Myers et al., 2008; Rehman et al., 2017) and bioconversion rates range from 2 to 6% DM (Miranda et al., 2019; Rehman et al., 2017). This compares with a 13% DM waste reduction and a 4% DM bioconversion rate observed in the present study. These differences confirm that predicting larval performance exclusively based on the type of biowaste is not reliable and can lead to greatly over- or underestimated performance. Such variation is likely to result not only from variable biowaste composition (i.e. nutrient and microbial numbers and communities) but also differences in experimental setups. To help address this, international standards for BSFL feeding experiments could allow for better comparisons across studies.

Treatment performance of biowaste formulations

The performance of the BSFL grown on the different waste formulations was significantly different despite targeting a similar protein and NFC content and ratio (Table 2.4; Figure 2.3). Overall, using a formulation significantly increased performance compared to individual wastes. Distributions were different between the waste formulations and individual wastes for survival rate, waste reduction, bioconversion rate and protein conversion efficiency but not for waste conversion efficiency.

Feeding BSFL with the waste formulations resulted in higher survival rates in comparison to the individual wastes, and ranged from 97 to 100%. Despite a 25% lower feeding rate, the median larval weight was 43.5 mg DM for the formulations and 40.1 mg DM for the individual wastes. The median of the survival rate was 99% for the formulations and 95% for the individual biowastes. Individual wastes resulted in the median waste reduction and bioconversion rate being lower, by 45.4 and 25.0%, respectively, compared to poultry feed. In comparison, the median waste reduction was only 18.5% lower, and the bioconversion rate even 8.6% higher, for the formulations compared to the poultry feed. The median protein conversion efficiency was 28.5% lower for the individual biowastes and 8.4% higher for the formulations comparison to the poultry feed. These results suggest that the performance of BSFL treatment facilities can be increased by designing biowaste mixtures based on similar protein and NFC contents.
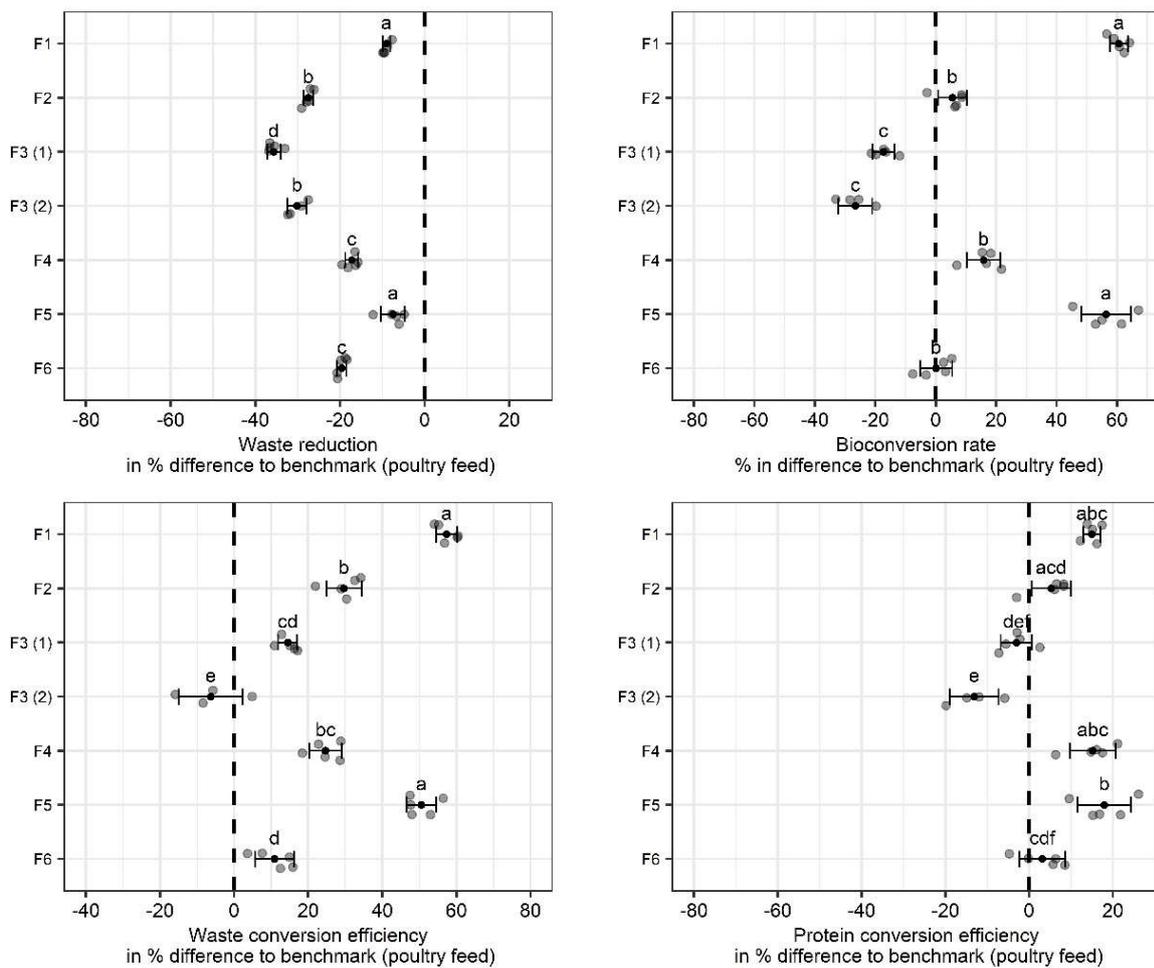
Figure 2.3 Effects of the different waste formulations on the waste reduction (top, left), bioconversion rate (top, right), waste conversion efficiency (bottom, left) and protein conversion efficiency (bottom, right) of BSFL in comparison to poultry feed (dashed vertical line). Means, standard deviations and results per replicate are displayed. Performance results with no shared letter are significantly different from each other. All results are given in dry mass.

By comparing Figure 2.2 and Figure 2.3, it also becomes apparent that the use of formulations decreased the variability in performance. In comparison to the poultry feed, the results for the

formulations had a range of 28% for waste reduction, 87% for bioconversion rate, 64% for waste conversion efficiency and 31% for protein conversion efficiency. In comparison, individual biowaste produced a range of 65% for waste reduction, 96% for bioconversion rate, 101% for waste conversion efficiency and 68% for protein conversion efficiency. This suggests that formulating different biowastes based on their initial nutrient composition can improve the reliability of BSFL treatment facilities. Although formulations, on average, contained less NFC, protein and lipids and more fibre than the individual wastes (see Table 2.2 and Table 2.3), they were more balanced in nutrients without the absence or excess of NFC (as was the case for human faeces, cow manure and poultry slaughterhouse waste), protein (poultry slaughterhouse waste) and fibre (cow manure and mill by-products). It should be stated that all of the formulations included at least 50% mill by-products or canteen waste or both, and these were the wastes that supported high BSFL performance when used individually.

Variability in the performance parameters was, nevertheless, higher than expected in the formulations; bioconversion rates were expected to be similar between the formulations as protein and NFC appear to have the greatest influence on larval development (Barragán-Fonseca et al. 2018b, Barragán-Fonseca et al. 2018c; Cammack and Tomberlin, 2017). However, variable bioconversion rates (by 87%) are not practical for BSFL treatment facilities. Such variability between formulations could be due to variable fibre and lipid contents. In the formulation feeding experiments, for example, lipid correlated positively and fibre negatively with waste reduction ($R^2=0.96$, $p<0.01$ for lipids, $R^2=-0.97$ and $p=<0.01$ for fibre) and the bioconversion rate ($R^2=0.96$, $p=<0.01$ for lipids, $R^2=-0.95$, $p<0.01$ for fibre). Formulations 1 and 5 resulted in the greatest amount of waste reduction and bioconversion rate. These formulations were highest in lipids and lowest in fibre (see Table 2.2) due to the high proportion of canteen wastes (see Table 2.1). In contrast, formulations 2 and 3 had the lowest lipid and highest fibre content due to a high proportion of human faeces and cow manure. This suggests that the variability in performance could be further reduced by keeping content of lipids and fibre within narrower limits. However, maintaining all macronutrients within fixed limits is difficult in practice considering that wastes typically have variable amounts of each macronutrient.

In addition to different lipid and fibre contents between the formulations, biowaste microbial numbers and communities could have been contributing to the variable BSFL treatment performance despite a similar protein and NFC content. This was not part of this study but can be expected considering that microbes can influence biowaste decomposition (De Smet et al., 2018; Gold et al. 2018a) and larval growth and typically differ between biowastes (Bruno et al., 2018; Ryckeboer et al., 2003; Wynants et al., 2018).

Similar to the larvae grown on the individual wastes, larval protein content was variable between the formulations (Table 2.4). Larvae fed on the formulations with a lower bioconversion rate tended to have a higher protein content. Protein efficiency was not significantly different between formulations 1, 4, and 5, and between formulations 2, 3, and 6, with the latter having a lower protein conversion efficiency, overall.

2.5   Conclusions

Given reliable biowaste compositional data, the formulation of mixed biowaste offers a promising systematic approach for the more efficient and predictable operation of black soldier fly larvae (BSFL) treatment facilities using a range of biowastes. Formulating biowaste mixtures in such a way that similar

protein and non-fibre carbohydrate (NFC) contents are achieved can be expected to increase BFSL treatment performance and to reduce performance variability. Performance variability could be further reduced by keeping lipids and fibre within narrower limits. Future research should investigate whether these bench-scale results are transferrable to industry-scale BSFL treatment plants with higher larval densities and feed temperatures. Benefits of biowaste formulations needs to be balanced with the additional resources required for biowaste analysis and the needed technologies to produce formulations as part of biowaste pre-treatment (e.g. scales, shredder, dewatering, mixer and tank).

Acknowledgements

3. Chapter – Efficient and safe substrates for black soldier fly biowaste treatment along circular economy principles

Unpublished manuscript

Moritz Gold[1,2], Davis Ireri[3], Christian Zurbrügg[2], Trevor Fowles[4], Alexander Mathys[1]

[1]ETH Zurich, Sustainable Food Processing, Department of Health Science & Technology, Institute of Food, Nutrition & Health, Zurich, Switzerland
[2]Eawag: Swiss Federal Institute of Aquatic Science and Technology; Department of Sanitation, Water and Solid Waste for Development (Sandec), Dübendorf, Switzerland
[3]Sanergy, New Technology Commercialization, Nairobi, Kenya
[4]University of California, Department of Entomology and Nematology, Davis CA, USA

3.1 Introduction

Biowastes constitute a large amount of the global waste problem (Wilson et al., 2015). Currently, biowaste is frequently not safely managed, wasting nutrients, energy and water (Diener et al., 2014; Hoornweg and Bhada-Tata, 2012). Thereby, biowaste management also contributes to global challenges such as poor urban health, degradation of the natural environment and climate change.

Black soldier fly larvae (BSFL) treatment is an emerging technology for biowaste treatment (Gold et al., 2018a; Zurbrügg et al., 2018). The technology uses the immature life stage of the black soldier fly (*Hermetia illucens*) that feeds on a large variety of biowastes and turns it into a compost-like residue. The insect biomass can be separated from the residue and processed into raw materials for lubricants and biodiesel, pharmaceuticals, and currently most promising, animal feed markets (e.g. poultry, fish, pigs, pets) (reviewed by Gold et al. 2018a).

In the past decades, research and implementation indicated that the technology has the potential to produce higher treatment product revenues, emit fewer greenhouse gas emission than other technologies such as composting, and produce insect-based feeds with a lower environmental impact than the status quo (Gold et al. 2018a; Mertenat et al., 2019; Smetana et al., 2019). In addition, the technology can operate at various scales, modularity, and levels of automations. These advantages are demonstrated by the global implementation of the BSFL technology in recent years (e.g. The Netherlands, China, Chile, USA, Germany, Kenya, Ghana, South Africa, and Indonesia). However, for BSFL treatment to have a significant global impact on waste management and the sustainability of the food system, more insect-based biowaste treatment facilities are needed.

One main challenge faced by utilities, municipalities, insect companies and entrepreneurs is the selection of suitable biowastes for BSFL treatment. Waste treatment performance, larval growth and biomass production vary between substrates as measured by bioconversion rate, larval mass, and larval biomass composition (e.g. protein and lipid content) (Gold et al. 2020a, Gold et al. 2018a). In addition, chemical and microbial contaminants in different biowastes require careful consideration to ensure product safety. Moreover, substrates may already have a value when already used otherwise. Or the provision of the waste has high costs due to decentralized collection, long distance to the production facility, or high amounts of inorganic materials (i.e. plastic, glass, paper) that need to be removed prior to BSFL treatment (Diener et al., 2015a; Dortmans et al., 2017).

We herein present a systematic approach to identify suitable substrates for BSFL treatment, considering waste availability and costs, the BSFL process performance of the biowaste, the waste recovery hierarchy and product safety. The applied approach is universal and enables the design of BSFL treatment facilities based on realistic performance estimates, production of safe insect-based products and environmental benefits of products in comparison to the status quo. An open-access web

application (moritzgold.shinyapps.io/BSF_app/) was developed with tools to explore waste compositional data and BSFL process performance, and formulate efficient biowaste mixtures.
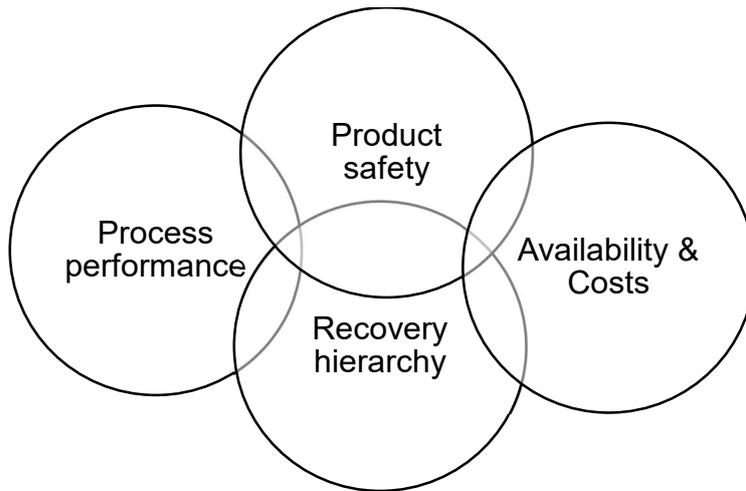
Figure 3.1 Criteria considered for selection of BSFL treatment substrate in Nairobi, Kenya.

3.2 Materials and Methods

This case study was executed in Nairobi, Kenya, using a combination of primary and secondary research. At an existing BSFL treatment facility, around 20 tonnes/day of human faeces, and fruit and vegetable waste were treated at the time of writing. The BSFL are processed into dry larvae and sold to feed millers, and the residue is composted and sold as a soil conditioner. The assessment was completed with the goal to source more biowaste for the upscaling of the facility.

Selection Criteria

The biowaste assessment approach considered four criteria (Figure 3.1): the waste availability and costs, the BSFL process performance of the biowaste, the waste recovery hierarchy and product safety. These criteria can be processed step-by-step, in parallel or iteratively. The process performance of the biowaste in BSFL treatment is important since it influences the treatment time and larval and residue amounts produced per unit of biowaste. Ultimately, process performance thereby influences operational costs and revenues from treatment products. It is important to consider the waste recovery hierarchy as well as the waste availability and potential waste costs, since wastes with high process performance may already have a use (e.g. animal feed). Therefore, conversion of the waste may not produce affordable treatment products, or wastes are already part of meaningful resource recovery (Smetana et al., 2019). Product safety is critical for BSFL treatment since treatment products are used for human food production, and wastes can include contaminants that are relevant to animal and human health (Van der Fels-Klerx et al., 2018).

Availability and costs

Availability and costs of wastes in Nairobi was assessed considering criteria proposed by Lohri et al. (2015) such as biowaste quantity, biowaste cost, waste characteristics (e.g. nutrient composition, mixing of organics with inorganics) and existing use of waste (e.g landfilling, composting). Data regarding these criteria was mainly collected from previous waste assessments conduced in Nairobi (Baud et al., 2004;

Njoroge et al., 2014; Kasozi and Von Blottnitz, 2010; Kirai et al., 2009). Data was validated by semi-structured key informant interviews and sight tours of restaurant and hotels, slaughterhouses, livestock farms, supermarkets and food industries. In addition, waste characteristics were assessed by analyses of wastes for gross nutrient composition. Crude protein, lipids, neutral detergent fibre (NDF), acid detergent fibre (ADF) and ash were analysed using standard procedures used for animal feeds outlined in detail in Gold et al. (2020a). Protein was estimated by multiplication of nitrogen with waste-specific factors (Chen et al., 2017; Gold et al., 2020a; Mariotti et al., 2008; Sriperm et al., 2011). Hemicelluloses were determined as the difference between NDF and ADF. ADF was assumed to be a reliable estimate of fibres (cellulose and lignin) content. Samples were pit latrine sludge (n=3), human faeces with sawdust (n=4), fruit and vegetable waste (n=1), vegetable waste (n=2), poultry feed (n=1), food waste from hotels (n=9), food waste from restaurants (n=2), brewery side streams (n=1), fruit waste (n=4) and slaughterhouse waste (n=2). To generalize our approach, literature values for the same parameters were additionally summarized (Giromini et al., 2017; Gold et al., 2020b, 2020a; Liu et al., 2018; Meneguz et al., 2018; Shumo et al., 2019; Vruggink, 2020).

Biowaste compositional data (n=67) were analysed using R version 312 3.6.2 (R Core Team, 2020). Data was manipulated using *tidyverse* (Wickham et al., 2019). Data was visualized by boxplots using *ggplot2* (Wickham, 2016) and in a two-dimensional pane following a principal component analysis (PCA) using the *FactoMineR* (Le et al., 2008) and *factoextra* package (Kassambara and Mundt, 2020). The analyses were translated into a web application (moritzgold.shinyapps.io/BSF_app/) using the *Shiny* (Chang et al., 2020) and *shinydashboard* package (Chang and Ribeiro, 2018). *Shiny*-based applications are interactive and change outputs (e.g. plots, descriptive statistics) based on the input by users.

Process performance

Process performance of the identified wastes with BSFL were estimated using literature values of typical BSFL performance metrics (e.g. larval mass, bioconversion rate, waste reduction) (Banks et al., 2014; Bava et al., 2019; Diener et al., 2009; Gold et al., 2020a; Jucker et al., 2017; Lalander et al., 2019; Liu et al., 2018; Meneguz et al., 2018; Miranda et al., 2019; Nguyen et al., 2013; Nyakeri et al., 2019; Rehman et al., 2017; Somroo et al., 2019; Spranghers et al., 2017; Tschirner and Simon, 2015). Feeding rate and treatment time within these studies were 40-250 mg waste/larva*day and 6-27 days, respectively. The summary of BSFL performance metrics (n=60) and the calculation of the nutrient content of biowaste mixtures were also included in the *Shiny*-based web application (Barragán-Fonseca et al., 2018b; Gold et al., 2020a).

Product safety

Biowaste contain various contaminants such as microbes (e.g. pathogenic bacteria and viruses), (heavy) metals and/or chemicals (e.g. pharmaceutical and pesticide residues) (Gold et al. 2018a; Van der Fels-Klerx et al., 2018). We used the available literature to estimate potential waste contaminants in the identified wastes, their fate in BSFL treatment and product processing, and compared risks to general animal feed safety considerations by the Food and Agriculture Organization of the United Nations (FAO) (FAO 1997).

Waste recovery hierarchy

BSFL treatment frequently aims to provide biowaste treatment and products with lower environmental impact than the status quo. Using the waste recovery hierarchy concept (UNEP, 2013) and published

life cycle assessments (LCAs) results (Bosch et al., 2019; Smetana et al., 2019) we discuss whether environmental benefits are likely associated with diverting the identified wastes to BSFL treatment.

3.3 Results and Discussion

Availability and cost

Organic solid waste can be broadly categorized into domestic and non-domestic waste (Kirai et al., 2009). Most organic waste is household waste from around 4.4 million people (KEBS, 2019) living in Nairobi. Considering the city-wide estimate of waste generation (0.65 kg/capita/day) and an average waste compositon (59% of waste is organic), around 1,675 tonnes of organic household waste are produced per day (Kasozi and Von Blottnitz, 2010). Organic household waste has prohibitively high collection and transport costs, and was therefore not further considered for upscaling of the BSFL treatment facility. Firstly, household waste collection is expensive (20-50 USD/tonne) (Gower and Schröder, 2018) and currently 30-50% of household waste is collected (Aryampa et al., 2019; Kasozi and Von Blottnitz, 2010). Secondly, collected organic waste is typically mixed with inorganic waste requiring prohibitively laborious separation before BSFL treatment.

Determination of amounts of don-domestic organic waste (e.g. from schools, restaurants and hotels, markets) is challenging due to an unknown number of sources (> 3,000) (Kirai et al., 2009) and variable waste generation rates per source (Table 3.1). About 20-30% of all organic waste generated in the city has previously been estimazted to be non-domestic waste (Kasozi and Von Blottnitz, 2010; Kirai et al., 2009). Consequently, using the estimate for domestic waste generation and average waste compositon (74% of waste is organic) (Kirai et al., 2009), around 529-906 tonnes of non-domestic organic waste is produced per day. Based on previous work by Kirai et al (2009) and our own intereviews and field visitis, non-domestic organic waste in Nairobi is fruit and vegetable wastes (e.g. from canneries, juice producers and retailers); animal manure (e.g. dairy and pig farms); food waste (e.g. retailers, markets, hotels, restaurants, shopping malls); cereal-based byproducts (e.g. spent grain from breweries, press cakes from oil production, byproducts from flour and animal feed mills); and slaughterhouse waste (e.g. rumen content and blood). Solids in wastewater and faecal sludge are an additional poorly managed and valorised domestic and non-domestic waste in Nairobi.

Similar to household waste, use of many non-domestic waste sources for BSFL production requires removal of inorganics. Even though non-domestic waste in Nairobi is less contaminated (26% inorganics) (Kirai et al., 2009) with inorganics than household waste (41% inorganics) (Kasozi and Von Blottnitz, 2010), most non-domestic waste includes inorganics. Market waste comprising most non-domestic organic waste contains on average around 5% inorganics. Kirai et al. (2009) estimated that 73% of supermarket waste is organic and that 50% of restaurants collect organics and inorganics separately. Non-domestic organic waste free of inorganics is typically already used (e.g. composting, biogas, animal feed, bone meal) and not available for BSFL production. For example, food scraps from large hotels and restaurants, wheat bran and maize germ from mills, and spent grain from breweries are used as animal feed, and waste from markets and food industry by-products (e.g. juice production) are used as soil conditioner (Baud et al., 2004; Kirai et al., 2009). The remaining waste is currently mostly (>70%) landfilled (Kirai et al., 2009). Based on this summary, our interviews and field visits and the waste already processed in the existing facility human faeces, animal manures, faecal sludge, fruit and vegetable wastes, slaughterhouse waste, as well as hotel, restaurant and supermarket food waste with

inorganics are substrates available for upscaling of the BSFL treatment facility (in descending order of estimated quantity).

Table 3.1 Don-domestic waste generation (in kg) per day and facility from different sources.

Source	Waste examples	Waste generation
Hotels	Food scraps	200-2,000[1,2]
Restaurants	Food scraps	2,100[2]
Markets	Food scraps, discarded food (e.g. fruit and vegetable peels)	> 19,000[2]
Shopping malls	Food scraps	1,100[2]
Commercial animal farms	Pig and cow manure	25,000[2]
Slaughterhouses	Blood, rumen content	45,000[1] 16,000[2]
Supermarkets	Discarded fruits and vegetables	2,500[2]
Food industry	Discarded fruits and vegetables, by-products from juice production, canneries and bread baking	0.5-100[1]

[1]own data
[2]Kirai et al (2009)

This study developed an open-access web application (moritzgold.shinyapps.io/BSF_app/) to summarize and visualize biowaste compositional and BSFL process performance data. Figure 3.2 shows the "Nutrient composition" tab. It summarizes the waste nutrient composition based on the BSFL substrate group (see Gold et al. (2018a)) and nutrient parameter selected by the user. The boxplots and PCA biplot demonstrate a large variability in nutrient compostion among samples of the same substrate group. This is to be expected as waste comes from different sources (e.g. different fruits or vegetables) and waste management systems (e.g. waste storage durations). Despite this variability, the vector directions of the PCA biplot allow categorization of wastes into broad categories based on nutrient composition. For example, considering the wastes available in Nairobi, animal manures and faecal sludge are high in fibres and low in lipids, and fruit and vegetable wastes are low in proteins and lipids. Food wastes are the substrate group with the largest variability, but tend to be high in lipids, protein and hemicelluloses, and low in fibres and ash. Since nutrients correlate with process performance (Gold et al., 2020a; Lalander et al., 2019), exploring the waste nutrient composition with the developed web application can support the selection of efficient biowastes and the formulation of biowaste mixtures. In this regard, compositional databases for food (ndb.nal.usda.gov), feed (feedipedia.org) and food wastes (foodwasteexplorer.eu) should also be considered.

Figure 3.2 The "Nutrient composition" tab of the web application summarizes the waste nutrient composition based on the substrate group and nutrient parameter selected by the user. Descriptive statistics are included in a table below the plots (not shown in this Figure).

Process performance

Figure 3.3 shows the "Conversion performance" tab of the web application. It summarizes performance metrics of BSFL treatment based on the substrate group and the performance metric selected by the user. The summary supports the selection of efficient substrates and provides realistic performance estimates for treatment design and estimation of treatment product quantities. In general, animal manures, faecal sludge, fruit and vegetable wastes and slaughterhouse waste available in Nairobi have a lower performance than food wastes, cereal-based byproducts or human faeces. A few wastes that tend to be unsuitable for BSFL treatment due to very low growth include grass, wastewater sludge or water hyacinths (Dieu et al., 2015; Lalander et al., 2019; Liu et al., 2018) were not included in the performance summary. Research is ongoing on increasing growth of BSFL on these substrates by pre-treatments (Isibika et al., 2019) and co-conversion with beneficial bacteria (Mazza et al., 2020; Rehman et al., 2019).
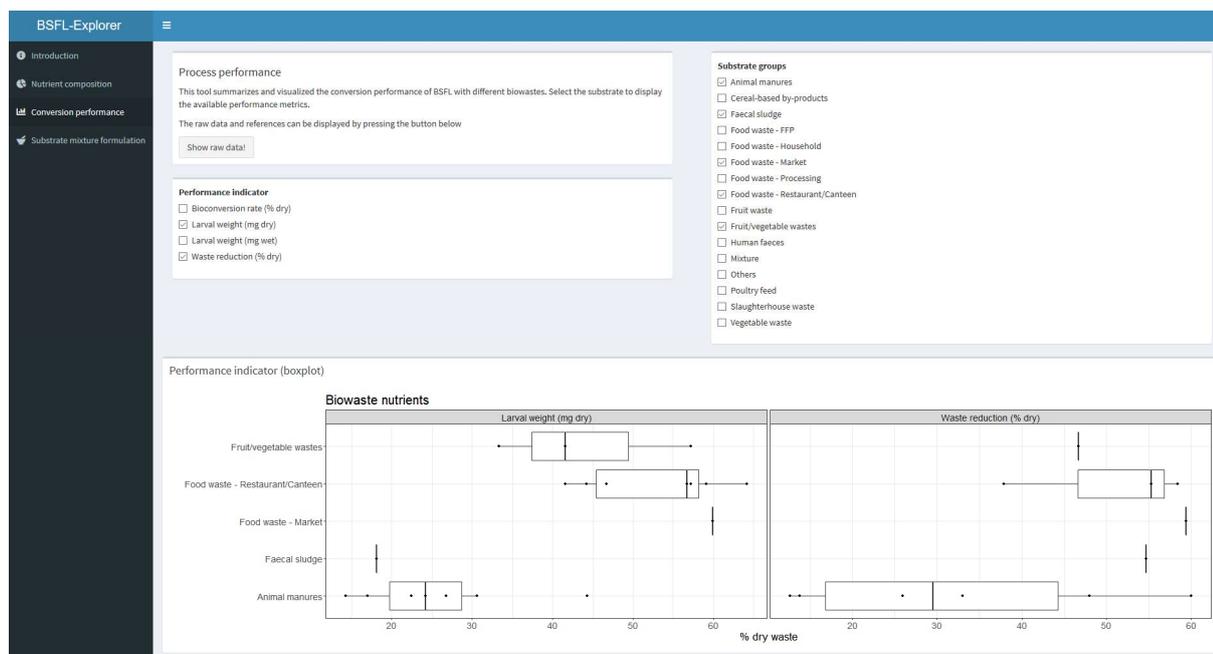
Figure 3.3 The "Conversion performance" tab of the web application summarizes process performance results based on substrate group and performance metric selected by the user. Descriptive statistics are included in a table below the boxplot (not shown in this Figure).

The process performance of individual wastes is of limited value in Nairobi, as a single waste stream cannot meet the entire treatment capacity (> 100 tonnes/day). In such a case, knowledge of an efficient biowaste mixture and the associated process performance is required. Typically, biowaste mixtures have been identified in trial-and-error feeding experiments with different biowaste mixtures (Nyakeri et al., 2019; Rehman et al., 2017). Formulating biowaste on the basis of nutritional composition (e.g. protein and carbohydrates), taking into account knowledge on the BSFL nutritional needs and digestion biology, has recently emerged as a more systematic approach (Barragán-Fonseca et al., 2018b; Gold et al., 2020a). However, this approach requires accurate waste compositional data. The "Substrate mixture formulation" tab (Figure 3.4) of the web application works towards closing this gap by calculating the nutrient composition of biowaste mixtures based on waste compositional data summary (Figure 3.2). The web application considers the nutritional variability by displaying the range of the nutritional composition of the biowaste mixture.
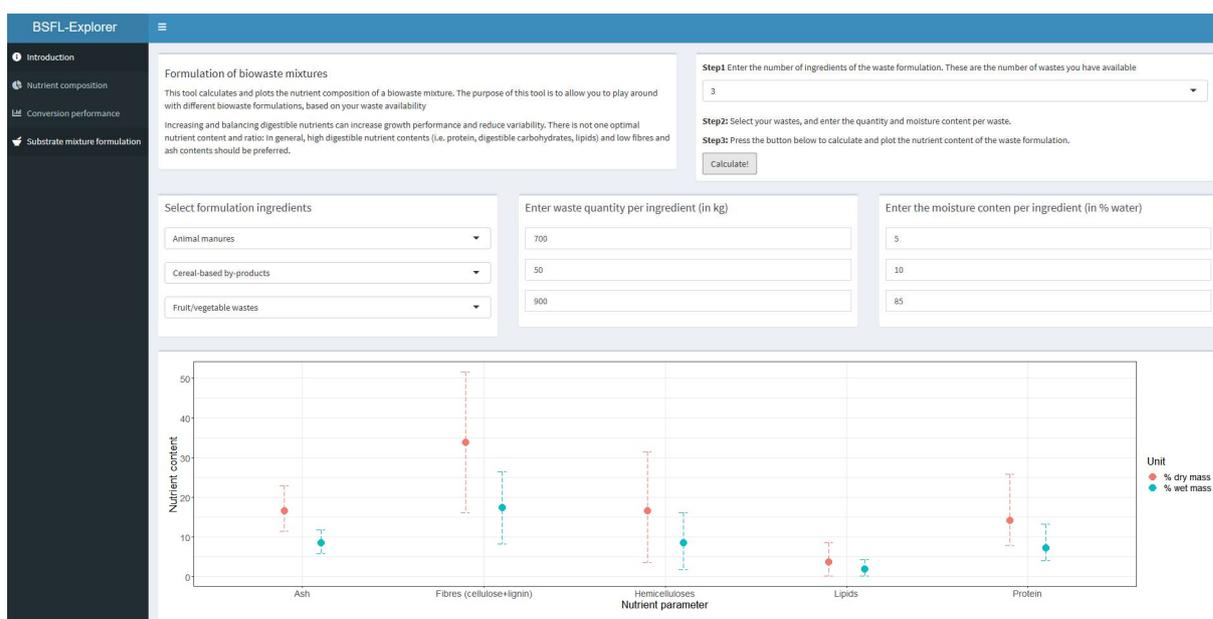
Figure 3.4 The "Substrate mixture formulation" tab of the web application calculates the nutrient composition of a biowaste mixture based on the substrate, quantity and moisture content provided by the user.

Product safety

Table 3.2 summarizes potential hazards in the BSFL substrates identified in Nairobi. Considering the current knowledge on the fate of condiments in BSFL treatment, heavy metals and arsenic in biowastes are a large hazard for animal feed safety. In contrast to most microbes (i.e., bacterial and viral pathogens) that are high in unprocessed BSFL, but can be inactivated by heat treatment, some heavy metals have been shown to bioaccumulate in the larval tissue (Diener et al., 2015b; Schmitt et al., 2019). Spore forming pathogens (e.g. *Bacillus cereus*, *Clostridium botulinum)* are also of specially concern since they can survive heat treatment. Aflatoxin B1 (a common mycotoxin) and human/veterinary drugs and chemicals were so far not detected in BSFL treatment on contaminated substrates (Bosch et al., 2017; Charlton et al., 2015; Lalander et al., 2016; Purschke et al., 2017).

Table 3.2 Potential hazards in BSFL substrates. A=mycotoxins, B=human/veterinary drugs, C=agricultural chemicals, D=microbial pathogens and their toxins (e.g. Botulinum toxin), E=metals, F=misfolded prion proteins (Bosch et al., 2017; FAO 1997; Gold et al. 2018a; Lalander et al., 2016; Purschke et al., 2017).

Waste	Hazard
Human faeces and excreta	B, D
Wastewater and faecal sludge	B, D, E
Animal manures	B, C, D
Slaughterhouse waste	D, F
Household waste	D, E
Cereals-based by-products	A, D
Food & restaurant waste	A, D
Food processing waste	A, D

Strategies to manage these hazards include the selection of traceable substrates with low contaminant concentrations, mixing of substrates with low and higher contaminant concentration, post-processing of treatment products and good hygiene practices (IPIFF 2019). For example, at the BSFL treatment facility in Nairobi, only human faeces collected from standardized and well-operated source-separating

toilets are used. Separation of faeces from urine means that an estimated 2/3 of the excreted drug residues (Lienert et al., 2007) are disposed elsewhere. Further, mixing of faeces with sawdust in the toilet, and co-processing of this mixture with discarded fruits and vegetables further reduce drug residue concentrations. In case drug residues are carried over to BSFL (which has not yet been shown in the few completed studies), heat treatment contributes to their inactivation (Tian et al., 2017).

Since all substrates identified in Nairobi may include microbial pathogens, BSFL should not be fed live to animals. In addition, more research is needed on how the hazards in wastewater sludge (e.g. heavy metals) and slaughterhouse waste (e.g. misfolded prion proteins) can be managed.

Recovery hierarchy

BSFL treatment is frequently selected with the aim to provide biowaste treatment and products with lower environmental impact than the status quo (Mertenat et al., 2019; Smetana et al., 2019). To deliver on this promise, the waste recovery hierarchy is a useful and established concept. It indicates the order of preferences of waste reduction and management measures. In the order of most preferred to least preferred measure, it includes prevention, reduction, recycling, recovery and disposal (UNEP, 2013). We propose to extend this concept to encompass BSFL production on biowastes (Figure 3.5). BSFL production should be the most preferred measure following waste prevention (e.g. by reducing food overproduction) and waste reduction (e.g. by use as livestock or pet feed). For the Nairobi case study, this means that cereal-based byproducts and some food and fruit/vegetable wastes should not be diverted to BSFL production due to their use as animal feed. In addition, BSFL treatment should be considered before other waste treatment technologies such as anaerobic digestion or composting to recycle nutrients back into the food chain. Thereby, biowastes (e.g. from the food processing industry) can enter a circular value chain (Cappellozza et al., 2019).
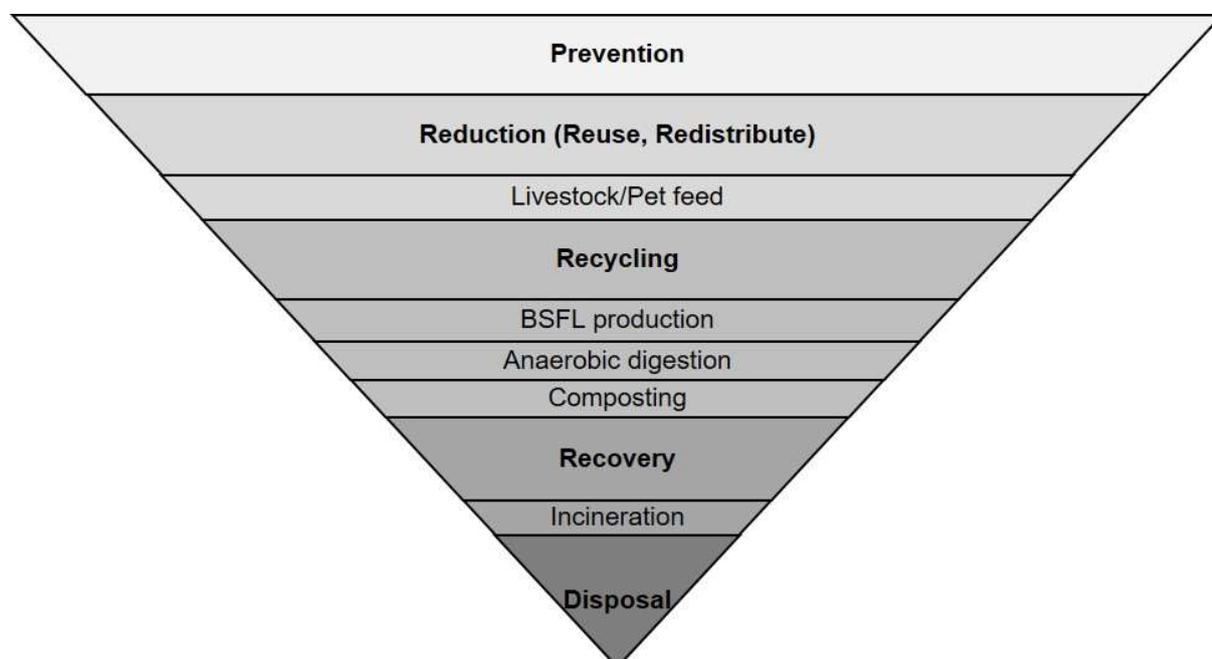
Figure 3.5 Biowaste recovery hierarchy including BSFL treatment, adapted from Papargyropoulou et al. (2014) and UNEP (2013).

Trade-offs between criteria

The case study of Nairobi demonstrates that selecting substrates for BSFL treatment is a trade-off between larval biomass production efficiency and substrate costs, while considering safety and sustainability of the larval-based products. Substrates that have the highest performance have typically high costs and are already used as animal feed, which is in line with the waste hierarchy concept and circular economy principles. The remaining substrates that are available at reasonable costs have a lower performance and require careful consideration to ensure product safety.

Based on the presented four-criteria assessment, a mixture of human faeces, animal manures, fruit/vegetable waste and food waste should be being pursued for upscaling of the BSFL treatment facility in Nairobi. Multiplication or further expansion of the facility requires changes in Nairobi`s waste management system such as more separation of biowaste from inorganic wastes, and financially viable collection services. 70-80% of Nairobi`s biowaste is household waste mixed with inorganics, making it unsuitable for BSFL treatment (Kirai et al., 2009). In addition, only 30-50% of waste in Nairobi is collected (Aryampa et al., 2019; Kasozi and Von Blottnitz, 2010). Making this a reality is challenging, considering that more than 50% of Nairobi`s population live in informal areas.

Acknowledgements

4. Chapter – Identification of bacteria in two food waste black soldier fly larvae rearing residues

Moritz Gold[1,2], Fabienne von Allmen[1,2], Christian Zurbrügg[2], Jibin Zhang[3], Alexander Mathys[1]

[1]Sustainable Food Processing Laboratory, Institute of Food, Nutrition and Health, Department of Health Science and Technology, ETH Zürich, Zurich, Switzerland
[2]Department Sanitation, Water and Solid Waste for Development (Sandec), Eawag: Swiss Federal Institute of Aquatic Science and Technology, Dübendorf, Switzerland
[3]State Key Laboratory of Agricultural Microbiology, National Engineering Research Center of Microbial Pesticides, College of Life Science and Technology, Huazhong Agricultural University, Wuhan, PR China

4.1 Abstract

Significant economic, environmental, and social impacts are associated with the avoidable disposal of foods worldwide. Mass-rearing of black soldier fly (*Hermetia illucens*) larvae using organic wastes and food- and agro-industry side products is promising for recycling resources within the food system. One current challenge of this approach is ensuring a reliable and high conversion performance of larvae with inherently variable substrates. Research has been devoted to increasing rearing performance by optimizing substrate nutrient contents and ratios, while the potential of the substrate and larval gut microbiota to increase rearing performance remains untapped. Since previous research has focused on gut microbiota, here, we describe bacterial dynamics in the residue (i.e., the mixture of frass and substrate) of black soldier fly larvae reared on two food wastes (i.e., canteen and household waste). To identify members of the substrate and residue microbiota, potentially associated with rearing performance, bacterial dynamics were also studied in the canteen waste without larvae, and after inactivation by irradiation of the initial microbiota in canteen waste. The food waste substrates had similar microbiota; both were dominated by common lactic acid bacteria. Inactivation of the canteen waste microbiota, which was dominated by *Leuconostoc*, *Bacillus*, and *Staphylococcus*, decreased the levels of all rearing performance indicators by 31-46% relative to canteen waste with the native microbiota. In both food waste substrates, larval rearing decreased the bacterial richness and changed the physicochemical residue properties and composition over the rearing period of 12 days, and typical members of the larval intestinal microbiota (i.e., *Providencia*, *Dysgonomonas*, *Morganella,* and *Proteus)* became more abundant, suggesting their transfer into the residue through excretions. Future studies should isolate members of these taxa and elucidate their true potential to influence black soldier fly mass-rearing performance.

4.2 Introduction

Significant economic, environmental, and social impacts are associated with the avoidable disposal of foods worldwide (Gustavsson et al., 2011; Papargyropoulou et al., 2014; Chen et al., 2020). Measures to reduce food loss and waste amount exceeding 1.3 billion tons per year (Gustavsson et al., 2011) include avoiding surplus food production, followed by redistribution and reuse of surplus foods (Papargyropoulou et al., 2014). Once produced, food loss and wastes should be recycled by using them as animal feed (Dou et al., 2018) or compost (Li et al., 2013), and extraction of energy should be the least preferred approach (Papargyropoulou et al., 2014). In recent years, mass rearing of the black soldier fly (*Hermetia illucens* L. (Diptera: Stratiomyidae)) larvae (BSFL) has emerged as an additional solution for food waste recycling (Gold et al., 2018a; Zurbrügg et al., 2018; Berggren et al., 2019; Varelas, 2019). BSFL converts a range of organic wastes (e.g., food waste, animal manure) and food- and agro-industry side products (e.g., breweries, food processing industry) (Nyakeri et al., 2017b; Barragán-Fonseca et al., 2018b; Lalander et al., 2019; Gold et al., 2020a) into larval biomass and a compost-like residue (i.e., mixture of frass and substrate). The larval biomass is rich in proteins and lipids, and thus, serves as a raw material for various applications within the food system, such as proteins and lipids in feeds for pets (Bosch et al., 2014) and livestock (Barragán-Fonseca et al., 2017; Wang and Shelomi, 2017), and processing of the larval exoskeleton into chitosan (Hahn et al., 2019). Next to this recycling of waste nutrients, according to circular economy principles (Cappellozza et al., 2019), waste treatment by BSFL (Ermolaev et al., 2019; Mertenat et al., 2019; Pang et al., 2020) and animal feed products (Smetana et al., 2016, 2019) with BSFL can have lower environmental impact than the status quo (i.e., composting and commercial feed ingredients such as fish meal).

One current challenge for BSFL rearing is to obtain reliable and high rearing performance (e.g., > 200 mg for harvested BSFL) (Gold et al., 2018a). Researchers have previously improved rearing performance by optimizing substrate nutrient contents and ratios (Nyakeri et al., 2017b; Barragán-Fonseca et al., 2018b; Gold et al., 2020a), however, few studies exist regarding the manifold roles in which BSFL-associated microbiota may influence rearing performance (De Smet et al., 2018). BSFL guts, rearing substrates and residues (i.e., the mixture of frass and substrate) all have rich and diverse microbiomes (Bruno et al., 2019; Klammsteiner et al., 2020) varying due to different biotic (e.g., initial rearing substrate microbiome) and abiotic (e.g., temperature) factors among rearing systems (Wynants et al., 2019; Raimondi et al., 2020). Similar to many insects (Douglas, 2009; Engel and Moran, 2013; Lee and Brey, 2013), Dipteran larvae such as those of *Drosophila melanogaster* (Diptera: Drosophilidae) and *Musca domestica* (Diptera: Muscidae) engage in complex interactions with their gut microbiota, as these influence larval immunity (Broderick and Lemaitre, 2012) and metabolism (Shin et al., 2016), growth signaling (Storelli et al., 2011), and nutrient provision (Zurek and Nayduch, 2016). Microbiota associated with BSFL may have similar functions (Ao et al., 2020) considering their similar ecological niche and phylogenetic order (Gold et al., 2018a; Zhan et al., 2020). Identification and manipulation (e.g., by addition of bacterial mixtures) of microbiota in BSFL rearing is an additional promising approach to increase rearing performance, next to the optimization of substrate nutrient contents and ratios.

Members of the BSFL gut microbiota (e.g., *Bacillus natto*, *Bacillus subtilis*, *Lactobacillus buchneri*, and *Kocuria marina*) can increase performance when added to rearing substrates (Yu et al., 2011; Xiao et al., 2018; Rehman et al., 2019; Somroo et al., 2019; Mazza et al., 2020). Members of the substrate and residue microbiota are an additional yet unexplored pool of potentially beneficial bacteria. Fly larvae may support and sustain certain microbiota in the substrate and residue to favor decomposition and digestibility (Zhao et al., 2017) and thus larval development (Storelli et al., 2018) and also protect from other insects and microbes competing for the same resources (Bernard et al., 2020). Bacterial candidates associated with rearing performance could be identified by studying microbiota throughout the rearing time in the substrate and rearing residue. For BSFL, previous studies have typically only determined the bacterial community in the initial substrate as well as the final residue. Jiang et al. (2019) were the first to determine bacterial community dynamics throughout one rearing cycle and found that BSFL rearing affects and alters the substrate bacterial community over time to increase the capacity to decompose the substrate. Lalander (2013b) and Erickson et al. (2004) also reported a reduction in certain bacteria during BSFL rearing, and Cai et al. (2018) and Liu et al. (2020) reported a reduction in antibiotic resistance genes in bacteria. These results contradict the findings of Bruno et al. (2019), who did not identify a significant influence of BSFL on substrate microbiota during rearing. Changing the bacterial community in the rearing substrate may also be beneficial for rearing of insects for food and feed applications, when considering that the substrate microbial community may include human and animal pathogens (e.g., *Bacillus cereus* and *Enterococcus faecalis*) (Lalander et al., 2013; Van der Fels-Klerx et al., 2018; Wynants et al., 2019).

The aim of this study was to identify groups of bacteria potentially associated with BSFL residues and the rearing performance. We assessed the bacterial community dynamics when using two food waste substrates during BSFL rearing. We also studied the bacterial community dynamics in food waste after inactivation by irradiation of the initial microbiota in food waste with and without BSFL. We hypothesized that an inactivation of the initial microbial community in the substrate should decrease

rearing performance, revealing that some important bacteria are associated with rearing performance. In addition, we hypothesized that certain groups of bacteria become more abundant during BSFL rearing in comparison to controls without larvae, and that these taxa are associated with rearing performance.

4.3 Materials and Methods

Food waste substrates

Two types of food waste were collected in containers treated with 70% ethanol. Canteen waste included a mixture of discarded pasta, meat, fish, bread, and vegetables from the Polyterasse canteen at ETH Zurich in Switzerland. Household waste included discarded fruit peels, vegetables, eggs, bread, herbs, and food leftovers collected from a household organic waste bin in Zurich. Each collected substrate mixture was homogenized with a kitchen blender to mimic typical waste processing before BSFL rearing (Dortmans et al., 2017). Pictures of the fresh and homogenized rearing substrates are included in Supplementary Material. Following homogenization, the rearing substrates were stored at 4°C for 48 h. During this storage time, part of the canteen waste was sterilized with a high-energy electron beam. This substrate was fed to BSFL in parallel to the non-sterile food wastes to assess the influence of the loss of the initial substrate microbiota on rearing performance and bacterial dynamics. Irradiation was completed by a commercial provider (Leoni Studer AG, Däniken, Switzerland) with a 10 MeV electron beam (Rhodotron TT300, IBA Corp, Louvain-La-Neuve, Belgium) at a dose of 32 kGy in accordance with the ISO 11137-3:2017 standard (ISO 2017). These treatment conditions produced sterile substrates without microbial growth (Gold et al., 2020c).

Since substrate composition influences microbial communities and BSFL rearing performance, substrate gross nutrient composition, pH, and moisture content were determined using standard procedures described in detail in Gold et al. (2020a). The pH was measured with a portable meter and the pH probe HQ40d (Hach Lange GmbH, Rheineck, Switzerland). Moisture and organic matter were determined using an automatic thermogravimetric instrument (TGA-701, Leco, St. Joseph, MI, USA). Nitrogen content was determined using a C/N analyzer (Type TruMac CN, Leco). Glucose (D-Glucose GOPOD K-GLUC, Megazyme, Wicklow, Ireland) and starch (Total Starch Assay K-TSTA, Megazyme), and fructose (Available Carbohydrates K-ACHDF, Megazyme) were determined using commercial enzyme assays. For fructose determination, absorbance differences of >0.07 were used instead of > 0.1, as recommended by the manufacturer. Neutral and acid detergent fibers were assessed using a fiber bag system (Fibretherm, Gerhardt Analytical Systems, Köningswinter, Germany). Lipids were analyzed by Eurofins Scientific, Schönenwerd, Switzerland, according to Regulation (EC) No 152/2009 (EC, 2009). Protein was estimated by multiplying the nitrogen results with 5.4 (based on results for meat, fish, cereals, and vegetables) (Mariotti et al., 2008), and the caloric content was estimated by multiplying the mean lipid, non-fiber carbohydrate, and protein results with their gross caloric content of 9.4, 5.4, and 4.1 kcal/g, respectively (Merrill, 1973; Wu, 2016). Hemicellulose content was determined as the difference between the neutral and acid detergent fibers. The sum of glucose, fructose, and starch was assumed to reflect the total non-fiber carbohydrate content.

Fly larva rearing

BSFL were reared on homogenized food waste substrates using the following experimental setup. BSFL were obtained from a colony operated at Eawag (Dübendorf, Switzerand) since 2017, based on the

protocol of Dortmans et al. (2017). The hatched larvae were fed *ad libitum* with poultry feed (UFA 625, UFA AG, Herzogenbuchsee, Switzerland) for 7-9 days to a weight of 0.5 mg dry mass (DM)/larva. Thereafter, larvae were manually separated from the poultry feed residue and 12 replicates with approximately 200 larvae per replicate were prepared for each treatment. To eliminate possible contaminations by airborne microbes, cross-contamination between substrates, and contamination with human microbes during rearing, larvae were reared in a one-time-feeding bench-scale batch experiment. Larvae were placed in sterile plastic containers (diameter: 100 mm, height: 80 mm) (O118/80, Eco2 NV, Ophasselt, Belgium) with substrates at a feeding rate of 22 mg DM/larva per day for twelve days, resulting in a larval density of 2.5 larvae/cm². Plastic containers were covered with lids (OD118 Filter XL, Eco2 NV, Ophasselt) permitting air flow, while being impermeable to microbes. Containers were placed in a random order in a climate chamber (HPP 260, Memmert GmbH, Büchenbach Germany), providing a microclimate of 28°C and 70% relative humidity. Temperature was automatically recorded every 10 min in the substrate/residue of one replicate per treatment (DS1922L iButton, Maxim Integrated, San Jose, CA, USA).

For each treatment, every three days, for a total of twelve days, three containers were removed from the climate chamber. One residue sample was collected per removed container to determine the physicochemical and microbial parameters in the residue. Larvae were manually separated from the residue, cleaned with tap water, and dried with paper towels. Larvae were counted, weighed, freeze-dried and then stored at 4°C before larval protein content measurement. Residue samples were analyzed for water activity and pH, thereafter freeze-dried and stored to later measure other physicochemical parameters. Weight loss in larvae and residue samples during freeze drying was used to correct all results for moisture content. All manipulations with rearing containers and collection of substrate/residue samples for microbial parameters were performed using sterile techniques under a laminar flow cabinet.

Controls with no larvae

Sterile and non-sterile canteen waste without larvae underwent identical experimental and environmental conditions to those used for BSFL rearing. After 12 days in sterile containers in the climate chamber, samples were collected and processed in the same way as the rearing residue samples.

Physicochemical properties and composition of the residue

Changes in the residue composition were measured through physicochemical parameters which are relevant for microbial growth. Portable meters were used to measure water activity (HygroPalm23-AW, Rotronic, Bassersdorf, Switzerland) and pH (HQ40d, Hach Lange GmbH) in the fresh residue samples. A thermogravimetric instrument (TGA-701, Leco) and a C/N analyzer (Type TruMac CN, Leco) were used to measure moisture and organic matter, and carbon and nitrogen on the freeze-dried residue samples, respectively.

Rearing performance

Residue and larval dry weights as well as larval protein content were used to calculate typical rearing performance indicators per replicate. Larval weight, bioconversion rate, and waste reduction were calculated according to Gold et al. (2020a). The total larval protein per biological replicate (which had 200 larvcae) was calculated using equation (7).

$$\text{Total larval protein} \left(\frac{\text{g DM protein}}{\text{replicate}}\right) =$$

$$\text{larval weight} \left(\frac{\text{g DM}}{\text{larva}}\right) \text{x larval protein content} \left(\frac{\text{g DM protein}}{\text{100 g DM}}\right) \text{x} \frac{\text{200 larvae}}{\text{replicate}} \qquad (7)$$

Each larval protein content was estimated as nitrogen content × 4.67, as proposed by Janssen et al. (2017). Nitrogen was measured on freeze-dried samples using a C/N analyzer (Type TruMac CN, Leco) and corrected for residual moisture with thermogravimetric determinator (TGA-701, Leco).

Microbial counts and bacterial communities

Microbial numbers (i.e., CFU: colony forming units) in the substrate and residue were estimated using plate counts from a dilution series. Microbes were extracted from samples (10 g) by 2 min Stomacher treatment in a medium for recovery of organisms (Difco Maximum Recovery Diluent, BD Diagnostics, Le Pont de Claix, France). For each sample, 50 µL of the dilution series were spread in duplicate on Petri dishes (diameter: 90 mm) divided into four quadrants. Since we partially recorded colonies within the representative range of 20-250 for different dilutions and replicate plates, counts in the Stomacher-homogenate were calculated using equation (8) (Maturin and Peeler, 2001)

$$\frac{\text{CFU}}{\text{mL}} = \frac{\sum c}{V \times (1n_1 + 0.1n_2) \times d} \qquad (8)$$

where $\sum c$ is the number of colonies on all plates, V is the volume added to each plate (0.05 mL), n_i is the number of quadrants counted of the i_{th} dilution, and d is the dilution. Total viable counts (TVC) were determined using standard agar (15 g/L Agar, VWR International, Leuven, Belgium; 30 g/L Tryptic Soy Broth No. 2, Sigma Aldrich GmBH, Buchs, Switzerland), lactic acid bacteria (LAB) on De Man, Rogosa, and Sharpe Agar (MRS, VWR International), and fungi on Dichloran Rose Bengal Chloramphenicol agar (DRBC, Sigma Aldrich GmBH) after incubation at 30°C for 20 to 48 h.

The bacterial community was determined by high-throughput 16S rRNA gene sequencing using the MiSeq Illumina platform. Total genomic DNA was extracted from 0.2 g of substrate or residue sample using the DNeasy PowerSoil Kit (Qiagen, Hilden, Germany) with one modification. To enhance DNA extraction, three sterile metal beads (diameter: 3 mm; Uiker AG, Freienbach, Switzerland) were added to each PowerBead Tube with the lysis buffer and homogenized with a BeadRuptor (Omni International, Kennesaw GA, United States; speed 5.5, 2 x 20 s with 30 s break between rounds). Purity (Nanodrop ND 1000 Spectrophotometer, Thermo Scientific, Wilmington MA, USA) and concentration (Qubit dsDNA HR Assay Kit on a Spark 10 M microplate reader (Tecan, Männedorf, Switzerland) of the extracted DNA was determined.

Library preparation followed a two-step protocol. Limited-cycle PCR was conducted in a 25 µL reaction volume using KAPA HiFi HotStart ReadyMix (12.5 µL) (Kapa Biosystems, Wilmington, MA, USA), template DNA (5 µL), forward and reverse primer (0.75 µL; 0.3 mM each), and molecular-grade water (6 µL). All primers included a hexanucleotide barcode and Illumina adapters (Illumina Inc., San Diego, CA, USA). The prokaryotic V3-V4 hypervariable region was amplified in triplicate using the primer pair 341F (5'- CCT ACG GGN GGC WGC AG 3') and 806R (5'- GGA CTA CNV GGG TWT CTA AT - 3'). PCR conditions were an initial denaturation at 95°C for 300 s, 1 cycle at 98°C for 60 s, 26 cycles of 98 °C for 20 s, 51 °C for 20 s, and 72 °C for 12 s, and a final extension at 72 °C for 120 s (Hugerth et al., 2014). Negative controls were run by replacing template DNA with molecular grade

water. A positive control was run by replacing template DNA with a known mixture of bacterial DNA (i.e., mock sample). Products from the first PCR were pooled, cleaned using solid-phase reversible immobilization beads (ETH Zurich Genetic Diversity Center, Zurich, Switzerland) and used as a template for the second PCR to attach dual indices using the Nextera XT Index Kit v2 (Illumina Inc., San Diego CA, USA). Index PCR included the first PCR product (2 µL), KAPA HiFi HotStart ReadyMix (10 µL), molecular grade water (4 µL), and Nextera indexing primers (2 µL). PCR conditions were an initial denaturation at 95°C for 180 s, 10 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, and a final extension at 72°C for 300 s. Index PCR products were cleaned and DNA concentration was determined using the High Sensitivity D1000 Kit on a 2200 TapeStation (Agilent Technolgies Inc., Santa Clara, CA, USA). Cleaned PCR products were pooled equimolar to a library concentration of 2 nM. The concentration and purity of the pooled library were controlled using a Qubit Fluorometer (Invitrogen Q32857, Carlsbad, CA, USA) and the TapeStation, respectively. Paired-end sequencing was performed using 19 pM of the prepared library in a single MiSeq 2 × 300 bp flow cell, using the MiSeq Reagent Kit v3 and a 10% PhiX concentration according to the manufacturer's directions (Illumina Inc.).

Bioinformatics

Initial quality control of the sequencing data was conducted using FastQC (version 0.11.2). Subsequent bioinformatics data preparation included trimming of read ends with USEARCH (version 11.0.667) and merging of pairs into amplicons with FLASH (Magoč and Salzberg, 2011). Following removal of primer sites with USEARCH, quality-filtering was performed with the PRINSEQ-lite (version 0.20.4) (Schmieder and Edwards, 2011). The resulting high-quality reads were de-noised and clustered into zero-radius operational taxonomic units (ZOTUs) using the UNOISE3 algorithm (Edgar, 2016b). The taxonomic origin of each ZOTU was determined with the SINTAX algorithm (version 11.0.667) (Edgar, 2016a) in USEARCH using Silva 16S (V128) as the reference database. Taxonomic assignments were considered reliable when bootstrap confidence values exceeded 0.85.

Downstream data analyses

Data were analyzed using R version 312 3.6.2 (R Core Team, 2017). Rare ZOTUs with less than 10 total counts and samples with less than 2000 reads (i.e., the highest number of reads in the control samples) were removed before downstream analyses. ZOTUs belonging to the phylum *Cyanobacteria* and to the family *Mitochondria* were also removed, as they likely belonged to eukaryotic 16S rRNA (Michelou et al., 2013), given the plant-based nature of the rearing substrates. We abstained from statistical analyses among sampling days for all parameters due to the small number of replicates (n=1-3). Instead, we compared the results visually or using the mean and standard deviation (when >2, difference between values for n=2). The mean and standard deviation were calculated for rearing performance indicators, physicochemical residue parameters, and microbial counts. Differences in rearing performance indicators between measurement days were calculated by subtracting the mean values. Pearson correlation coefficients ($p < 0.01$) were calculated following visual assessment of normality (see Supplementary Material) to identify linear dependencies between rearing performance indicators and the physicochemical residue composition.

Heat maps at the phylum and genus levels were produced in *ampvis2* (Andersen et al., 2018) after conversion of reads into percent abundance per sample. The same package was used to identify shared ZOTUs between samples with Venn diagrams (frequency cutoff > 80% and abundance

cutoff > 0.01%). Alpha diversity (i.e., observed richness, Chao1, Shannon index, and Simpson Index) and beta diversity (i.e., non-metric multidimensional scaling (NMDS)) were calculated in *phyloseq* (McMurdie and Holmes, 2013). NMDS was used to illustrate the bacterial dynamics of ZOTUs that accounted for more than 1% of relative abundance in all samples using weighted UniFrac distance to account for phylogenic distances between ZOTUs. Distance-based redundancy analysis (dbRDA) was performed on the same data in *vegan* (Oksanen et al., 2019) with the capscale function using the Bray-Curtis Dissimilarity matrix to determine correlations between physicochemical residue properties and composition, rearing performance, and bacterial community dynamics. Prior to analysis, parameters with co-linearity were excluded from the data with a Pearson correlation matrix (see Supplementary Material) and variance inflation factors (VIF) using the *usdm* function. A VIF value > 5 indicated multi-collinearity. The remaining parameters were scaled and centered. The significance of the model and the correlation of each parameter with the bacterial community was determined by the permutation test (1000 iterations) with a p-value < 0.05, denoting significance.

4.4   Results

Waste nutrient composition

The household and canteen waste rearing substrates had similar protein contents, but canteen waste was richer in hemicellulose, lipids, and non-fiber carbohydrates (Table 4.1). Household waste had more cellulose and lignin, as well as glucose and fructose, that was almost absent in the canteen waste.

Rearing performance

Considering the mean and standard deviation, the bioconversion rate and larval protein were similar between the two rearing substrates (Figure 4.1B and 1C), but larval weight (Figure 4.1A) and waste reduction (Figure 4.2A) were higher for household than for canteen waste. Following twelve days of rearing, mean (standard deviation) bioconversion rate and larval protein were 28.0 (2.7) % DM and 4.1 (0.4) g protein/replicate for household waste, and 23.5 (2.1) % DM and 3.6 (0.3) g protein/replicate for canteen waste. Larval weight and waste reduction were higher for household than for canteen waste: 77.6 (7.3) mg DM and 68.6 (1.2) % DM for household waste, and 61.0 (5.2) mg DM and 58.6 (1.0) % DM for canteen waste.

Inactivation of the initial canteen waste microbiota by irradiation reduced rearing performance (Figure 4.1 and Figure 4.2). In rearing with sterile canteen waste, larval weight, bioconversion rate, larval protein levels, and waste reduction were reduced by 18.7 mg DM, 7.3 % DM, 1.3 g protein/replicate, and 26.8 % DM, respectively. Without larvae, 15.3 (1.0) % DM was lost from canteen waste, and -3.9 (1.6) % DM was lost from sterile canteen waste (Figure 4.2B).

Physicochemical residue composition

Rearing performance indicators were positively correlated with residue moisture content (r=0.75-0.86, p < 0.01) and pH (r=0.76-0.81, p < 0.01) (Table 4.2). Moisture and nitrogen content (Table 4.2) increased throughout the rearing experiment in the canteen and household waste residue in comparison to the substrates. The residue pH decreased from the substrate value in the first half of rearing, and then increased above the initial substrate value in the second half of rearing. Residue organic matter had different trends for the two rearing substrates. It decreased from the value in the household waste, but not in the canteen waste substrate. Values for carbon (range: 49.2-56.0% DM), water activity (range:

0.95-0.99), and temperature (range: 27.5-29.9°C) showed low variability throughout the rearing experiment from the initial value (≤ ± 5%, see Supplementary Material). The residue composition changed much less when the initial canteen waste microbiota was inactivated. Except for the residue moisture content and the carbon to nitrogen ratio, the residue composition was ≤ ± 5% relative to the initial value in the substrate.
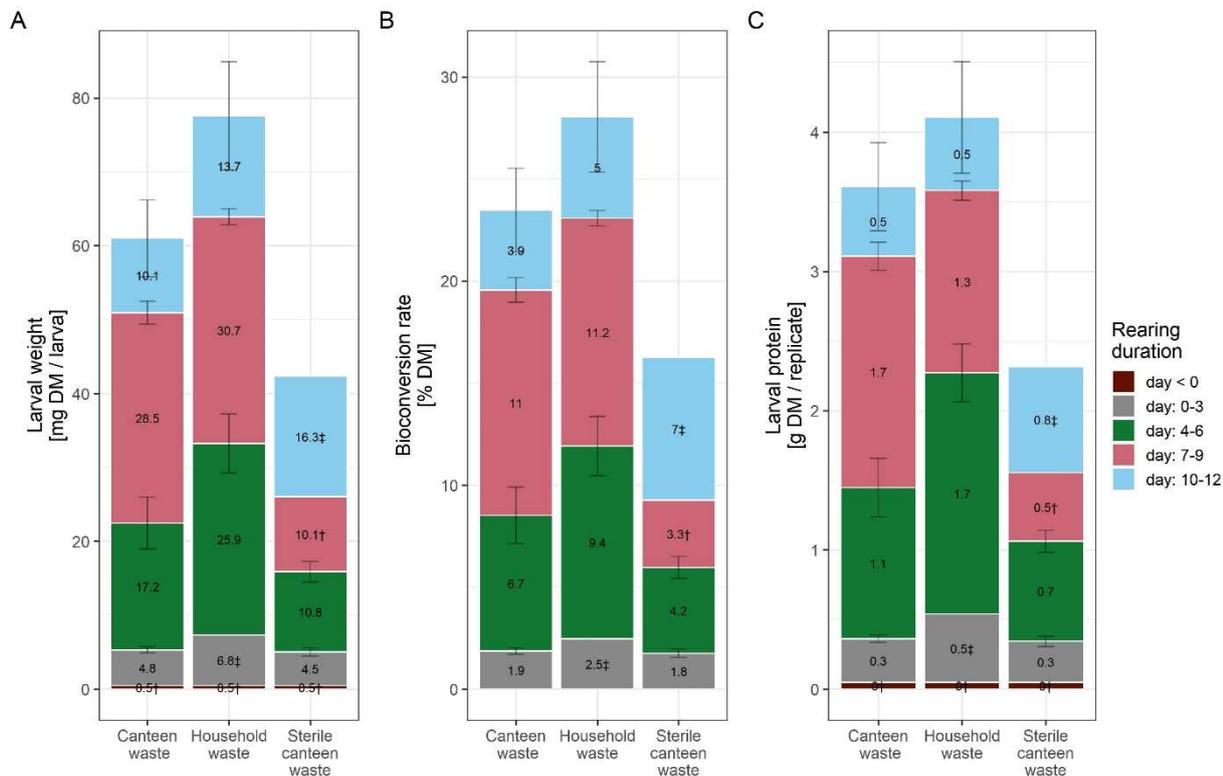
Figure 4.1 Rearing performance of BSFL on canteen and household food waste. Bar plot of larval weight (A), bioconversion rate (B), and larval protein per replicate (C). The barplot label shows the mean increase in the levels of the performance indicator between the measurement days. † (n=1) and ‡ (n=2) indicate results with fewer than three biological replicates.

Table 4.1 Mean nutrient composition of canteen and household waste used for rearing. Nutrients are in percent dry mass and caloric content in kcal per 100 g dry waste.

| | Protein | Non-fiber carbohydrates | | | | Fiber | | | Lipids | P:NFC[1] ratio | Caloric[2] content |
		Total	Glucose	Fructose	Starch	Total	Hemicellulose	Cellulose & lignin			
Canteen waste	15.3 (0.4)	38.6 (0.1)‡	0.3 (0.1)	0 (0.0)‡	37.2 (2.0)	36.1 (2.3)‡	26.9 (1.2)‡	9.1 (1.1)‡	24.5†	1:2	471
Household waste	16.3 (1.1)	29.5 (4.1)‡	5.9 (0.7)	7.5 (1.5)‡	16.5 (2.4)	22.9 (1.5)‡	8.0 (1.1)‡	14.9 (0.4)‡	18.1†	1:2	412

in parenthesis: standard deviation for samples where n ≥ 3 and differences between analyses where n=2

[1] P:NFC = ratio of protein to non-fiber carbohydrates (NFC)

[2] gross caloric content of protein, NFC, and lipids

† n=1, ‡ n=2
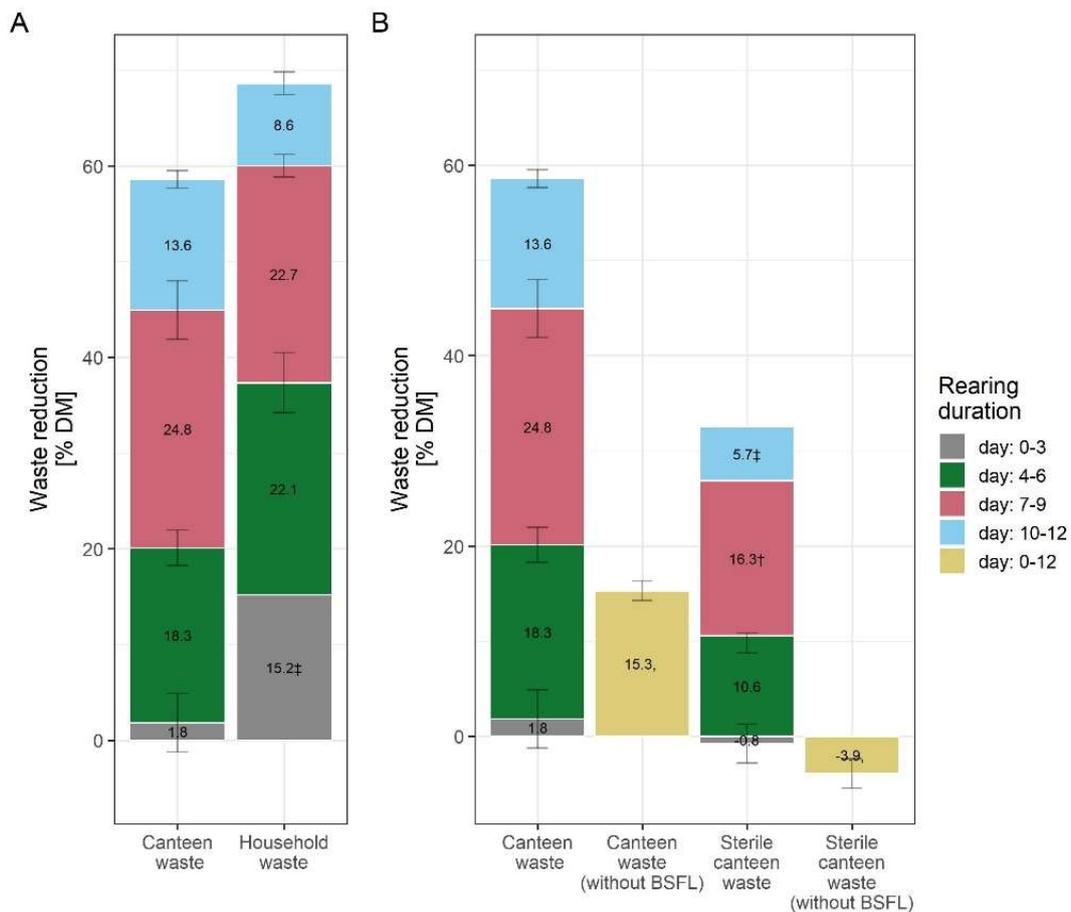
Figure 4.2 Waste reduction during BSFL rearing with canteen and household food waste (A), and on canteen waste without larvae, and sterile canteen waste with and without larvae (B). The barplot label shows the mean increase in the levels of the performance indicators between the measurement days. † (n=1) and ‡ (n=2) indicate results with fewer than three biological replicates.

Microbial dynamics

Canteen and household waste rearing substrates

Canteen and household waste had similar counts of TVC, LAB, and fungi (Table 4.2). Throughout the rearing experiment, residue microbial counts deviated by ±1-3 $\log_{10}$ CFU/g from the counts in the substrate. At the end of the rearing experiment, TVC in the canteen waste residue was 1 $\log_{10}$ CFU/g higher and in the household waste residue 1 $\log_{10}$ CFU/g lower than that in the substrate. LAB counts decreased by 1-2 $\log_{10}$ CFU/g and fungal counts increased by 2-3 $\log_{10}$ CFU/g in the residue in comparison to the counts in the substrate.

Considering all samples, gene sequencing using DNA produced a total of 1,783,860 reads, with an average of 44,597 reads/sample, and a total of 275 ZOTUs. Rarefaction curves (see Supplementary Material) demonstrate that samples were sequenced to an extent sufficient to approximate true diversity.

Table 4.2 Physicochemical properties, composition, microbial numbers, and bacterial community alpha diversity (i.e., richness and diversity) in the substrates and residues. See Supplementary Material for the carbon, water activity, and temperature results.

	Day	Moisture content	pH	Nitrogen	C/N	Organic matter	TVC	LAB	Fungi	Observed richness	Chao 1	Shannon Index	Simpson`s Index
		%	-	%DM	-	%DM	log10/g	log10/g	log10/g	-	-	-	-
BSFL rearing													
Canteen waste	0	69.5 (0.2)‡	4.4 (0.0)‡	2.9†	19.3†	95.9 (0.0)‡	9.2 (0.2)	9.2†	5.2 (0.5)	119†	140†	2.4†	0.8†
	3	69.0 (1.0)	3.8 (0.0)	3.5†	15.2†	95.8 (0.1)	8.4 (0.2)	8.4 (0.2)	6.8 (0.0)	113 (14)	148 (45)	2.4 (0.2)	0.8 (0.0)
	6	71.8 (0.3)	3.7 (0.1)	3.4 (0.3)	16.2 (1.4)	95.2 (0.2)	7.7 (0.0)	7.7 (0.1)	6.7 (0.1)	92 (5)	103 (10)	2.3 (0.1)	0.8 (0.0)
	9	77.4 (1.0)	4.6 (0.1)	3.5 (0.1)	16.0 (0.4)	94.1 (0.2)	8.2 (0.0)	8.2 (0.1)‡	7.1 (0.1)	73 (6)	95 (14)	2.5 (0.1)	0.9 (0.0)
	12	81.1 (0.7)	5.6 (0.2)	3.9 (0.1)	14.3 (0.4)	95.0 (0.2)	8.6 (0.5)	8.1 (0.1)	8.7 (0.1)	57 (5)	76 (11)	2.5 (0.1)	0.9 (0.0)
Sterile canteen waste	0	69.5 (0.2)‡	4.4 (0.0)‡	2.9†	19.3†	95.9 (0.0)‡	n.a.	n.a	n.a	125†	143†	2.4†	0.8†
	3	68.2 (0.5)	3.9 (0.1)	3.1 (0.2)	17.6 (1.4)	95.9 (0.4)	8.6 (0.0)	8.6 (0.1)	6.4 (0.1)	78 (12)	101 (10)	1.5 (0.2)	0.6 (0.1)
	6	69.5 (0.2)	3.8 (0.0)	3.0 (0.1)	18.2 (0.7)	95.4 (0.1)	8.5 (0.1)	8.5 (0.1)	6.9 (0.2)	62 (5)	84 (20)	1.4 (0.2)	0.6 (0.1)
	9	72.0†	3.9†	3.0†	18.0†	95.3†	8.3†	8.0	7.2†	53†	66†	2.0†	0.8†
	12	73.6 (1.2)‡	4.2 (0.0)	3.1 (0.1)	17.9 (0.8)‡	95.8 (0.1)‡	9.3 (0.1)‡	8.2 (0.1)‡	9.0 (0.1)‡	63 (14)‡	89 (30)‡	2.4 (0.1)‡	0.9 (0.0)‡

In parenthesis: standard deviation for samples where n ≥ 3, differences between analyses where n = 2

† n=1, ‡ n=2, n.a. = not analyzed

C/N: carbon to nitrogen ratio

Richness: The number of ZOTUs determined with 16S rRNA gene sequencing based on DNA extracted from samples.

Chao 1: the total number of ZOTUs estimated by infinite sampling. A higher number indicates a higher richness (Chao, 1984).

Shannon Index: Measure richness and evenness. Increases with community richness and evenness (Shannon and Weaver, 1949; Lemos et al., 2011).

Simpson`s Index: Measures community evenness. Index increases as diversity decreases (Simpson, 1949; Lemos et al., 2011).

The canteen waste and household waste bacterial community consisted of 119 and 125 ZOTUs, respectively (Table 4.2). Canteen waste was dominated by a few genera of *Firmicutes*, and household waste by *Firmicutes* and *Proteobacteria* (Figure 4.3). Dominant genera were *Leuconostoc*, *Bacillus*, *Staphylococcus* in the canteen waste and *Leuconostoc*, *Weissella*, *Pseudomonas*, *Oxalobacteraceae*, and *Lactococcus* in the household waste. Sixty two percent of the relative abundance of the two wastes was due to 28 shared ZOTUs, while 49 ZOTUs were unique to canteen waste and 34 ZOTUs were unique to household waste (see Venn diagram in Supplementary Material). Unique high-abundance (>10%) species were from *Pseudomonas* and *Oxalobacteraceae* in household waste and *Staphylococcus* and *Bacillus* in canteen waste.
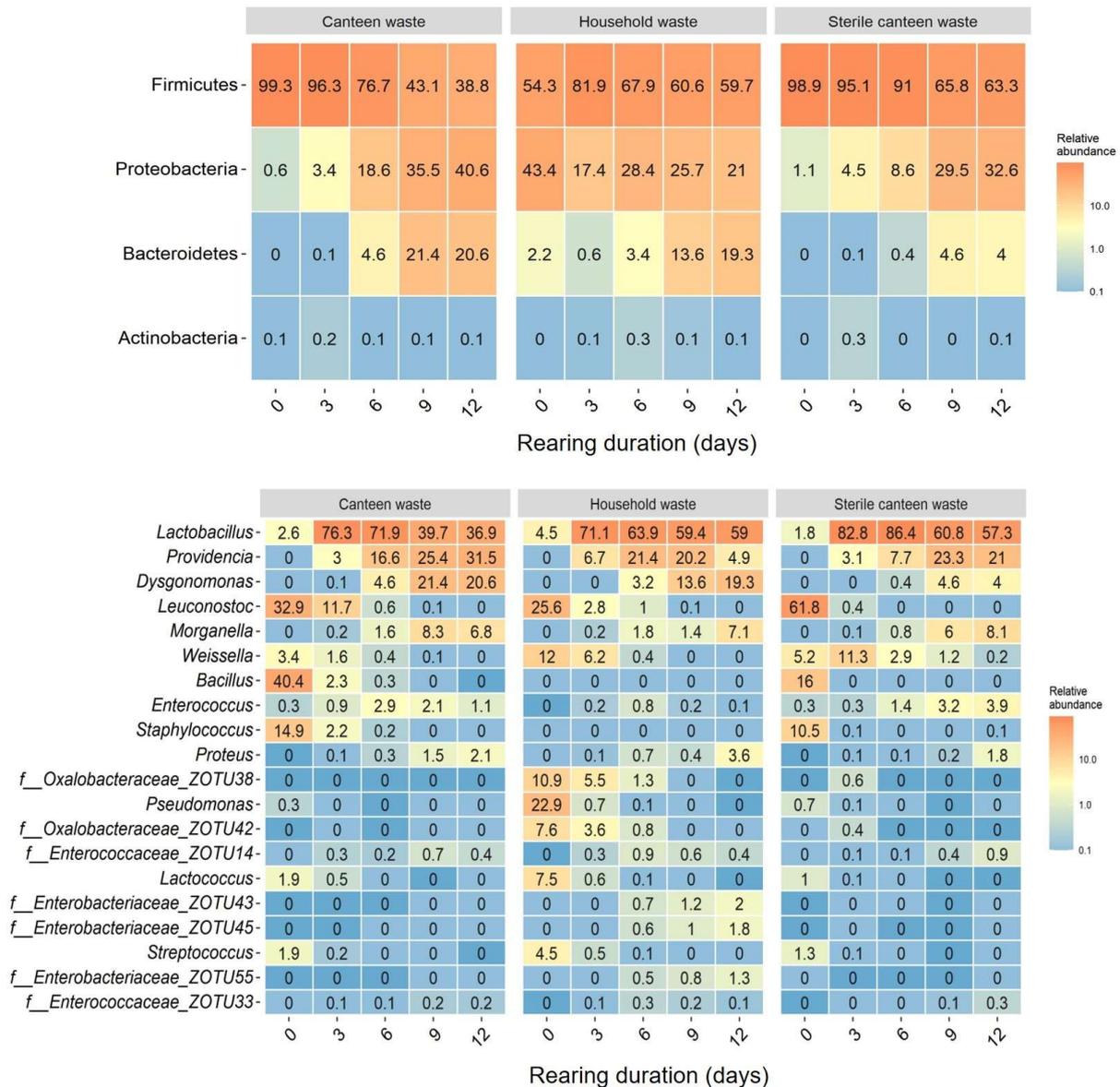
Figure 4.3 Substrate and residue bacterial community at different days (0-12 days) of BSFL rearing. Heatmaps of the top phyla (top) and top 20 genera (bottom) based on the relative abundance of ZOTUs in all samples. Relative abundances are the mean of replicate samples rounded to one digit. If no clear assignment to a genus was possible, the family assignment is shown together with the ZOTU.

Table 4.2 (continuation) Physicochemical properties, composition, microbial numbers, and bacterial community alpha diversity (i.e., richness and diversity) in the substrates and residues. See Supplementary Material for the carbon, water activity, and temperature results.

	Day	Moisture content	pH	Nitrogen	C/N	Organic matter	TVC	LAB	Fungi	Observed richness	Chao 1	Shannon Index	Simpson`s Index
		%	-	%DM	-	%DM	log10/g	log10/g	log10/g	-	-	-	-
BSFL rearing													
Household waste	0	76.7 (0.1)‡	4.8 (0.0)‡	3.1†	16.7†	94.3 (0.1)‡	9.2 (0.1)	9.0 (0.3)‡	5.4 (0.2)	122†	147†	2.6†	0.9†
	3	79.4 (0.2)‡	3.9 (0)‡	3.3†	15.8†	93.7 (0.0)‡	8.6 (0.1)‡	8.7 (0.1)‡	6.6 (0.1)‡	122 (52)‡	142 (61)‡	2.0 (0.6) ‡	0.7 (0.2)‡
	6	83.0 (0.5)	3.9 (0.0)	3.2 (0.1)	16.5 (0.4)	92.1 (0.4)	8.0 (0.1)	7.9 (0.1)	6.3 (0.2)	99 (7)	122 (13)	2.3 (0.1)	0.8 (0.0)
	9	88.2 (0.4)	4.5 (0.1)	3.2 (0.1)	15.7 (0.5)	88.8 (0.4)	7.6 (0.1)	7.6 (0.1)	5.0 (0.1)	67 (9)	89 (14)	2.1 (0.0)	0.8 (0.0)
	12	90.2 (0.3)	6.5 (0.6)	3.6 (0.1)	13.8 (0.5)	85.5 (0.6)	10.0 (0.5)	7.5 (0.0)	7.5 (1.3)‡	62 (5)	88 (9)	2.2 (0.1)	0.8 (0.0)
Without BSFL													
Canteen waste	12	72.2 (0.3)	4.8 (0.3)	3.8 (0.0)‡	14.8 (0.2)‡	95.2 (0.1)‡	n.a	n.a	n.a	106 (4)‡	122 (10)‡	2.7 (0.0)‡	0.9 (0.0)‡
Sterile canteen waste	12	66.9 (0.4)	4.0 (0.1)	3.1 (0.1)	17.2 (0.5)	95.7 (0.0)‡	n.a	n.a	n.a	97 (9)	133 (11)	1.6 (0.3)	0.6 (0.1)

In parenthesis: standard deviation for samples where n ≥ 3, differences between analyses where n = 2

† n=1, ‡ n=2, n.a. = not analyzed

C/N: carbon to nitrogen ratio

Richness: The number of ZOTUs determined with 16S rRNA gene sequencing based on DNA extracted from samples.

Chao 1: the total number of ZOTUs estimated by infinite sampling. A higher number indicates a higher richness (Chao, 1984).

Shannon Index: Measure richness and evenness. Increases with community richness and evenness (Shannon and Weaver, 1949; Lemos et al., 2011).

Simpson`s Index: Measures community evenness. Index increases as diversity decreases (Simpson, 1949; Lemos et al., 2011).

The results for the bacterial community alpha and beta diversity demonstrated that the addition of BSFL to the substrates dramatically changed the bacterial richness (Table 4.2) and community (Figure 4.4 and Figure 4.5). Community richness decreased on both wastes throughout rearing, and the bacterial community between the two substrates became more similar. Replicate samples clustered well based on UniFrac distances according to rearing day and substrate. After three days of rearing, the bacterial communities were more similar to each other than the initial wastes (Figure 4.4A). Changes in the bacterial community were the largest within the first six days. The similarity between bacterial communities decreased again following nine days. In both residues, most bacteria belonged to the genus *Lactobacillus*. Throughout the rearing period, the phyla *Proteobacteria* and *Bacteroidetes*, and the genera *Providencia*, *Dysgonomonas*, *Morganella*, and *Proteus* became more abundant than in the substrate.

Sterile canteen waste

Despite complete microbial inactivation by irradiation, the sterile canteen waste substrate had a similar bacterial community as the non-sterile canteen waste (Table 4.2, Figure 4.3 and Figure 4.5). Sterile canteen waste and canteen waste shared ZOTUs that accounted for 99.5% of the relative abundance (see Venn diagram in Supplementary Material). Bacterial dynamics were also similar between sterile and non-sterile canteen waste (Figure 4.4B). The addition of BSFL to the sterile canteen waste led to a repopulation of the substrate to microbial numbers similar to those determined in the non-sterile canteen waste (Table 4.2). *Lactobacillus* was also highly abundant in the sterile canteen waste residues, decreased in abundance during rearing, and *Providencia*, *Dysgonomonas*, *Morganella*, and *Proteus* became more abundant, but to a smaller extent compared to non-sterile canteen waste.

Substrates without larvae

The bacterial community of sterile canteen waste was similar to the initial bacterial community of the canteen waste substrates, after 12 days of storage (Figure 4.4B). However, for both sterile and non-sterile canteen waste, it differed noticeably from the bacterial communities in the rearing residues (Figure 4.5). The substrates stored without larvae had a higher bacterial richness (Table 4.2) and *Leuconostoc*, *Stenotrophomonas*, *Hafnia-Obesumbacterium*, *Lactococcus*, and *Enterobacteriaceae* were highly abundant but absent in the rearing residues (Figure 4.5). Genera that became more abundant in the rearing residues (i.e., *Providencia*, *Dysgonomonas*, *Morganella*, and *Proteus)* were absent from the substrates stored without larvae.

Correlation among bacterial community, rearing performance, and residue composition

Bacterial community dynamics correlated with rearing performance and physicochemical properties and composition of the residue (Figure 4.6). Of all parameters, larval weight, and residue pH, carbon, nitrogen, and water activity had the lowest co-linearity and were used in distance-based redundancy analysis (dbRDA). The global dbRDA model and the first two axes were statistically significant and explained 83.5% of the variability in the bacterial community.

A

B
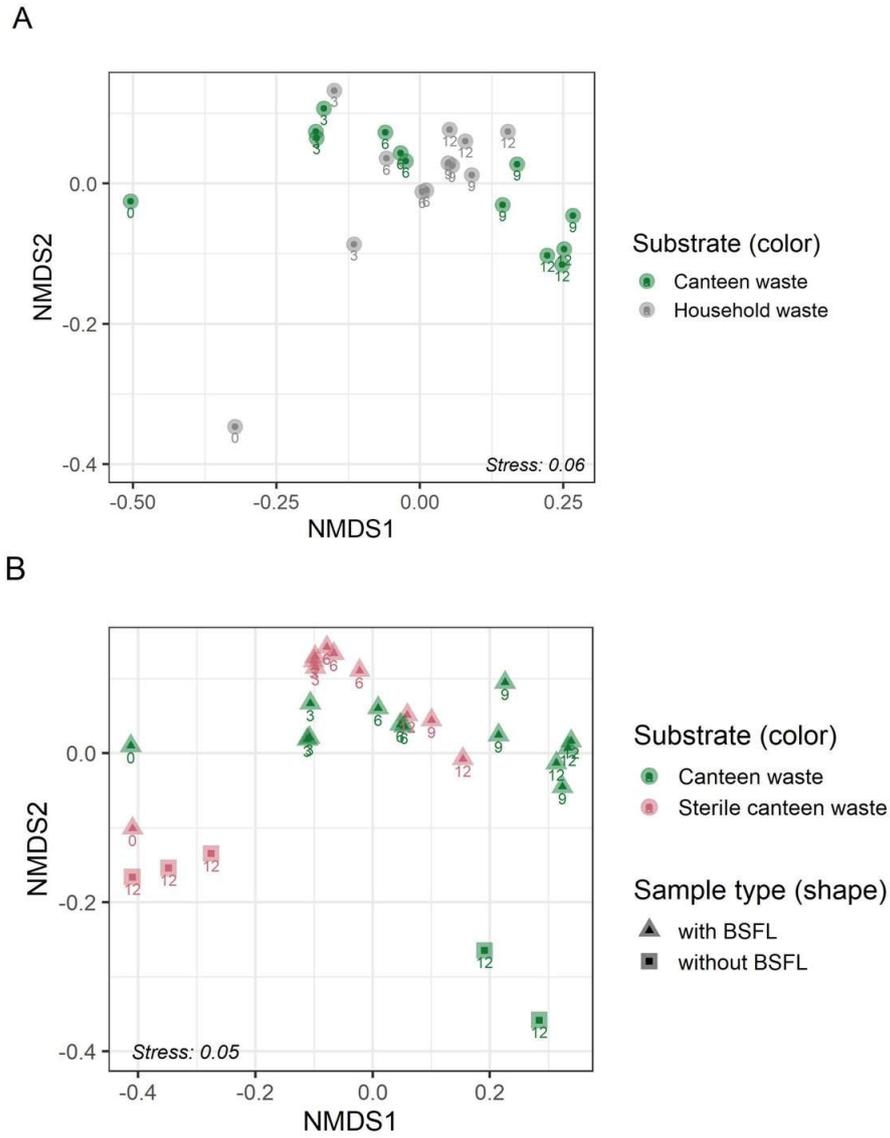
Figure 4.4 Residue bacterial community dynamics in the two food wastes (A) and in canteen waste (sterile, non-sterile) (B) during rearing illustrated by non-metric multidimensional scaling (NMDS) of bacterial communities in the substrate and residue based on weighed UniFrac dissimilarity. Numbers adjacent to the symbols indicate the sampling day during BSFL rearing (0–12 day).
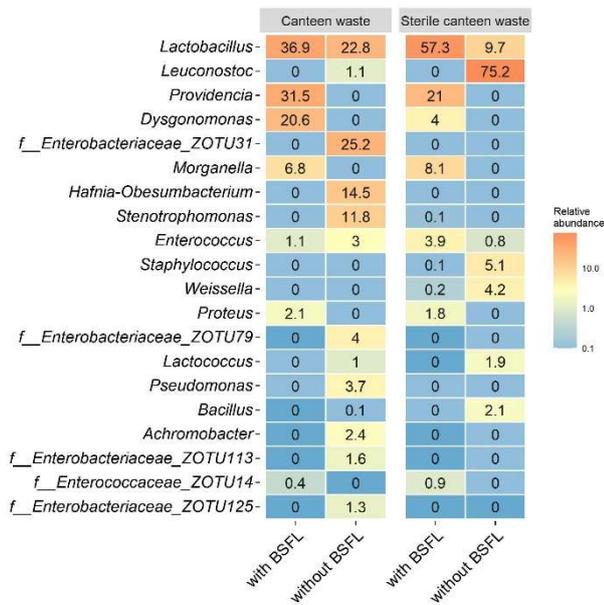
Figure 4.5 Bacterial community in canteen waste (sterile, non-sterile) with and without larvae following 12 days of rearing or storage. Heatmaps of the top 20 genera based on the relative abundance of ZOTUs in all samples. Relative abundances are the mean of replicate samples rounded to one digit. If no clear assignment to a genus was possible, the family assignment is shown together with the ZOTU.
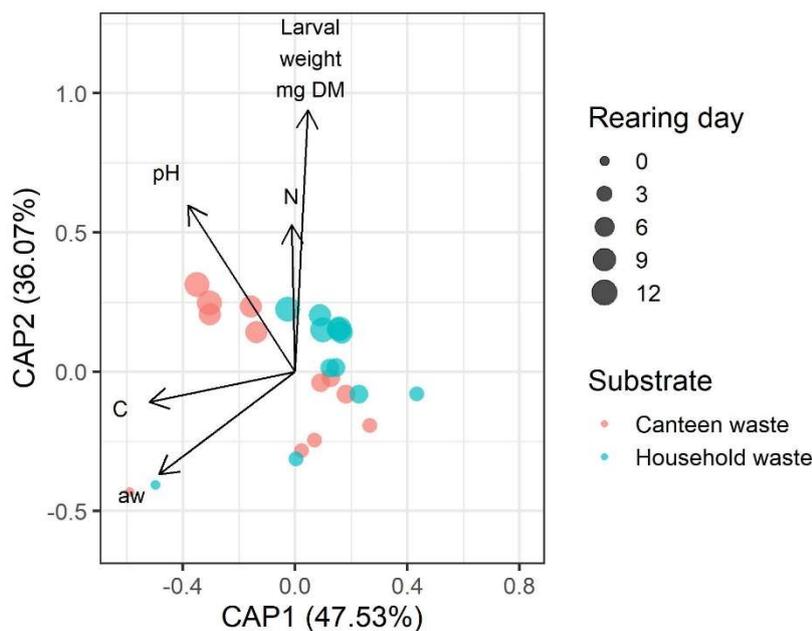
Figure 4.6 Distance-based redundancy analysis (dbRDA) biplot of canteen waste and household waste samples showing the correlation between the physicochemical properties and composition of the residue (pH, N, C, aw), larval weight, and the bacterial community. The length of the vectors indicates the relative importance of the parameter. The vector angle between variables indicates a correlation. Variables with smaller angles between vectors have a closer positive correlation. Perpendicular vectors indicate that there is no correlation. Vectors pointing in opposite directions indicate a negative correlation. Shorter distance between points indicates similarity between bacterial communities.

4.5   Discussion

Substrate microbiota contributes to rearing performance

We compared results for substrate and residue bacterial communities and BSFL rearing performance indicators between sterile and non-sterile canteen waste. We hypothesized that inactivation of the initial bacterial community in the rearing substrate will decrease rearing performance and reveal important members of the microbiota influencing improved rearing performance.

Our hypothesis was confirmed as results show that inactivation of microbes in the substrate reduced rearing performance (Figure 4.1 and Figure 4.2), suggesting that the initial substrate microbial community contributes to substrate decomposition and/or larval growth. The initial canteen waste microbial community was dominated by LAB (Figure 4.3), which is typical for fermented foods (Wu et al., 2018) and grain-based substrates (Wynants et al., 2019) with high contents of digestible carbohydrates (i.e., starch) (Table 4.1) and low pH (Table 4.2). LAB that have been shown to promote the growth of *Drosophila melanogaster* (Shin et al., 2016; Storelli et al., 2018), are routinely used as feed additives to promote the growth of farmed animals (e.g., poultry, pigs, and cattle) (Vieco-Saiz et al., 2019), and the addition of LAB (e.g., *Lactobacillus buchneri*) to the substrate has been shown to increase BSFL rearing performance (Somroo et al., 2019; Mazza et al., 2020). The mechanisms by which LAB promote growth are debated and still part of ongoing research, but suggestions include an increase of the metabolic capacity by fermentation of substrate carbohydrates into short chain fatty acids (as indicated by the low pH in the residue, Table 4.2), growth signaling, immunity, and protection and maintenance of stable gut microbiomes (Holzapfel et al., 2001). Interestingly, bacteria and fungi in the digestive tract or on the surface of larvae repopulated the high-energy electron beam treated canteen waste during rearing (Table 4.2) but rearing performance indicators remained nevertheless lower given the loss of the initial microbial community (Figure 4.1 and Figure 4.2).

Intact DNA after high-energy beam treatment interfered with further interpretation of bacterial dynamics in the sterile canteen residue. Despite lethal irradiation doses (Gold et al., 2020c), similar bacterial community between sterile and non-sterile canteen waste (**Figure 4.5**) after 12 days indicates that some bacterial DNA remained intact and was considered in the bacterial community based on DNA sequencing. As the bacterial communities identified in the sterile canteen waste residue may also include members without any major metabolic functions, these results should be interpreted with caution and are not further discussed. This finding agrees with recent research on the effect of irradiation on bacteria. Hieke and Pillai (2018) reported that *Escherichia coli* maintain their cell integrity post-irradiation, which protects DNA from denaturation. Since larvae and the associated microbiota digest bacteria (Gold et al., 2018a), it is possible that the determined bacterial community becomes more representative of the viable bacterial community with increasing rearing duration.

Rearing performance between the two food wastes

While it has been recognized that both the rearing substrate nutrient composition and the microbial community (De Smet et al., 2018) composition influence rearing efficiency and reliability, previous studies typically emphasized only one aspect (Bruno et al., 2019; Klammsteiner et al., 2020) or considered both aspects but in isolation (Wynants et al., 2019; Gold et al., 2020a). We determined both substrate nutrient contents and bacterial communities and this over the rearing duration. We purposely used two rearing substrates with similar nutrient contents. Thereby we expected that the differences in rearing performance could be more easily attributed to the different substrate bacterial communities and could reveal members associated with rearing performance rather than showing the effect of nutritional differences.

Rearing performance was high with both food wastes (Figure 4.1 and Figure 4.2). We expected this based on the high nutrient contents (Table 4.1). Previous studies have reported lower bioconversion rates for food waste of 13.9-22.7% DM (Nyakeri et al., 2017b; Lalander et al., 2019; Gold et al., 2020a) as compared to 23.5-28.0% DM in this study. Waste streams with low nutrient contents, such as digested waste water sludge, or cow and poultry manure, typically have even much lower bioconversion rates of 2.2-3.8% DM (Lalander et al., 2019; Gold et al., 2020a). Since the bioconversion rate is a key indicator determining the economics of insect rearing and the environmental sustainability of insect-derived products (Smetana et al., 2019), the results show that food waste could be an especially viable substrate for BSFL rearing.

Rearing performance was similar between the two rearing substrates with regard to conversion efficiency (Figure 4.1). However, larval biomass production (Figure 4.1) and waste reduction (Figure 4.2A) were higher for household than for canteen waste substrates. As demonstrated by the reduction in rearing performance due to the loss of the initial microbial community, differences in the initial substrate bacterial community could have contributed to the differences in this rearing performance. Notably, between the two substrates, there was a considerably higher waste reduction within the first three days of rearing (Figure 4.2A). However, as the two substrates shared most taxa, no conclusions can be drawn on bacteria that may explain the differences in rearing performance between substrates. It appears to be more likely that the disparity in rearing performance between substrates is due to different content and digestibility of nutrients. For example, non-fiber carbohydrates in household waste are likely more digestible for BSFL, considering that they were mostly comprised of glucose and fructose (Table 4.1). Pimentel et al. (2018) demonstrated that *Musca domestica* larvae can directly absorb glucose in the anterior and posterior midgut. Starch however requires catalysis before absorption and comprises all non-fiber carbohydrates in the canteen waste (Table 4.1) therefore being less directly digestible. In addition, household waste digestibility may have been increased by the onset of microbial substrate decomposition during storage (i.e., in the order of hours to days) at the household level (Albuquerque and Zurek, 2014). In contrast, canteen waste was collected on the same day of waste generation and stored at 4°C.

In summary, these findings demonstrate the challenge of unambiguously identifying the causes for differences in rearing performance despite comprehensive analysis of nutrient contents and bacterial communities. Differences in rearing performance could be due to differences in the low-abundance taxon or due to differences on the species level for the same genus. In addition, although Bruno et al. (2019) and Klammsteiner et al. (2020) concluded that bacterial gut communities are rather similar

between substrates with broadly similar nutrient contents (Table 4.1), differences in bacterial community structure and function among substrates, as demonstrated by Zhan et al. (2020), may also have contributed to rearing performance differences.

Common fly-associated bacteria dominate the rearing residue

Microbial dynamics in the rearing residues of BSFL are still poorly understood. We studied bacterial community dynamics to identify taxa that were more abundant during BSFL rearing and that were absent in the controls (i.e., substrates stored under the same environmental conditions but without larvae). As suggested by previous researchers (Zhao et al., 2017; Ao et al., 2020), we hypothesized that bacteria enriched in the rearing residue contribute to substrate decomposition. This could imply that pure-culture bacteria and/or defined bacterial mixtures comprised of these bacteria from the residue could potentially increase large-scale BSFL rearing.

Previous studies have reported inconsistent results on the significance of altered substrate bacterial community during BSFL rearing (Bruno et al., 2019; Jiang et al., 2019; Cifuentes et al., 2020). Our study confirmed that BSFL rearing dramatically changes the substrate bacterial community and physicochemical properties and composition. For example, consistent with previous research, the pH increased in the residue during BSFL rearing (Table 2) (Erickson et al., 2004; Lalander et al., 2014; Ma et al., 2018; Jiang et al., 2019; Wynants et al., 2019; Klammsteiner et al., 2020). Characteristic for food waste decomposition, the residue pH initially decreased due to the hydrolysis of proteins (Wu et al., 2018). Following 6 days of rearing, the pH increased beyond the initially value of the substrate, presumably due to the excretion of nitrogenous compounds by BSFL (e.g. uric acid) (Klammsteiner et al., 2020). Similar to the results of Jiang et al. (2019), ordination plots (Figure 4.4) revealed an obvious succession of the bacterial community throughout BSFL rearing. Consistent with previous research, this study observed a reduction in bacterial richness (Table 4.2) (Jiang et al., 2019; Wynants et al., 2019) and an increase in residue pH (Table 4.2) (Erickson et al., 2004; Lalander et al., 2014; Ma et al., 2018; Jiang et al., 2019; Wynants et al., 2019; Klammsteiner et al., 2020) in the substrate and residue over the rearing duration. In contrast to Wynants et al. (2018) and Jiang et al. (2019), Bruno et al. (2019) did not observe significant differences in the bacterial community between substrates and residues, and Cifuentes et al. (2020) observed an increase in bacterial richness. Different abiotic (e.g., temperature, substrate nutrient content, and pH) and biotic (e.g., initial substrate bacterial communities) factors known to influence microbial ecology and the presence and stability of antimicrobial proteins by BSFL (De Smet et al., 2018; Vogel et al., 2018) may provide some explanation for discrepancies in findings among studies. Wynants et al. (2018) studied BSFL rearing with a variety of mostly grain-based substrates in several laboratory and industry-scale settings, Jiang et al. (2019) with food waste in an industry-scale setting, and Bruno et al. (2019) with a standard substrate for fly rearing, vegetables and fish waste in a laboratory setting (Table 4.3). Considering that substrate digestion by BSFL (Cai et al., 2018; Bruno et al., 2019) and other fly larvae (e.g., *Lucilia sericata,* Diptera: Calliphoridae) (Mumcuoglu et al., 2001; Lerch et al., 2003) decreases bacterial richness along the digestive tract (Gold et al., 2018a; Vogel et al., 2018), different feeding rates, intervals, and larval densities among studies could be especially relevant (Table 4.3). One could expect that the substrate and residue bacterial community is altered to a greater extent by BSFL when less feed is provided per larvae (i.e., lower feeding rate and/or higher larval density). Our study and that of Jiang et al. (2019) had a 2-3-fold higher larval density and feed was provided less frequently when compared to Bruno et al. (2019), thus allowing more time for larval digestion (Table 4.3). Cifuentes et al. (2020) provided an insufficient rearing protocol to allow

comparison with the other studies. A further study focusing more on microbial dynamics in BSFL under different rearing parameters is therefore recommended. However, considering the results of this and previous studies, and that large-scale rearing facilities may have higher larval densities (e.g., 4 larvae per cm² by Dortmans et al. (2017)), a lower number of bacterial species can be expected in well-digested residues in comparison to the initial substrates.

Genera in the residues at the time of larval harvest are ubiquitous in the environment (e.g., soil, water, digestive tracts of humans and farmed animals) and have been previously identified in different life stages of the BSF (Table 4.3) (Bruno et al., 2019; Cifuentes et al., 2020; Huang et al., 2020; Klammsteiner et al., 2020; Raimondi et al., 2020; Zhan et al., 2020), *Musca domestica* (Zurek et al., 2000; Su et al., 2010; Gupta et al., 2012), and *Lucilia sericata* (Singh et al., 2014). Bacteria commonly associated with BSFL residues (Table 4.3) are from the *Lactobacillaceae*, *Bacillaceae*, *Enterobacteriaceae*, *Planococcaceae*, and *Flavobacteriaceae* families. One possible explanation for the recurrence of these taxa in fly larvae residues is through the transfer of intestinal commensal bacteria with larval secretions and excretions into the residue (Zhao et al., 2017; Storelli et al., 2018). The results of this study support this hypothesis. Recurring taxa in the residues belong to the phyla *Firmicutes*, *Proteobacteria*, and *Bacteroidetes*, which are the main intestinal bacteria in fly larvae (Zurek et al., 2000; Jeon et al., 2011; Boccazzi et al., 2017; Scully et al., 2017; Cifuentes et al., 2020; Liu et al., 2020). *Providencia* spp., *Dysgonomonas* spp., *Morganella* spp., and *Proteus* spp. present in the BSFL residues, and absent in controls without larvae, are highly abundant in the BSFL guts (Ao et al., 2020; Cifuentes et al., 2020; Huang et al., 2020; Klammsteiner et al., 2020; Raimondi et al., 2020). Identification of *Dysgonomonas*, *Providencia*, *Morganella*, and *Proteus* in the posterior midgut of BSFL suggest that members of these genera may survive gut passage (Bruno et al., 2019). The one-time feeding regime, low feeding rate, and high larval density in our experiments could have contributed to the more pronounced appearance of these genera than in previous studies (Bruno et al., 2019; Wynants et al., 2019), as the residue presumably passed the digestive tract more often than that at higher feeding rates and frequency and lower larval densities. However, it is important to bear in mind that these genera are typically present at low abundance (<0.01% in this study) in BSFL substrates (see Figure 4.3) (Bruno et al., 2019; Shelomi et al., 2020). Consequently, the proliferation of these genera during BSFL rearing could also have been in part due to the observed changes in the residue physicochemical properties and composition, and not only the secretions/excretions of BSFL. For example, members of the genera *Proteus*, *Providencia*, and *Morganella* are involved in urea hydrolysis and thus may benefit from the nitrogenous compounds excreted by BSFL (Manos and Belas, 2006; Klammsteiner et al., 2020).

Table 4.3 Literature summary of bacterial communities in BSFL rearing residues. Families and genera identified in at least two studies are highlighted in bold. The bacterial community was identified in all studies by 16S rRNA gene sequencing

Reference	Substrate	Major families*	Major genera**	Feeding rate mg DM/day	Feeding interval -	Larval density BSFL/cm²
This study	Canteen waste Household waste	***Lactobacillaceae*, *Enterobacteriaceae*,** *Porphyromonadaceae*	***Lactobacillus*, *Providencia*,** *Dysgonomonas*, ***Morganella***	22	One time	2.5
Jiang et al. (2019)	Food waste	*Corynebacteriaceae*, ***Bacillaceae***	*Corynebacterium*, ***Bacillus*, *Lactobacillus*,** *Enterococcus*	125	Every 2 d	2.9
Bruno et al. (2019)	Substrate for fly rearing Vegetable waste	***Flavobacteriaceae*,** *Comamonadaceae*, *Sphingobacteriaceae*, *Stenotrophomonas* ***Lactobacillaceae*,** *Methylocystaceae*	*Flavobacteriaceae*, *Comamonas*, ***Sphingobacterium*,** *Stenotrophomonas* ***Lactobacillus*,** *Methylocystaceae*	*ad libitum*	Every 1-2 d	1.0
	Fish waste	***Planococcaceae*,** *Clostridiales*, *Bacilli*, ***Peptostreptococcaceae*,** *Clostridiaceae*	*Planococcaceae*, *Clostridiales*, *Bacilli*, *Tissierella*, ***Clostridium***			

*relative abundance > 10%, in order of descending relative abundance
**relative abundance > 5%, in order of descending relative abundance; whenever the genera could not be identified, the lowest available taxonomic rank (i.e. family or class) is reported

Dysgonomonas, *Providencia*, *Proteus,* and *Morganella* have important functions in the life cycle of fly species. Despite their prominence in the digestive tracts and residues, the ways in which they influence larvae and substrate decomposition are still poorly understood. Studies focusing on the role of these bacteria in BSFL rearing do not exist. *Morganella* spp. and *Providencia* spp. are typically transferred between generations in several fly species (Su et al., 2010), and *Proteus spp.* have been isolated from the egg surface of the BSF (Mazza et al., 2020) and the digestive tract of *Musca domestica* (Su et al., 2010; Gupta et al., 2012). One function of these bacteria appears to be stimulation of the fly ovipositor by the release of volatile compounds. Different species of these genera, such as *P. mirabili,* have been shown to control fly ovipositon in *Lucilia sericata* (Ma et al., 2012; Tomberlin et al., 2012; Uriel et al., 2020) and *Cochliomyia hominivorax* (Diptera: Calliphoridae*)* (DeVaney et al., 1973; Eddy et al., 1975). In addition, *P. mirabili* may repel bacteria that are detrimental to larval development. *P. mirabilis* is associated with *Lucilia sericata* and exerts bactericidal effects (Erdmann and Khalil, 1986), but the antimicrobial excretion/secretion from *Lucilia sericata* is not active against *P. mirabilis,* suggesting a symbiotic host-microbe relationship (Barnes et al., 2010). In addition, bacteria may support the decompostion of substrate constituents (Zhao et al., 2017). Bruno et al. (2019) identified *Dysgonomonas* and Aoi et al. (2020) *Providencia* as major genera in the BSFL digestive tracts and proposed that their members could be involved in the digestion of hemicellulose and proteins and lipids, respectively. Similarly, *Dysgonomonas* and *Providencia* could contribute to nutrient decomposition in the residue.

However, it should be noted that there is considerable uncertainty with these claims as they are based on findings for phylogenetically different well-studied insects (e.g., honeybees and termites), correlations between bacterial communities and environmental parameters (e.g., substrate nutrients), or functional predictions based on DNA sequencing, which may not provide direct evidence for bacterial community functional capacities.

We demonstrated that there is considerable overlap between bacterial communities in BSFL residues and digestive tracts. We hypothesize that some members of these genera may influence substrate decomposition and larval development, and therefore have the potential to increase the performance of large-scale BSFL rearing. A natural progression of this work is to isolate members of these genera from residues or larval digestive tracts, and assess their potential to increase rearing performance by adding them to the rearing substrates *in vivo* (Yu et al., 2011; Xiao et al., 2018; Rehman et al., 2019; Somroo et al., 2019; Mazza et al., 2020) and *in vitro* (Gold et al., 2020d). Further research is needed to better understand the variable effectiveness among bacterial species (Mazza et al., 2020), and among strains of the same species (Yu et al., 2011), in influencing rearing performance under variable biotic and abiotic conditions typical in practice. These studies should use or imitate large-scale rearing conditions (e.g., larval densities) to ensure maximum transfer of results into practice. Even though we used realistic rearing conditions (i.e., feeding rates and larval densities), residue temperatures (27-30°C) influencing bacterial communities in BSFL (Raimondi et al., 2020) were below those found in large-scale rearing (e.g., 33-45°C) (Bloukounon-Goubalan et al., 2020) due to the bench-scale nature of our study.

Table 4.3 (continuation) Literature summary of bacterial communities in BSFL rearing residues. Families and genera identified in at least two studies are highlighted in bold. The bacterial community was identified in all studies by 16S rRNA gene sequencing

Reference	Substrate	Major families*	Major genera**	Feeding rate mg DM/day	Feeding interval -	Larval density BSFL/cm²
Wynants et al. (2018)	Fruit and vegetable waste	**Enterobacteriaceae**, **Bacillaceae**, *Planococcaceae*	*Cosenzaea*, **Bacillus**, **Morganella**, **Providencia**, **Sporosaricina**	*ad libitum*	Every 3-4 d	-
	Supermarket/restaurant waste	*Lactobacillaceae, Enterobacteriaceae*	**Lactobacillus**, **Morganella**, **Providencia**			
	Poultry blood	**Lactobacillaceae**, **Enterobacteriaceae**, *Clostridiaceae*, **Peptostreptococcaceae**	**Lactobacillus**, *Buttiauxella*, **Clostridium**, *Pediococcus, Peptostreptococcus*			
	Poultry manure	**Bacillaceae**, *Carnobacteriaceae*	**Gracilibacillus**, *Virgibacillus, Lentibacillus*, **Atopostipes**, **Amphibacillus**, **Bacillus**, **Oceanobacillus**			
	Brewery waste, fruit waste	*Pseudomonadaceae, Sphingobacteriaceae*, **Flavobacteriaceae**, **Bacillaceae**	*Pseudomonas*, **Sphingobacterium**, **Flavobacterium**, *Myroides*, **Bacillus**, **Oceanobacillus**, *Mucilaginibacter*			
	Vegetable waste, mill by-products, yeast	**Bacillaceae**, **Planococcaceae**	**Gracilibacillus**, **Oceanobacillus**, **Sporosaricina**			
	Fruit and vegetable waste, brewery waste, former food products, food waste	**Bacillaceae**, *Pseudomonadaceae, Carnobacteriaceae*, **Flavobacteriaceae**, **Bacillaceae**	**Bacillus**, **Amphibacillus**, *Thiopseudomonas*, **Atopostipes**, **Flavobacterium**, *Gracilibacillus*			
Shelomi et al. (2020)	Soy pulp, canteen waste	*Cellvibrionaceae, Cytophagaceae, Caulobacteraceae*	*Cellvibrio, Leadbetterella, Brevundimonas*	*ad libitum*	Daily	-

*relative abundance > 10%, in order of descending relative abundance
**relative abundance > 5%, in order of descending relative abundance; whenever the genera could not be identified, the lowest available taxonomic rank (i.e. family or class) is reported

Implications for product safety

Despite not being the main focus of this research, our results present relevant findings regarding BSFL rearing product safety. BSFL substrates may have pathogenic microbes (Erickson et al., 2004; Lalander et al., 2013) and since BSFL live within their rearing substrate and pass it through their digestive tract, pathogenic microbes inside or on the harvested larvae are a hazard for product safety. Such pathogenic microbes in the harvested larval biomass can be eliminated by thermal or non-thermal inactivation technologies. An alternative approach could be the inactivation of pathogenic microbes in the substrate before BSFL rearing, for instance by irradiation. Our results suggest that such an approach may greatly decrease rearing performance. Future research should be undertaken to mimic more realistically rearing facility substrate inactivation technologies (e.g., pasteurization) and conditions (e.g., time, temperature). Some technologies may only lead to partial microbial inactivation and at the same time reduce particle size and increase nutrient digestibility, impacting positively on rearing performance. However, considering that fly larvae may live in close association with pathogens such as *Providencia rettgeri*, *Proteus mirabilis*, and *Morganella morganii*, post-harvest treatment of the residue (e.g., composting) and larval biomass (e.g., heat treatment such as pasteurization) may still be the most efficient and reliable approach.

4.6   Conclusions

Sustainable mass rearing of BSFL for feed and food applications requires efficient and reliable process performance. Complementing previous work on the larval microbiota, this study set out to identify bacterial taxa in two food waste rearing substrates and residues that are potentially associated with rearing performance. As expected, considering their high nutrient content, rearing performance was high with canteen and household food waste substrates, underlining their potential for efficient insect production. A loss of the initial food waste microbiota, dominated by lactic acid bacteria, decreased rearing performance, indicating that initial substrate microbiota influence the complex bioconversion process. Furthermore, the rearing performance could also be influenced by bacteria in the rearing residue. Rearing duration decreased the bacterial richness and changed the physicochemical properties and composition of the residue, and typical members of the larval intestinal microbiota (that is, *Providencia, Dysgonomonas, Morganella*) became more abundant, suggesting their transfer into the residue through excretions. The present study provides a scientific basis for future studies that should isolate these bacteria and assess their true role in influencing rearing performance.

Acknowledgments

5. Chapter – Effect of rearing system and microbial inoculants on black soldier fly growth and microbiota with two agro-food byproducts

Unpublished manuscript

Moritz Gold[1,2,†], Trevor Fowles[3,†], Jesus D. Fernández-Bayo[4], Lydia Palma Miner[4], Christian Zurbrügg[2], Christian Nansen[3], Heather N. Bischel[5], Alexander Mathys[1]

[1]ETH Zurich, Sustainable Food Processing, Schmelzbergstrasse 9, 8092 Zurich, Switzerland
[2]Eawag, Department of Sanitation, Water and Solid Waste for Development (Sandec), Überlandstrasse 133, 8600 Dübendorf, Switzerland
[3]University of California, Department of Entomology and Nematology, Briggs Hall, 95616 Davis CA, USA
[4]University of California, Department of Biological and Agricultural Engineering, Bainer Hall, 95616 Davis CA, USA
[5]University of California, Department of Civil and Environmental Engineering, Ghausi Hall, 95616 Davis CA, USA

†These authors have contributed equally to this work

5.1 Introduction

Recycling of nutrients in agri-food byproducts is crucial in our time of significant population growth and environmental degradation (Willett et al., 2019). Currently, many agri-food byproducts are only partially valorised or wasted, leading to losses in nutrients and resources, which negatively impacts the environment (Chen et al., 2020; Gustavsson et al., 2011). An emerging approach for valorisation of agri-food byproducts is their conversion into insect biomass for use as a raw material for food and feed (Barragán-Fonseca et al., 2017), biotechnical (Hahn et al., 2019), cosmetic (Almeida et al., 2020) and pharmaceutical (Vilcinskas, 2013) applications.

Larvae of the black soldier fly (BSFL), *Hermetia illucens* L. (Diptera: Stratiomyidae), are one of the most promising insect species for recycling of nutrients (Gold et al., 2018a). However, some of the most abundant and affordably sourced agri-food byproducts (e.g. fruit and vegetable pomace, maize straw, almond hulls) result in a low or variable rearing performance (i.e. larval growth, substrate reduction) (Gold et al., 2018a; Lalander et al., 2019). Since performance is decisive for the affordability and environmental impact of BSFL-based products (Smetana et al., 2019), approaches are needed to increase the rearing of BSFL on agri-food byproducts.

Previous studies improved rearing by optimizing larval densities, feeding rate and feeding regime (e.g. batch vs. continuous) (Barragán-Fonseca et al., 2018c; Diener et al., 2009). Palma et al. (2018) introduced the first method of BSFL cultivation in closed containers with aeration. This system supported BSFL growth but the study failed to establish whether such a system is comparable or superior to the existing rearing in open beds, buckets or bins. Altered exchange of water, air and volatile organic compounds between the open and closed system could influence larval behaviour, microbiota, and residue temperature and pH. These parameters were previously deemed influential for rearing performance (Callegari et al., 2020; Meneguz et al., 2018; Raimondi et al., 2020).

Rearing performance was also previously improved by inoculation of substrates with pure-culture bacteria (Rehman et al., 2019; Somroo et al., 2019; Yu et al., 2011) or defined bacteria mixtures (Callegari et al., 2020; Mazza et al., 2020). Certain fly-, soil- or manure-associated bacteria (e.g. *Bacillus natto, Bacillus subtilis, Lactobacillus buchneri,* and *Kocuria marina*) reduced development time, and increased larval growth and substrate reduction. However, cultivation of pure bacterial cultures alongside insect rearing is not always practical due to the required laboratory capacities. A simpler method could be the use of the residue or a residue-concentrate as inoculum. During growth, certain bacteria are excreted by larvae and become more abundant in the residue (i.e. substrate and frass) (Gold et al., 2020b; Raimondi et al., 2020). Previous researchers hypothesized that excreted microbes contribute to substrate decomposition and larval growth (Bruno et al., 2019; Chen et al., 2017; Gold et al., 2020b). Consequently, similar to the fermentation of foods (e.g. sauerkraut and sourdough) (Kim et al., 2018), addition of these microbes to the substrate of the next rearing cycle could increase rearing performance.

The aims of this research was to validate the rearing system designed by Palma et al. (2018) and develop a simpler method to produce microbial inoculants by use of the rearing residue. These objectives address possible solutions for the often low or variable performance of BSLF reared on many agri-food byproducts. We hypothesized that the rearing system alters residue properties and microbiota, and thereby performance, and that residue-derived inoculants increase rearing performance. In controlled feeding experiments, BSFL were reared on tomato pomace (TP) and white wine pomace

(WWP), and larval weight, substrate reduction, residue properties (i.e. pH, temperature, moisture content), and microbiota were determined. By optimizing rearing conditions and inoculating substrates with microbes, this research sought to increase the valorisation of low-value agri-food byproducts in BSFL rearing.

5.2 Materials and Methods

Agri-food byproducts

BSFL were reared on two agricultural byproducts prevalent in the California Central Valley, USA, as well as one control substrate. TP consists mainly of crushed skins and seeds and was collected from Campbell Soup Supply Company (Dixon, CA, USA). WWP consists mainly of unfermented skins, pulp, seeds and stems and was collected from the UC Davis Teaching and Research Winery (Davis, CA, USA). Because BSFL grow usually best on food waste (Gold et al., 2020a; Lalander et al., 2019), enzymatically digested food waste (DFW) from supermarkets collected from California Safe Soils (Sacramento, CA, USA) (Jinno et al., 2018) was used as a high performance control. Following collection in non-sterile containers, all substrates were frozen and stored at -20 °C until the start of the feeding experiments.

Prior to feeding experiments, the wastes were thawed at 4 °C for 24 h and Milli-Q was added to elevate the substrate moisture content into the range (60-80%) typical for BSFL digestion (Dortmans et al., 2017; Gold et al., 2020a). Milli-Q quantities (0.45 ml/g TP; 0.35 ml/g WWP) were selected based on the perceived absorption capacity of the substrate. The moisture content was elevated from 63 to 71% for TP and from 60 to 65% for WWP. DFW had a moisture content of 68%. WWP was also homogenized with a kitchen blender to increase the palatability by BSFL.

The substrate gross nutrient composition, moisture content, and pH was determined using standard procedures (AOAC, 2006, AOAC, 1997) (see Supplementary Material for detailed method references). The pH was determined in a solution of 1 g of sample and 9 ml of Milli-Q water. Moisture content was determined as the gravimetric loss during drying at 80 °C for 24 h. Nitrogen was determined by combustion and protein estimated by multiplication of the nitrogen value with waste-specific factors. Based on the review of factors by Mariotti et al. (2008), 4.4 was used for TP and WWP based on results for vegetables and mushrooms, and 5.4 for DFW based on results for meat, fish, cereals and vegetables. Lipids were estimated by extraction with ethyl ether. Fibre fractions including amylase-treated neutral (NDF) and acid detergent fibre (ADF) were assessed by treating samples with a neutral and acid detergent. Hemicelluloses were estimated as the difference between NDF and ADF, and ADF was assumed a reliable estimate of cellulose and lignin content. Ash was determined based on the gravimetric loss during combustion at 550°C for at least 3 h.

Experiments

Two experiments were conducted to assess the influence of the rearing system (experiment 1) and the addition of residue-derived inoculants (experiment 2). In the first experiment, BSFL were reared on each substrate in parallel in the open (Gold et al., 2020a) and closed rearing systems (Palma et al., 2018). The open rearing system consisted of a plastic container (diameter: 9 cm, height: 14 cm) covered with a paper towel. The closed rearing system consisted of a sealed plastic bags (approximately 1,500 ml) supplied with compressed humidified air at 40 ml/min, or 0.7 ml/min/g dry mass (DM).

In the second experiment, BSFL were reared in substrates following addition of a microbial inoculant produced with the residue from the first experiment. BSFL were also reared in parallel in the open and closed system to validate the results of the first experiment. DFW was excluded from the second experiment because the first experiment confirmed that this substrate was performing well and does not need further improvement.

The production of microbial inoculants followed an approach similar to that common for pure-bacteria cultures. 3-10 g of residue from the experiment stored for 24 hours at 4°C was mixed with 40 ml sterile phosphate buffered saline (PBS) in a 50 ml falcon tube at room temperature (21°C) for 20 min. Large particles were removed with a 40 µM sterile cell strainer (Corning, New York, NY, USA), and the filtrate was diluted 100-fold. Three replicates of filtrate (1 ml) were incubated at 30°C overnight in sterile nutrient broth (5 ml, Difco Nutrient Broth, Becton, Dickinson and Company, Le Pont de Claix, France) under continuous shaking (120 rpm) (Max4000, Thermo Scientific, Waltham, MA, USA). 1 ml of this culture was added to 9 ml nutrient broth and incubated for another 4 h. Triplicate cultures were pooled and total viable counts (TVC) enumerated by single dilution series on triplicate agar plates as described below.

Microbial inoculant (10^9 TVC/ml) was added to each substrate with the Milli-Q water used to increase palatability (see description of rearing substrates) immediately prior to the feeding experiments with BSFL. The inoculant was dosed in TP at 3 ml/100 g DM for TP. Based on these results, the dose was increased to 10 ml/100 g DM for WWP. In the controls, the inoculant was sterilized by autoclaving before addition to substrates.

Fly larva rearing

BSFL used in the two experiments were obtained from a colony operated at UC Davis since April 2018. The hatched larvae were fed *ad libitum* with poultry feed (60% moisture content) (Purina Mills LLC., Purina Layena Pellets and Crumbles, Gray Summit, MI, USA) to 0.8-1.1 mg DM/larva. Thereafter, larvae were separated manually from the poultry feed residue. Three to four replicates were prepared for each treatment (i.e. rearing system, microbial inoculant) with approximately 200 larvae per replicate. At the beginning of the feeding experiment, BSFL were placed on 60 g DM substrate and reared in an incubator (Isotemp 637D, Fisher Scientific, Waltham, MA, USA) at 28 °C. The rearing duration was selected based on the larval mass on DFW. Since larvae on WWP were very small when harvesting larvae on DFW, the rearing duration was extended to facilitate larval-residue separation and accurate determination of performance metrics. BSFL were reared for 6 days on TP and DFW and 9-10 days on WWP. Temperature was automatically recorded every 10 min in the substrate/residue (DS1922L iButton, Maxim Integrated, San Jose, CA, USA). At the end of the experiment, containers/bags were removed from the incubators and a residue sample was collected for measurement of pH, TVC, and moisture content. Larvae were manually separated from the residue, rinsed with tap water and counted. Larvae were stored at -20 °C before determination of larval dry mass and DNA-based sequencing.

Rearing performance metrics

Larval mass and substrate reduction were the evaluated rearing performance metrics. Larval dry mass was determined for each biological replicate by dividing the dry mass of all larvae by the larval number. Substrate reduction was determined for each biological using Equation (9), as the ratio of residue dry

mass (residue$_{mass}$) to the dry mass of total substrate (substrate$_{mass}$) provided at the beginning of the experiment.

$$\text{Substrate reduction (\% DM)} = \left(1 - \frac{residue_{mass}\ (g\ DM)}{substrate_{mass}\ (g\ DM)}\right) \times 100 \qquad (9)$$

The residue dry mass was determined by correcting the residue mass removed from each biological replicate for moisture content. Larval dry mass and residue moisture content were determined following drying in a laboratory oven at 80°C for 24-48 hours.

Microbial numbers and bacterial communities

TVC were estimated in the substrates, residues, and inoculants by the spread plate method. *In situ* bacteria were eluted from solids and serial dilutions prepared using the same procedure as the inoculant preparation. 100 μL of each dilution was spread in triplicate on Petri dishes with standard agar (Difco Nutrient Agar, Becton, Dickinson and Company, Le Pont de Claix, France) and incubated at 30°C for 24-36 hours. Since we partially recorded colonies within the representative range of 20-250 for different dilutions and replicate plates, counts were calculated using Equation (10) (Maturin and Peeler, 2001), where $\sum c$ is the number of colonies on all plates, V is the volume added to each plate (0.1 mL), n_i is the number of plates counted of the i_{th} dilution, d is the dilution and E is the eluent concentration (i.e. ratio of sample mass and volume of PBS).

$$TVC\left(\frac{counts}{g\ sample}\right) = \frac{\sum c}{V \times (1n_1 + 0.1n_2) \times d} \; x \; \frac{1}{E} \qquad (10)$$

Bacterial communities were characterized by high-throughput 16S rRNA gene sequencing. Total genomic DNA was extracted from 0.2 g of substrate (duplicate), larval digestive tract (triplicate per treatment) and residue (triplicate per treatment) sample using the DNeasy PowerSoil Kit (Qiagen, Hilden, Germany). Samples of the digestive tract were BSFL following removal of the exoskeleton with micro scissors and tweezers (Bonelli et al., 2019; Gold et al., 2020d). The utensils were treated with 70% ethanol between dissections. DNA purity (Nanodrop ND 1000 Spectrophotometer, Thermo Scientific, Wilmington MA, USA) and concentration (Qubit dsDNA HR Assay Kit on a Spark 10 M microplate reader, Tecan, Männedorf, Switzerland) of the extracted DNA was determined. Library preparation followed the two-step protocol described in detail by Gold et al. (2020b) amplifying the prokaryotic V3-V4 hypervariable region using the primer pair 341F (5'- CCT ACG GGN GGC WGC AG 3') and 806R (5'- GGA CTA CNV GGG TWT CTA AT - 3'). PCR conditions were an initial denaturation at 95°C for 300 s, 1 cycle at 98°C for 60 s, 26 cycles (residue samples) and 33 cycles (larval samples) of 98 °C for 20 s, 51 °C for 20 s, and 72 °C for 12 s, and a final extension at 72 °C for 120 s (Hugerth et al., 2014). Index PCR conditions were an initial denaturation at 95 °C for 180 s, 10 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, and a final extension at 72 °C for 300 s. Molecular grade water was used as a no-template control. A bacterial community standard (Zymo D6311, Zymo Research, Irvine CA, USA) was used as the template DNA for positive control. Paired-end sequencing was performed on the library according to the manufacturer's directions using the MiSeq Illumina platform (Illumina Inc., San Diego, CA, USA). Bioinformatics were completed with the same protocol used by Gold et al. (2020b). High-quality reads were clustered into zero radius operational taxonomic units (ZOTUs) and their taxonomic origin was determined using Silva 16S (V128) as the reference

database. Taxonomic assignments were considered reliable when bootstrap confidence values exceeded 0.85.

Downstream data analyses

Data was analyzed in R version 3.6.2 (R Core Team, 2020). The hourly mean was calculated from raw temperature readings in the residue. We abstained from statistical analyses among the different treatments for all parameters due to the small number of biological replicates (n=3-4). Instead, we analyzed results and calculated descriptive statistics (e.g., median, mean, standard deviation). Heatmaps of bacterial communities were produced in *ampvis2* (Andersen et al., 2018) after conversion of reads into percent abundance per sample. Alpha diversity (i.e. Chao1 and Shannon index) and beta diversity were calculated in *phyloseq* (McMurdie and Holmes, 2013). The Unweighted pair group method with arithmetic averages (UPGMA) using weighted UniFrac distances of ZOTUs was applied to cluster samples based on their dis(similarity) of bacterial communities. Robust clusters of similar residue/intestinal bacterial communities were identified with the three step protocol proposed by García-Jiménez et al. (2019). First, the number of clusters with the highest silhouette width score were identified with the *fviz_nbclust* function in *factoextra* (Kassambara and Mundt, 2020). Second, the robustness of this clustering was confirmed with the *prediction.strength* function in the *fpc* package (threshold > 0.80) (Hennig, 2020). Third, the Jaccard score was calculated with the *clusterboot* function (threshold > 0.75). The UPGMA-UniFrac clustering was visualized in a two-dimensional pane after Principal coordinate analysis (PCoA) of bacterial communities.

5.3   Results

Effect of rearing system (experiment 1)

Considering the mean and standard deviation, the closed rearing system had a better performance regarding larval growth on WWP and substrate reduction on TP (Figure 5.1). Larval mass on WWP was 20.4 (0.5) mg DM in the closed system and 15.3 (0.4) mg DM in open system. Substrate reduction on TP was 58.6 (1.7) % DM in the closed system and 46.9 (0.8) % DM in the open system. DFW reduction was notably lower in two replicates in the closed system than the open system.
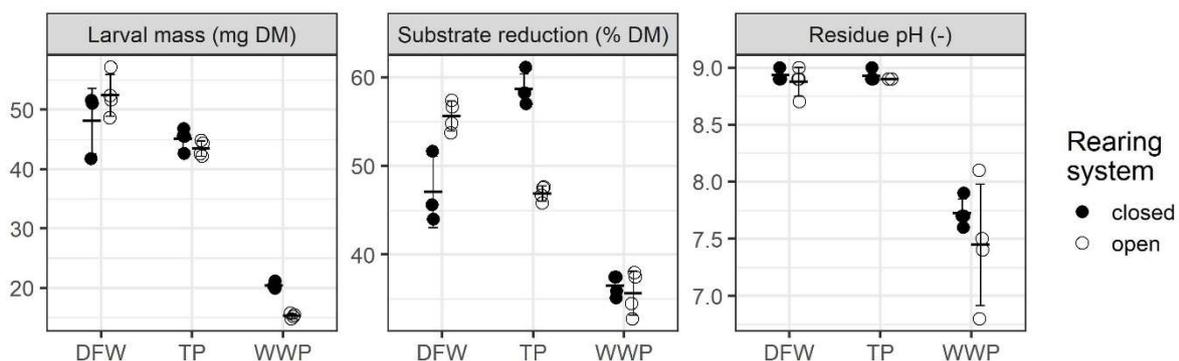
Figure 5.1 Effect of the rearing system on larval mass, substrate reduction and pH (experiment 1). Means (horizontal lines), standard deviations and results per biological replicate (n=3-4, filled circles = closed system, hollow circles = open system) are displayed.

The rearing system also had an apparent effect on the residue moisture content (Figure 5.2) and temperature (Figure 5.3). Considering the results for both experiments, the mean residue moisture content was higher in the closed system by 7.5-12.5% on TP, 25.6-50.4% on WWP and 17.1% on DFW. The residue temperature was higher in the open system than in the closed system for DFW and TP, but not WWP. The median temperature in the open and the closed system was 34.8 °C and 30.8 on DFW and 35.3 °C and 31.2 °C on TP.
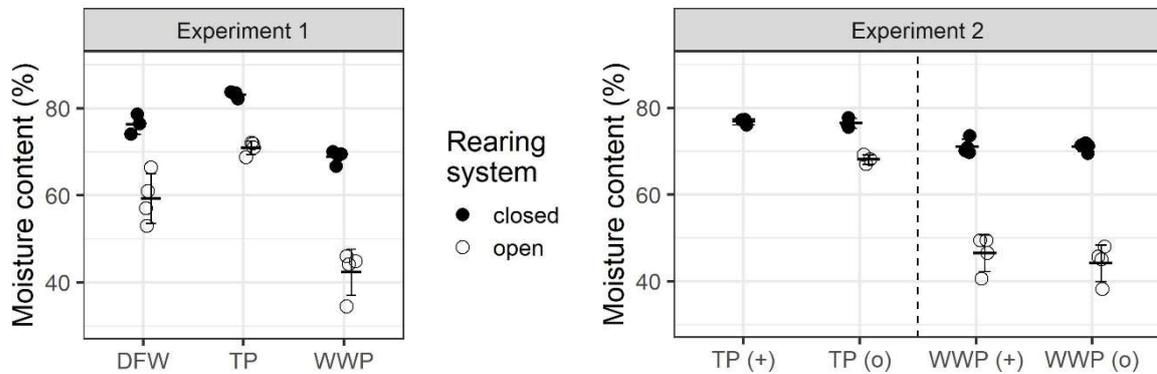
Figure 5.2 Effect of the rearing system (experiment 1 and 2) and the microbial inoculant addition (experiment 2, o = sterile inoculant, + = inoculant) on the residue moisture content. Means (horizontal lines), standard deviations and results per biological replicate (n=3-4, filled circles = closed system, hollow circles = open system) are displayed.
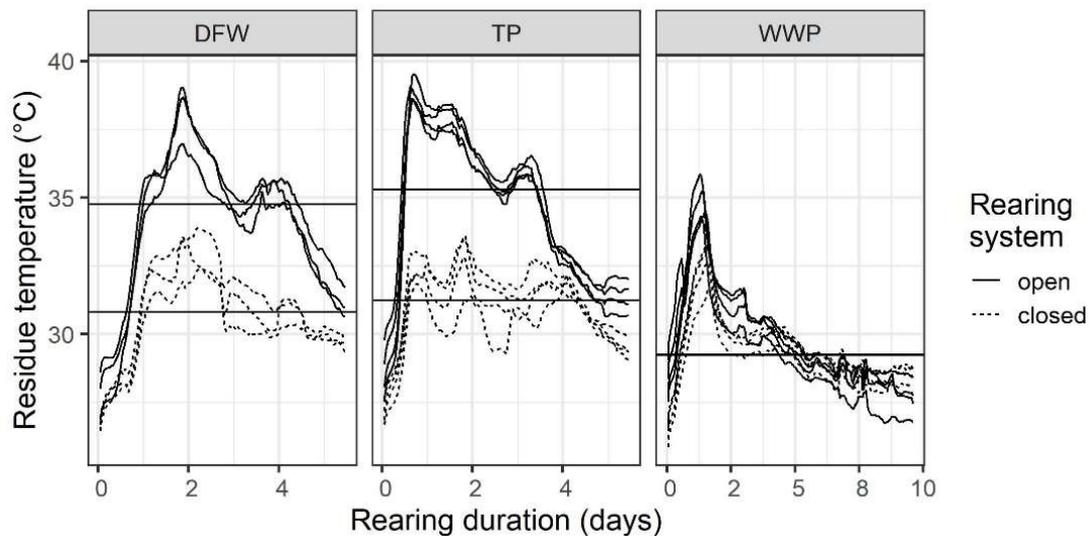
Figure 5.3 Effect of the rearing system on the residue temperature. Horizontal lines are the median temperatures for all replicates between the open and closed system.

The intestinal and residue bacterial community was determined in the two systems due to the possible impact on larval growth and substrate decomposition. Considering all samples, sequencing using extracted DNA produced 9,439,368 reads, with an average of 86,600 reads/sample, and a total of 2,204 ZOTUs. Rarefaction curves (see Supplementary Material) demonstrate that samples were sequenced to an extent sufficient to approximate true diversity. Since these results do not give any precise information about the microbial numbers, the TVC in the residue was additionally estimated, which were similar between systems (Table 5.1).

Table 5.1 Total viable counts (TVC, $\log_{10}$/g) in the residue of the open and closed rearing system.

	Open system	Closed system
TP	8.0 (0.3)‡	7.9 (0.1)‡
WWP	> 9.5 (0.0)°*	9.4 (0.2)*
DFW	7.8 (0.1)†	7.7 (0.4)†

in parenthesis: standard deviation for samples where n ≥ 3 and differences between analyses where n=2
*n=2, †n=3 and ‡n=4
°counts above countable range
TP: Tomato pomace
WWP: White wine pomace
DFW: Digested food waste

Alpha diversity metrics (i.e. Chao 1 and Shannon Index) show a similar species richness and evenness of the intestinal and residue bacterial community between the two systems (Figure 5.4). Small differences in mean species richness and evenness (i.e. Shannon Index) between systems were measured for the intestinal bacterial community on TP (Figure 5.4A), and the residue bacterial community on TP and DFW (Figure 5.4B). (Dis)similarities in the bacterial community between the open and closed system were further explored by hierarchical clustering (UPGMA) and multidimensional scaling (PcoA) using weighted UniFrac distances, to account for the phylogenetic relatedness between ZOTUs. These analyses show separate clusters between the open and closed rearing system for the intestinal bacterial community on DFW (Figure 5.4A), and residue bacterial community on DFW and WWP (Figure 5.4B). Overall, the distance of clusters, indicating dissimilarity between bacterial communities of the open and closed system, was small. The largest difference between systems was observed for the intestinal bacterial community on DFW. When microbial inoculants were added to the substrate (experiment 2), no effect of the rearing system on process performance (Figure 5.6), residue temperature (see Supplementary Material) and bacterial community (Figure 5.6) was observed.
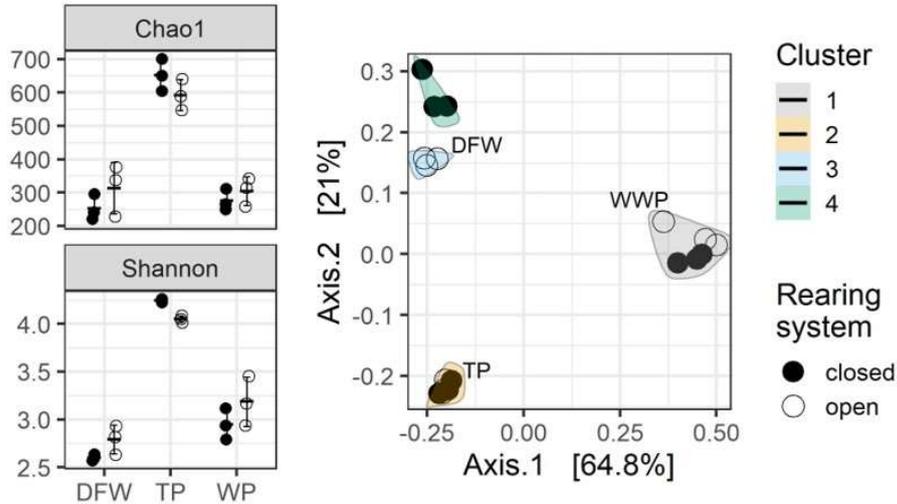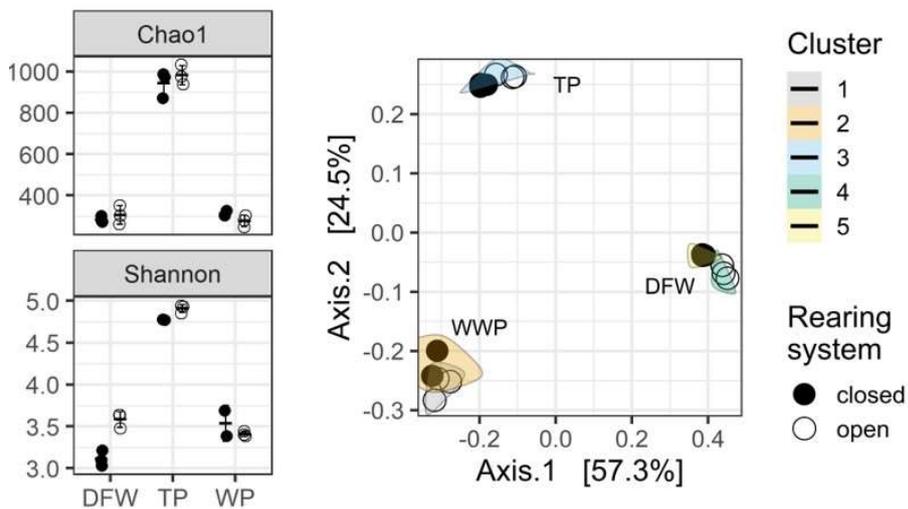
Figure 5.4 Effect of the rearing system on intestinal (A) and residue (B) bacterial community alpha and beta diversity metrics. Alpha diversity metrics display the mean (horizontal lines), standard deviations and results per biological replicate (n=3-4, filled circles = closed system, hollow circles = open system). Beta diversity is illustrated by Principal coordinate analysis (PCoA) of bacterial communities based on weighed UniFrac dissimilarity. Samples (n=3-4) were clustered with the Unweighted pair group method with arithmetic averages (UPGMA).

Effect of residue-derived bacterial inoculants (experiment 2)

The inoculants derived from the residue of the first experiment had a much lower bacterial community richness than the residue from the first experiment. The mean community richness decreased from the residue to the inoculant from 963 to 310 for TP and from 292 to 189 for WWP. The bacterial community was dominated (relative abundance > 5%) by members from the genera *Acinetobacter*, *Lysinibacillus*, *Myroides* and *Vaccocus* in the TP inoculant and by *Acinetobacter* and members of the family *Enterobacteriaceae* in the WWP inoculant (Figure 5.5).
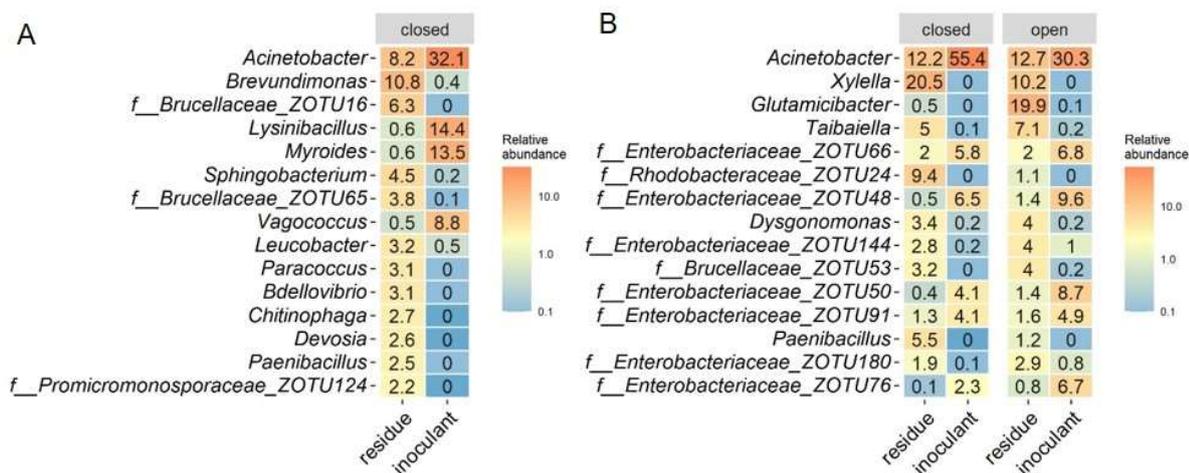
Figure 5.5 Bacterial community of TP (A) and WWP (B) bacterial inoculants and residues used for their production. Heatmaps of the top 15 genera of grouped samples based on the relative abundance of ZOTUs. Relative abundances are the mean of replicate samples (n=3-4 for residue, n=2 for inoculant) rounded to one digit. If no clear assignment to a genus was possible, the family assignment is shown together with the ZOTU.

The addition of the residue-derived inoculants to the substrate did not influence rearing performance and residue properties in comparison to the addition of sterile inoculant (Figure 5.2, Figure 5.6). The inoculant did also not influence bacterial numbers and bacterial diversity, richness and community (Figure 5.7) TVC (n is the number of biological replicates with countable plates) in the treatment (microbial inoculant) and control (autoclaved microbial inoculant) were 8.5 $\log_{10}$/g (n=1) and 9.0 (0.5) $\log_{10}$/g (n=4) for TP, and 9.1 (0.1) $\log_{10}$/g (n=4) and 9.5 (0.0) $\log_{10}$/g (n=2) for WWP. Our clustering approach identified two clusters - all TP and all WWP samples. The distance of samples demonstrates that inoculant addition to the substrate did increase the bacterial community variability among samples of the same treatment and rearing system type in comparison to the first experiment (Figure 5.4, Figure 5.7).

Table 5.2 Nutrient composition, pH, moisture content and bacterial counts of the rearing substrates (n=1).

	pH	Protein	Lipids	Ash	Cellulose & lignin	Hemi-celluloses	TVC
	-	%DM	%DM	%DM	%DM	%DM	$\log_{10}$/g
TP	5.8	15.7	14.1	3.2	44.8	1.6	8.4
WWP	4.7	9.7	9.4	7.7	34.2	20.7	4.2
DFW	5.8	33.8	12.1	24.6	11.5	57.7	7.0

TP: Tomato pomace
WWP: White wine pomace
DFW: Digested food waste
TVC: Total viable counts

Effect of substrate

The substrate type had a much larger influence on all metrics measured in this study than the rearing system and residue-derived inoculant addition to the substrate. DFW and TP were richest in protein and lipids and had similar microbial numbers (Table 5.2). DFW had the least cellulose and lignin, and TP had little ash. WWP had the lowest pH and had had much lower microbial numbers than TP and DFW.

Low microbial numbers in WWP presumably resulted in too few reads from gene sequencing to estimate the bacterial communities in the substrate before BSFL rearing. TP and the DFW differed in community richness and composition (Figure 5.8). TP had a rich and diverse community, dominated by species from nine bacterial classes. In contrast, few high-abundant genera (i.e., *Bacillus*, *Lactobacillus*, *Leuconostoc*) characterized DFW.
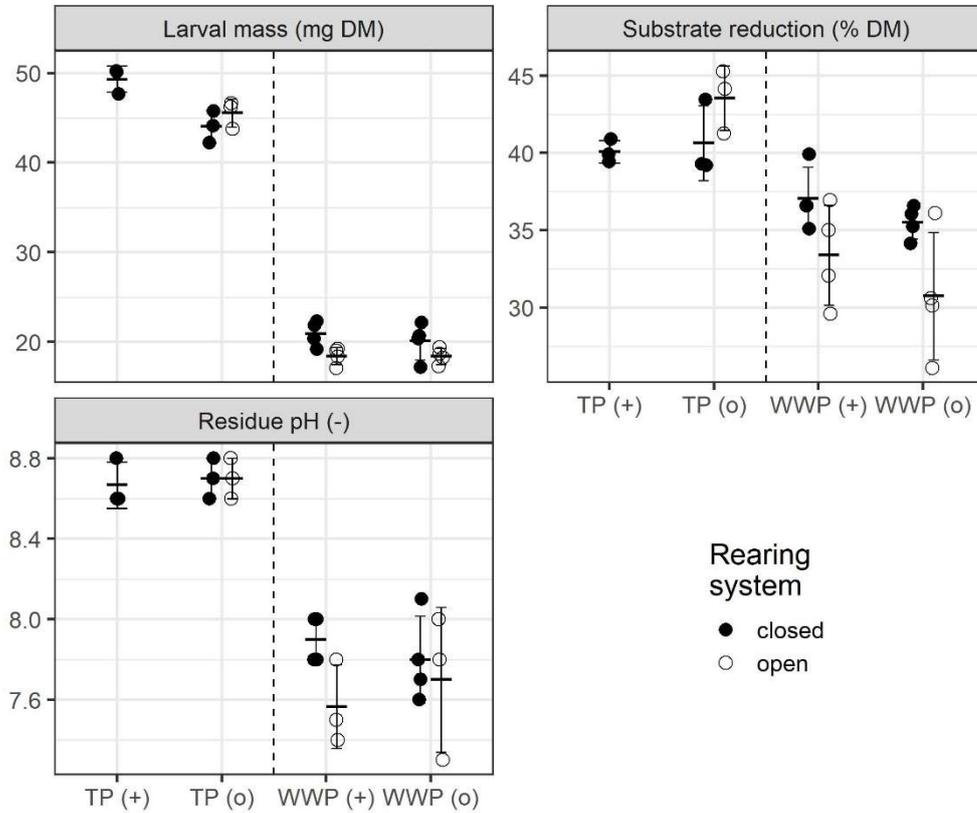
Figure 5.6 Effect of the bacterial inoculant addition (+ = inoculant, o = sterile inoculant) on larval mass, substrate reduction and residue pH (experiment 2). Means (horizontal lines), standard deviations and results per biological replicate (n=3-4, filled circles = closed system, hollow circles = open system) are displayed.
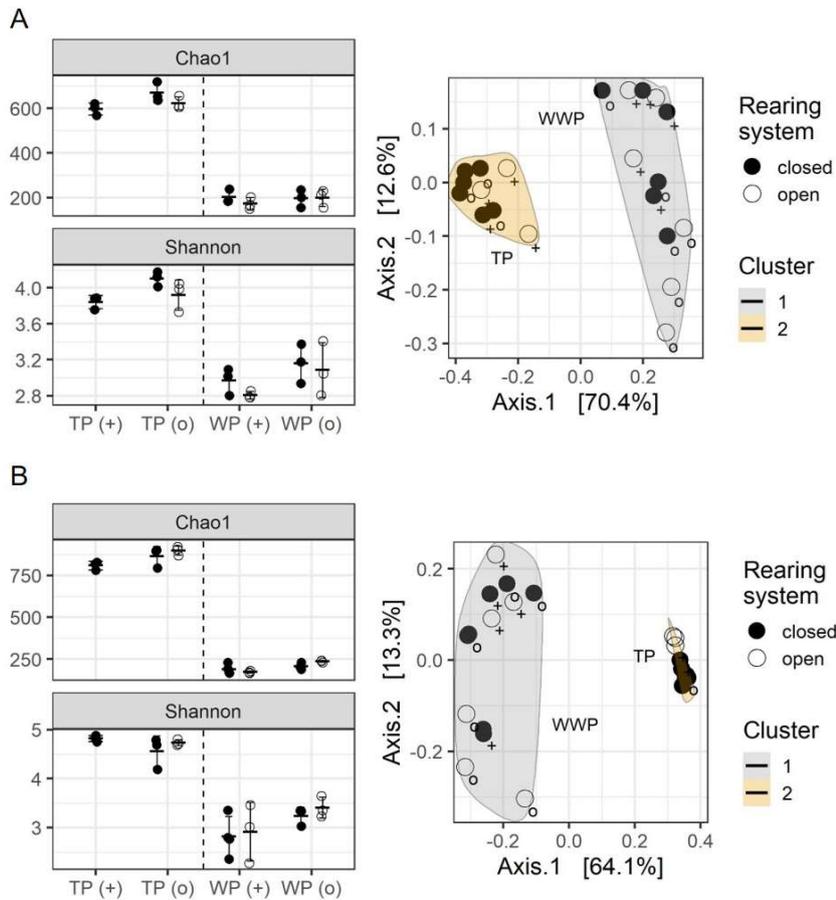
**Figure 5.7** Effect of the microbial inoculant addition (o = sterile inoculant, + = inoculant) on intestinal (A) and residue (B) bacterial community alpha and beta diversity metrics. Alpha diversity metrics display the mean (horizontal lines), standard deviations and results per biological replicate (n=3-4, filled circles = closed system, hollow circles = open system). Beta diversity is illustrated by Principal coordinate analysis (PCoA) of bacterial communities based on weighed UniFrac dissimilarity. Samples (n=3-4) were clustered with the Unweighted pair group method with arithmetic averages (UPGMA).
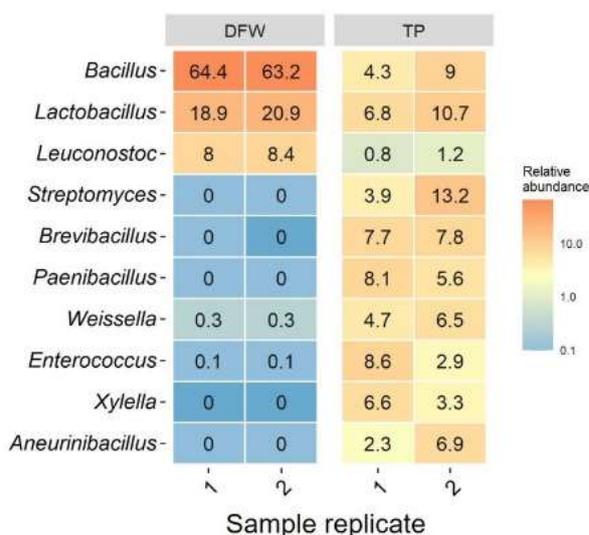
Figure 5.8 DFW and TP bacterial community. Heatmaps of top 10 genera in both substrates based on the relative abundance of ZOTUs rounded to one digit.

TP had a comparable rearing performance to DFW. Larval mass and substrate reduction (pooled results for both rearing systems) were 44.3 (1.7) mg DM and 52.8 (6.4) % DM on TP, and 50.6 (4.7) mg DM and 52.0 (5.3)% DM on DFW. Despite a 3-4 day longer rearing duration, larval mass and substrate reduction was lower on WWP. Larval mass and substrate reduction were 17.8 (2.8) mg DM and 36.1 (1.8) % DM, respectively.

Considering alpha and beta diversity metrics, the substrate had an apparent effect on the intestinal and residue bacterial richness and community. Similar to the substrate, the intestinal and residue bacterial communities were richest when TP was the substrate. Community richness was comparable between WWP and DFW (Figure 5.4, Figure 5.7). UniFrac distances and heatmaps demonstrate unique bacterial communities between the intestinal and residue for the same substrate, sharing few taxon on the family level (Figure 5.9A). Among substrates, the intestinal and residue bacterial communities also differed, with few shared taxon on the genus level (Figure 5.9D). Intestinal samples shared members of *Dysgonomonas*, *Enterococcaceae* and *Enterococcus*, and residue samples members of *Glutamicibacter*.
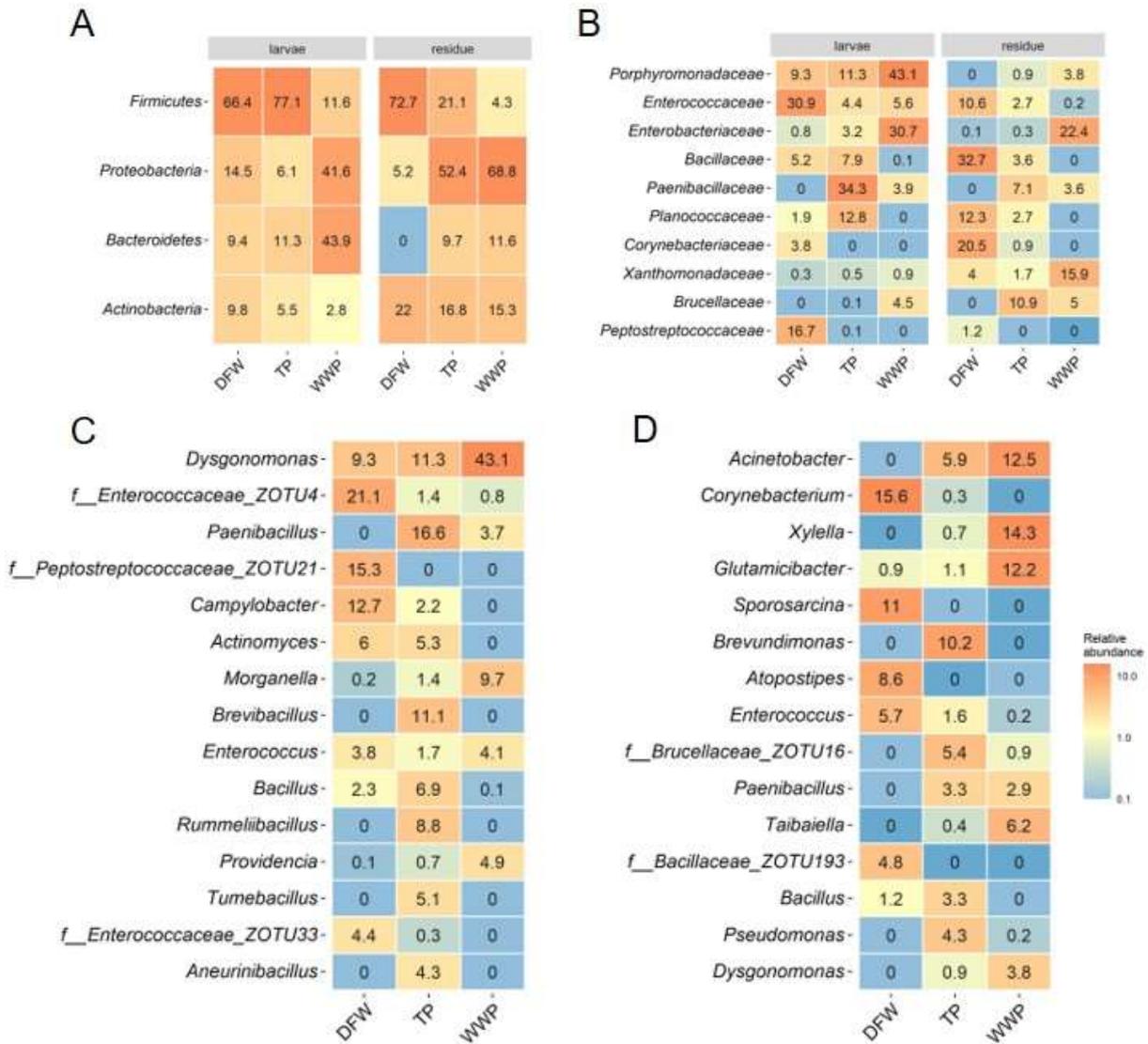
Figure 5.9 Bacterial community in larvae and residues reared on DFW, TP and WWP on the phylum (A), family (B) and genus level (C, D). Heatmaps of most abundant ZOTUs among grouped samples. Relative abundances are the mean of replicate samples (n=3-4) rounded to one digit. If no clear assignment to a genus was possible, the family assignment is shown together with the ZOTU.

5.4 Discussion

The goal of this research was to explore potential solutions to increase the performance of BSFL on abundant and affordably sourced agri-food byproducts that represent a challenge (and opportunity) in valorisation. As such, we used WWP and TP as model substrates. Specifically, we aimed to 1) validate whether the novel rearing system designed by Palma et al. (2018) for almond hulls is beneficial for BSFL rearing; and 2) develop a method to produce microbial inoculants from the rearing residue. We hypothesized that both the rearing system and introduction of residue-derived inoculants yield an increase in rearing performance.

We found the rearing system to influence performance (Figure 5.1). On average, BSFL reared in the closed system on WWP were 5.1 mg DM heavier than BSFL reared in the open system, and TP reduction in the closed system was 11.7% DM higher than in the open system. Surprinsingly, higher WWP larval mass and TP substrate reduction did not results in higher WWP substrate reduction and TP larval mass. An advantage of the closed system seems to be that the sealed bags and humidified airflow maintains a residue moisture content (Figure 5.2, 71-77% on TP and WWP) in the range considered optimal (70-80%) for BFSL (Dortmans et al., 2017). The slightly reduced larval mass on WWP in the open system could be due to the low moisture content in the residue (Figure 5.2, 42%) decreasing WWP palatability by BSFL. The higher TP reduction in the closed system is surprising as the median temperature in the residue was 4°C lower (Figure 5.3) than in the open system. The lower temperature in the closed system can be explained by continuous aeration of the substrates with air at ambient temperature. Since a rise in residue temperature presumably increases the activity of larval digestive enzymes (Bonelli et al., 2019), one could have expected higher TP reduction in the open system. A possible explanation for the higher TP reduction in the closed system could be more aeration than in the open system, resulting in enhanced larval/microbial substrate decomposition (Palma et al., 2018). It remains unclear, however, why this effect in substrate reduction between system was not observed on WWP or when the residue-derived inoculant was added to the TP substrate (Figure 5.6). A disadvantage of the closed system is insufficient aeration of pasty substrates, such as DFW, as highlighted by the increase in anaerobic bacteria of the family *Peptostreptococcaceae* (Slobodkin, 2014) in the intestinal bacterial community and a septic smell. This could explain the notably lower DFW substrate reduction in two out of three replicates for the closed system, compared to the open system (Figure 5.1). Considering this shortcoming and the higher operational costs (e.g. aeration, closing of containers, harvesting), benefits for industrial application of the closed system remain unclear.

Based on the results, we rejected the hypothesis that our method of incorporating residue-derived inoculants back into substrate improves rearing performance. This was a surprising result considering that previous studies showed clear improvement in rearing efficiencies by the addition of bacteria (Rehman et al., 2019; Somroo et al., 2019; Xiao et al., 2018; Yu et al., 2011), and that even rudimentary use of fermentate is ubiquitous in accelerating fermentation of foods. Possible explanations for this result are that the residues did not include probiotic bacteria. We expected that fly-associated bacteria from the genera *Lactobacillus, Bacillus, Dysgonomona, Morganella, Proteus* and/or *Enterococcus* were abundant in the residue on all substrates (Ao et al., 2020; Bruno et al., 2019; Gold et al., 2020b)*,* Typical intestinal bacteria were indeed present in the DFW residue belonging to *Enterococcus* and WWP residue belonging to *Dysgonomona and Providencia,* and the family *Enterobacteriaceae* to which *Proteus spp.* and *Morganella spp.* belong (Figure 5.5). However, these genera had an abundance < 6%, and were absent in the TP residue. Variable bacterial communities in residues and abundance of intestinal bacteria has also been reported by previous researchers (Wynants et al., 2019), and attributed to different initial substrate bacterial communities and nutrient contents, as well as operating parameters (e.g., feeding rate) (Gold et al., 2020b; Wynants et al., 2019). A further possible explanation of our results is the insufficient replication of the residue bacterial community by the applied cultivation method. For example, *Dysgonomonas* considered supporting in hemicellulose digestion decreased in abundance (Bruno et al., 2019). Bacteria abundant in the TP (*Acinetobacter, Lysinibacillus, Myroies, Vagococcus*) and WP (*Acinetobacter, Enterobacteriaceae)* inoculants (Figure 5.5) did not elicit any apparent positive effect on larval growth and substrate reduction (Figure 5.6). Future studies should isolate members of taxon potential beneficial and elucidate their true potential to influence BSFL rearing performance. In addition, recirculation of

bacteria could be improved by optimizing cultivation conditions (e.g. medium, oxygen conditions) and doses.

Even though our addition of the residue-derived inoculate resulted in no apparent increase in performance, our results are not completely unexpected. Performance improvements in rearing performance have also been absent or minimal in other studies with fly-associated bacteria. Callegari et al. (2020) isolated BSFL intestinal bacteria and showed a positive influence on larval growth with the addition of *Escherichia coli* and *Bacillus licheniformis* but not *Stenotrophomonas maltophilia*. Similarly, Mazza et al. (2020) inoculated chicken manure with pure-culture bacteria and bacteria mixtures isolated from eggs and the BSFL digestive tract. Four out of seven bacteria influenced larval mass by less than ± 2%, and three out of nine bacteria mixtures decreased larval mass. Many questions remain for how the inoculation of substrates can reliably increase rearing performance, but variable results could be partially explained by the different digestive/metabolic capacities of microbes and variable nutritional requirements of BSFL depending on operational rearing parameters. It remains to be confirmed whether the added bacteria colonize the residue or digestive tract, and whether viable bacteria are responsible for the reported improvements. This was the first study to use sterile inoculant instead of sterile water as a negative control. Autoclaving of the bacterial inoculant could have increased the digestibility for BSFL and be one explanation for the higher larval mass of the control in comparison to the treatment on TP.

Our results shows that the substrate type, i.e. substrate composition, including nutrient, pH, bacterial numbers and community, as well as metrics of palatability not quantified in this research, had a larger effect on rearing of BSFL than the rearing system (Figure 5.1) or addition of residue-derived inoculants (Figure 5.6). The nutrient composition (i.e. protein and lipid content, Table 5.2) and rearing performance metrics confirm that despite the enzymatic digestion process, DFW is a high-performing BSFL substrate. This was to be expected as the digested food waste was shown to have promise as pig feed (Jinno et al., 2018). Also, the DFW was high in *Lactobacillus* and, *Bacillus* that previously had positive effects on larval growth (i.e. *Bacillus natto, Bacillus subtilis, Lactobacillus buchneri)* (Rehman et al., 2019; Somroo et al., 2019; Xiao et al., 2018; Yu et al., 2011). Despite a much lower nutrient content than DFW, and with a high content of cellulose and lignin (44.8% DM, Table 5.2), TP had a rearing performance comparable to that of DFW (Figure 5.1), the substrate that frequently has the highest BSFL rearing performance (Gold et al., 2020a; Lalander et al., 2019). The low rearing performance of WWP could be due to the low protein (9.7 %DM, Table 5.2) and high fibre (34.2% DM) content. In addition, potential insecticidal and bactericidal properties of secondary metabolites in WWP (i.e. phenolic acids) (Katalinić et al., 2010) could also have affected larval growth and microbiota (Isibika et al., 2019; Pavela, 2011). Finally, pasteurizing prior to BSFL rearing could have also increased the digestibility of the DFW and TP by BSFL (Jinno et al., 2018). In comparison, WWP that was pressed mechanically at a winery was not subjected to heat treatment prior use for BSFL rearing. Yet, despite pasteurization both substrates had a high microbial number and bacterial community richness (Table 5.2, Figure 5.4).

5.5   Conclusions

Efficient rearing of BSFL on agri-food byproducts requires solutions to improve performance. This study examined whether the rearing system and the addition of residue-derived inoculants increased the performance on TP and WWP. The closed rearing system had an equal or superior performance

compared to the conventional open system. Sufficient aeration on pasty rearing substrates and efficient harvest of larvae from the residues with high moisture contents are research areas that are working towards an industrial application of BSFL rearing in closed systems. Returning potentially beneficial microbes with an inoculum made from the residue had no impact on performance, residue properties, and microbiota. This approach could be improved by studying the effects of bacteria thought to be benefical for rearing performance and developing cultivations method to reproduce them from the residue community.

Acknowledgements

6. Chapter – Estimating black soldier fly larvae biowaste conversion performance by simulation of midgut digestion

Moritz Gold[1,2], Julia Egger[1,2], Andreas Scheidegger[3], Christian Zurbrügg[2], Daniele Bruno[4], Marco Bonelli[5], Gianluca Tettamanti[4], Morena Casartelli[5], Eric Schmitt[6], Ben Kerkaert[7], Jeroen De Smet[7], Leen Van Campenhout[7], Alexander Mathys[1*]

[1]ETH Zurich: Swiss Federal Institute of Technology Zurich, Institute of Food, Nutrition and Health, Sustainable Food Processing Laboratory, Schmelzbergstrasse 9, 8092 Zurich, Switzerland
[2]Eawag: Swiss Federal Institute of Aquatic Science and Technology, Department Sanitation, Water and Solid Waste for Development (Sandec), Überlandstrasse 133, 8600 Dübendorf, Switzerland
[3]Eawag: Swiss Federal Institute of Aquatic Science and Technology, Department Systems Analysis, Integrated Assessment and Modelling, Überlandstrasse 133, 8600 Dübendorf, Switzerland
[4]University of Insubria, Department of Biotechnology and Life Sciences, via J.H. Dunant 3, 21100, Varese, Italy
[5]University of Milan, Department of Biosciences, via Celoria 26, 20133, Milan, Italy
[6]Protix: Industriestraat 3, 5107 NC, Dongen, the Netherlands
[7]KU Leuven: Department of Microbial and Molecular Systems (M2S), Lab4Food, Campus Geel, Kleinhoefstraat 4, 2440 Geel, Belgium

6.1 Abstract

Black soldier fly larvae treatment is an emerging technology for the conversion of biowaste into potentially more sustainable and marketable high-value products, according to circular economy principles. Unknown or variable performance for different biowastes is currently one challenge that prohibits the global technology up-scaling. This study describes simulated midgut digestion for black soldier fly larvae to estimate biowaste conversion performance. Before simulation, the unknown biowaste residence time in the three midgut regions was determined on three diets varying in protein and non-fiber carbohydrate content. For the static *in vitro* model, diet residence times of 15 min, 45 min, and 90 min were used for the anterior, middle, and posterior midgut region, respectively. The model was validated by comparing the ranking of diets based on *in vitro* digestion products to the ranking found in *in vivo* feeding experiments. Four artificial diets and five biowastes were digested using the model, and diet digestibility and supernatant nutrient contents were determined. This approach was able to distinguish broadly the worst and best performing rearing diets. However, for some of the diets, the performance estimated based on *in vitro* results did not match with the results of the feeding experiments. Future studies should try to establish a stronger correlation by considering fly larvae nutrient requirements, hemicellulose digestion, and the diet/gut microbiota. *In vitro* digestion models could be a powerful tool for academia and industry to increase conversion performance of biowastes with black soldier fly larvae.

6.2 Introduction

Black soldier fly larvae (BSFL), *Hermetia illucens* L. (Diptera: Stratiomyidae), treatment is an emerging technology for the conversion of biowaste (Dortmans et al., 2017) into marketable high-value products according to circular economy principles (Bortolini et al., 2020; Cappellozza et al., 2019; Leong et al., 2016; Setti et al., 2019; Vilcinskas, 2013; Wang and Shelomi, 2017). In addition to the recycling of nutrients into raw materials for fertilizer, lubricants and biodiesel, pharmaceuticals, and animal feeds markets, BSFL treatment can have environmental benefits. For example, depending on their operational conditions, BSFL conversion can reduce emissions from biowaste treatment in comparison to composting (Ermolaev et al., 2019; Mertenat et al., 2019; Pang et al., 2020), and animal feed products can have a lower environmental impact than conventional feeds when produced with BSFL (Smetana et al., 2019, 2016).

One current challenge for the replication and up-scaling of BSFL biowaste treatment is the often unknown or variable treatment performance (Gold et al. 2020a, Gold et al. 2018a). Metrics that correlate with treatment performance, similar to those determined for other biowaste treatment technologies, such as the biomethane potential for the design of anaerobic digesters, and the carbon and nitrogen ratio for composting, are lacking of BSFL treatment but could provide parameters for the reliable design and operation of BSFL treatment. Such metrics could be delivered by *in vitro* digestion model systems widely applied in research on humans (Brodkorb et al., 2019; Minekus et al., 2014) and farmed animals (Boisen and Eggum, 1991; Cheli et al., 2012; Fuller, 1991). Static *in vitro* digestion models are particularly popular and useful because of their simplicity, reproducibility, and low cost while still providing results predicting *in vivo* digestion outcomes (Bohn et al., 2018). They have been widely used to predict the quality and digestibility of animal diets with the goal of optimizing health and growth of farmed monogastric (e.g., poultry, pigs) and ruminant (e.g., cows) animals (Boisen and Fernández, 1997; Noblet and Jaguelin-Peyraud, 2007; Tilley and Terry, 1963; Van Soest, 1994).

Simulation of digestion could have similar applications in BSFL treatment. Nutrient contents in products following *in vitro* digestion could be used to predict *in vivo* performance (e.g., larval growth, waste reduction) (Kiers et al., 2000; McClements et al., 2009; Oomen et al., 2002, 2003). Thereby, simulation of BSFL digestion may enable direct and cost-effective comparison of biowastes with different compositions (e.g., nutrient contents, microbial loads, textures, moisture contents, and bulk densities).

BSFL digestion could be mimicked in the laboratory by exposing diets to pH values, temperatures, enzyme activities, and residence times similar to those found in the digestive tract of BSFL (Brodkorb et al., 2019; Hur et al., 2011; McDonald et al., 2011; Minekus et al., 2014). As the digestive tract of fly larvae presents some similarities to that of humans (Bonelli et al., 2019; Gold et al. 2018a; Jiang et al., 2015), a starting point for the development of BSFL *in vitro* simulations could be the static human *in vitro* digestion model, for which an international standard method was recently proposed (Brodkorb et al., 2019). In addition, the knowledge on the BSFL biology should be considered. The midgut is the most important organ for digestion in insects (Caccia et al., 2019). The midgut in BSFL is divided into three regions, i.e., anterior midgut (AMG), middle midgut (MMG), and posterior midgut (PMG), each with its own peculiar morphofunctional features (Bonelli et al., 2019; Bruno et al., 2019). Therefore, the *in vitro* digestion model should mimic this organization. Bonelli et al. (2019) reported the pH values of the lumen in the different midgut regions, and the activity of the enzymes involved in protein, carbohydrate, and lipid digestion, with results being in line with fly larvae digestion (Gold et al. 2018a). Even though this information is crucial for developing a static *in vitro* digestion model, reliable information regarding residence times of biowaste in the three midgut regions is also indispensable and, thus far, is not available for BSFL.

The aim of this research was to implement and validate a static *in vitro* model of the BSFL midgut. Before *in vitro* digestions, diet residence time was determined as a missing input parameter. Digestion was simulated to assess whether nutrient contents in *in vitro* digestion products could be indicative of *in vivo* feeding experiment outcomes. Despite the simplicity of the model which among limitations only mimics one digestion and does not consider temperature dynamics, we hypothesized that *in vitro* diet digestibility and supernatant nutrient content correlate with *in vivo* waste reduction and larval weight. Thereby, an *in vitro* model could be a powerful tool in academia and industry to increase the understanding and performance of the BSFL treatment process.

6.3 Materials and Methods

This research consisted of four parts: i) Determination of diet residence times in the three midgut regions; ii) *in vitro* simulation of BSFL digestion using the midgut region residence time estimates; iii) *in vivo* feeding experiments; and iv) comparison of *in vitro* and *in vivo* results.

Determination of diet midgut residence times and diet intake

Artificial diets

Residence times of diet in the three midgut regions was determined as a missing input parameter for a static *in vitro* model mimicking BSFL digestion. Gut residence times have previously be shown to vary for many organism with the diet nutrient content (Karasov et al., 2011). To receive a representative estimate for *in vitro* simulation, midgut residence time was determined with three artificial diets varying in nutrient contents. The diets were similar to those previously used by Cammack and Tomberlin (2017) and had the same lipid, vitamin, and mineral content, but two different protein and three different NFC contents (Table 6.1), Supplementary Material). Contents were selected to reflect the range of protein and NFC contents typical for BSFL substrates. The differences in protein and NFC were balanced with cellulose, which was mostly indigestible under the experimental conditions used in this study (Gold et al. 2018b). Protein:NFC:Fibre contents in % dry mass (DM) were 13:8:76, 13:47:37, and 7:47:42 in the artificial diets; henceforth, these diets are referred to as $P^{13}NFC^8$, $P^{13}NFC^{47}$ and P^7NFC^{47}, respectively. Based on preliminary experiments, the dry artificial diets were mixed with different amounts of water to account for different diet water adsorptions (Stana-Kleinschek et al., 2002) to 70% (for $P^{13}NFC^{47}$ and P^7NFC^{47}) and 80% (for $P^{13}NFC^8$) moisture content.

Table 6.1 Mean nutrient composition of diets used in this study.

	Protein	NFC	Lipids	Fiber	Organic matter	P:NFC* ratio
	% DM	% DM	% DM	% DM	% DM	-
Artificial diets						
$P^{13}NFC^8$	12.6	7.8	0.6	75.7	96.7	2:1
$P^{13}NFC^{47}$	12.6	46.8	0.6	36.7	96.7	1:4
P^7NFC^{78}	7.0	77.8	0.6	11.3	96.7	1:11
P^7NFC^{47}	7.0	46.7	0.6	42.4	96.7	1:7
Biowastes						
Cow manure	11.1 (0.4)	1.8 (0.6)	4.4	58.4 (0.4)	80.7 (0.5)	7:1
Mill by-products	14.5 (0.3)	23.2 (0.2)	3.0	51.7 (0.9)	93.8 (1.3)	1:2
Canteen waste	32.2 (0.8)	7.5 (0.7)	34.9	36.2 (1.4)	93.0 (0.7)	4:1
Poultry slaughterhouse waste	37.3 (0.5)	0.3 (0.1)	42.9	20.8 (1.9)	94.0 (1.3)	152:1
Vegetable canteen waste	12.1 (0.1)	15.5 (0.9)	28.9	31.5 (1.8)	92.4 (0.5)	1:1

In parenthesis: standard deviation for samples where n ≥ 3 and differences between analyses where n = 2
Composition of artificial diets was calculated based on the proportion/composition of the artificial diet ingredients (see Supplementary Material)
Composition of biowastes taken from Gold et al. (2020a)
*P:NFC = protein to NFC

Dye

Residence times were determined by mixing the artificial diets with the dye Blue 1 (Deshpande et al., 2014; Espinoza-Fuentes and Terra, 1987; Kim et al., 2018; Rodrigues et al., 2015) and observing the foremost dye position (i.e., interface between artificial diet without blue dye and artificial diet with blue dye, see Supplementary Material) over time via larval dissection. Preliminary experiments with poultry feed (UFA 625, UFA AG, Switzerland) confirmed that the effect of Blue 1 on larval weight and waste reduction was not significant (one-way ANOVA, $p < 0.05$).

Black soldier fly larvae (BSFL)

Larvae used in the experiments were from a BSFL research colony at Eawag. Eggs were placed for 24 hours on commercial poultry feed (UFA 625, UFA AG, Switzerland). Afterwards, BSFL were fed *ad libitum* with poultry feed for 13 to 14 days. In the entire study, BSFL were separated from the diet/residue using sieves, washed with deionized water, and briefly dried using paper towels. As diet residence time can be expected to depend on an organisms life stage (Chapman, 2013; Edgecomb et al., 1994), following separation, larvae were used for determination of morphometric parameters. Larval weight (n = 75, n = total number of larvae) was determined with a precision balance (BM-65, Phoenix instrument, Germany). The length of the midgut regions (n = 10) and cephalic capsule width (n = 10) were determined via a stereomicroscope (Olympus, SZX10) and the corresponding image analysis software (Olympus, cellSens Dimensions, Version 1.18). The larval instar was determined using the cephalic capsule measurement and the classification proposed by Barros et al. (2018).

Feeding experiment

Since changing the diet from poultry feed to the artificial diets may influence the midgut residence time, larvae were placed for 24 hours on the artificial diets containing no dye following separation from the poultry feed. The larvae were then separated from the artificial diets and placed into new containers with the respective artificial diets containing 0.05% (w/v) Blue 1. Three plastic containers (diameter: 7.1 cm, height: 11 cm) were prepared for each artificial diet containing 70 larvae (larval density: 1.8 larvae/cm²) and 50 g artificial diet (wet weight). Subsequently, after 10 (only experiment two and three), 20, 40, 60, 90, 120, 150, and 180 min, 10 larvae were randomly removed from each colored artificial diet using tweezers. The larvae were separated from the diet/residue and directly placed at -20 °C. The experiment was replicated in singlets three times. All feeding experiments were performed without light in a climate chamber (HPP 266, Memmert GmbH, Germany, 28°C, 70% relative humidity).

Visual determination of the foremost dye position

The foremost position of the dye (see Supplementary Material) in the midgut was determined with a stereomicroscope (Olympus, SZX10) following dissection of the larvae. Directly before dissection, the larvae were removed from the freezer and kept on ice. For each diet, time point, and experiment, ten larvae were analyzed. The entire gut was removed from the frozen larvae, placed in phosphate buffered saline, and the fat body and trachea surrounding the gut were removed. To define the position of the dye along the midgut, the different regions of this organ (AMG, MMG, and PMG) were identified (see Supplementary Material). To increase the resolution of diet residence time determination, PMG, which is the longest region of the midgut, was further subdivided into PMG1 and PMG2. This classification of the midgut into different regions followed the morphofunctional midgut characterization by Bonelli et al. (2019). Larvae that did not eat, or for which no clear allocation in the above mentioned regions

was evident, were excluded from the analysis. These larvae accounted for 12.6% of total larvae dissected.

Modelling of diet midgut residence time distributions

The data on the foremost dye position in the midgut were used to model the midgut residence time distributions. The observation (t, j) from the dissection of individual BSFL provides the information that the diet has reached the jth region at time t. The residence time in the ith gut region, $i = 1 \ldots 4$, is represented by the random variable X_i with probability distribution $p_i(:; \theta_i)$ parametrized by θ_i. Assuming the residence times are independent, the total time for the diet to travel from the beginning until the end of the jth midgut region is the sum of the corresponding residence times according to Equation (11). It is possible that diet residence times in gut regions are dependent on those of previous regions, but data of this study was insufficient to estimate these conditional probabilities.

$$T_j = \sum_{i=1}^{j} X_i \tag{11}$$

The probability distribution T_j, which is a random variable, was derived numerically by the R package *distr* (Ruckdesche and Kohl, 2014) to construct the following survival functions:

$$\text{Prob(at time } t \text{ that the diet has not yet passed region } j) =$$

$$\text{Prob}(t < T_j) = S_j(t; \theta) \tag{12}$$

where θ contains the (unknown) parameters of all the underlying residence time distributions. From equation (12), the likelihood function for a single observation (t, j) can be expressed as:

$$\text{Prob((t,j)|}\theta) = \text{Prob}(T_{j-1} < t \leq T_j | \theta) = \begin{cases} S_1(t; \theta) & j = 1 \\ S_j(t; \theta) - S_{j-1}(t; \theta) & \text{else} \\ 1 - S_3(t; \theta) & j = 4 \end{cases} \tag{13}$$

Finally, the likelihood function for all observations $[(t, j)_1, \ldots, (t, j)_N]$, is given by:

$$L(\theta) = \prod_{n=1}^{N} \text{Prob}((t, j)_n | \theta) \tag{14}$$

This enables the estimation of the parameters θ by maximizing the likelihood function numerically using the R package *maxLik* by Henningsen and Toomet (2011). Of all tested distribution families for the residence times $p_i(\cdot; \theta)$, the Gamma distributions were selected as they led to the highest log likelihood (Gamma: -511.4, Lognormal: -514.7, Weibull: -513.1).

As no observation of the time at which the diet left PMG2 was available, the residence time in this region was estimated by scaling the residence time of PMG1 based on the mean region lengths:

$$X_4 = \frac{l_{PMG2}}{l_{PMG1}} X_3 \tag{15}$$

where the mean length of PMG2 (1.6 cm) is denoted by l_{PMG2} and the mean length of PMG1 by l_{PMG1} (2.8 cm).

Determination of diet intake

Diet intake is the amount of diet ingested by the larvae at different time points and can be estimated by measuring the absorbance of the dye in BSFL gut samples at 628 nm (Kim et al., 2018; Rodrigues et al., 2015). At the time points 20, 90, and 180 min, for each artificial diet and experiment, five midguts from randomly selected larvae were placed in separate 1.5 mL tubes containing 300 μL phosphate buffered saline and two metal beads (diameter: 3 mm; Uiker AG, Switzerland). Tissues were homogenized by mixing the tubes on a vortex for 30 s. Following homogenization, tubes were centrifuged at 20,000 × g for 10 min at 4 °C, and the supernatant was stored at -20 °C until spectrometric analysis.

Following thawing, the absorbance was measured in triplicate with 300 μL of each gut homogenate at 628 nm and 750 nm using a spectrometer (Thermo Fisher Scientific, Genesys 10S). The mean absorbance at 750 nm was subtracted from the mean absorbance at 628 nm to eliminate the absorbance offset induced by other constituents in the sample (Wetzel and Likens, 2000). This net absorbance was converted into mg of wet and dry diet intake using a calibration curve. The calibration curve was produced by linear regression from absorbance values measured in triplicate for the three artificial diets containing the same amount of water as in the feeding experiments and 0.0001, 0.0002, 0.0004, and 0.0008% (w/v) Blue 1 (see Supplementary Material). The protein and NFC intake were calculated by multiplying the diet intake with the protein and NFC content of the respective artificial diet (Table 6.1) and the caloric intake was estimated according to Gold et al. (2020a). It should be noted that determining diet and nutrient intake with this approach assumed that the entire diet was ingested by BSFL.

Statistical analysis

Analysis of variance (ANOVA) followed by pairwise Tukey post-hoc comparisons were conducted to test i) the effect of the experimental repetition on the larval morphometric parameters (i.e., cephalic capsule width, wet larval weight, length of midgut regions, and total midgut length), ii) the effect of the artificial diet nutrient composition on diet, energy, protein, and NFC intake. The homogeneity of variance and normality between the different groups of comparison were assessed graphically (see Supplementary Material). A p-value < 0.05 was chosen to denote significance. Two data points, identified as outliers, were removed from the diet intake data before statistical analyses using the Median Absolute Deviation (MAD) method and a threshold of three MADs (Leys et al., 2013). The analysis was conducted using R version 3.6.2 (R Core Team, 2017).

In vitro digestions

Diets

In vitro digestions were performed using the same artificial diets used for determination of diet midgut residence times. To validate the midgut simulations with different diet contents, additionally, an artificial diet with 7:78:11% DM protein:NFC:fibre, referred to as P7NFC78, and five biowastes previously used in *in vivo* feeding experiments were used (Gold et al., 2020a) (Table 6.1, Supplementary Material).

Digestion protocol

The *in vitro* simulations of BSFL midgut digestion were based on the INFOGEST 2.0 method developed to mimic human gastrointestinal food digestion (Brodkorb et al., 2019). This method, was adapted considering the available information for BSFL digestion (i.e., pH and enzymes: Bonelli et al. (2019), temperature: Bloukounon-Goubalan et al. (2020), Table 6.2) and the diet residence times estimates. In addition, bile salts and pepsin were not considered as they are not present in insects or not yet studied in BSFL (Behmer and Nes, 2003). Gastric lipase was also not considered despite its presence in the BSFL AMG and PMG (Bonelli et al., 2019). Simulated digestion fluid amounts were changed as no information is available on the digestion fluids of BSFL, and use of the same fluid volume as in the human digestion model would increase the costs of enzymes and compromise the determination of supernatant nutrients. In comparison to the doubling of the simulated digestion fluids with each gut region starting from a ratio of 1:1 (w diet/w fluid) in the INFOGEST 2.0 method, 1 mL of simulated digestion fluid was added in this research per midgut region.

The *in vitro* digestions were carried out with chemicals of analytical grade in duplicate on artificial diets and in triplicate on biowastes with the conditions summarized in Table 6.2. For each digestion, approximately 1 g of diet was placed into a 50 mL falcon tube and digested in a water bath (Corio C, Julabo AG, Germany) at 37 °C, within the range of typical substrate temperatures in BSFL treatment (33-45 °C, Bloukounon-Goubalan et al. (2020). The artificial diets were mixed with deionized water to 80% moisture content, and biowastes were used as received (see Gold et al., 2020a for details). The pHs in the three midgut regions were adjusted with 2 M HCl and 0.1-6 M NaOH (Slimtrode, Metrohm AG, Switzerland). The enzymes used were α-amylase from human saliva (EC 3.2.1.1), trypsin from porcine pancreas (EC 3.4.21.4), and α-chymotrypsin from bovine pancreas (EC 3.4.21.1) (Brodkorb et al., 2019). Further details regarding enzyme activity and the *in vitro* digestion protocol are included in the Supplementary Material.

Table 6.2 *In vitro* digestion conditions used in this study.

	Digestion fluid[1]		Enzyme concentration	pH[2]	CaCl$_2$[1]	MiliQ water	Residence time[3]
	Type	Volume					
Region	-	mL	U/mL[2]	-	μL	mL	min
AMG	Salivary	0.8	6.4[A]	6	5 (0.3 M)	Top up to total fluid volume addition of 1 mL per midgut region	15
MMG	Gastric	0.8	-	2	0.8 (0.3 M)		45
PMG	Intestinal	0.35	4[A] 9.5[T] 53[C]	8.5	4 (0.3 M)		90

[1]Brodkorb et al. (2019), [2]Bonelli et al. (2019), [3]Results of this study (Table 6.6)
A: Amylase
T: Trypsin
C: Chymotrypsin

Following digestion in the PMG, 6 M HCl was added to stop enzyme activity. The digestion fluids were centrifuged at 14,000 × g for 45 min at 4 °C. The supernatants were immediately frozen with liquid nitrogen and stored at -20 °C until further analyses. The pellets were dried in a laboratory oven at 80 °C for 24 h.

Digestion products

A typical approach for evaluating *in vitro* digestion model outcomes is determination of nutrients in digestion products (Kiers et al., 2000; McClements et al., 2009; Oomen et al., 2002, 2003). We determined diet digestibility, and supernatant maltose, glucose, total organic carbon (TOC), total nitrogen (TN) and amino acids. These parameters were selected as estimates of NFC (i.e., maltose and glucose), protein (i.e., total nitrogen and amino acids) and organic matter (i.e., total organic carbon). NFC, protein and organic matter have previously been shown to be important for BSFL development (Barragán-Fonseca et al. 2018b, Barragán-Fonseca et al. 2018c; Beniers and Graham 2019; Gold et al., 2020a; Lalander et al. 2019).

Maltose and glucose were determined as D-glucose using a commercial enzymatic kit (Megazyme, Total Starch Assay K-TSTA-50A). The supernatant was mixed with 4.5 mL of 100 mM sodium acetate buffer (pH 4.5) and 0.1 mL amyloglucosidase (3,300 U/mL) and incubated at 50 °C for 30 min. D-glucose was quantified at 510 nm against a standard, following incubation with 3.0 mL GOPOD reagent at 50 °C for 20 min. The TN and TOC were determined with the 680°C combustion catalytic oxidation method according to the manufacturer's instructions (TOC-L Analyzer, Shimadzu Corporation, Japan). Amino acids (except for tryptophan) were analyzed in the pool of the supernatants from the *in vitro* digestion of the biowaste with a Waters AccQ-Tag Ultra Derivatization Kit and Waters UPLC system with an Acquity UPLC Binary Solvent Manager and Sample Manager, based on the manufacturer instructions and previous research (see detailed protocol in the Supplementary Material). The TN and TOC results of *in vitro* digestion samples without diet were used to correct TN and TOC results for nitrogen and organic carbon included in the digestion fluids and enzymes. Maltose and glucose, TOC, and TN concentrations were normalized (g/L × g DM) based on the amount of diet digested using equation (16):

$$\text{Supernatant sugar, TN, TOC} = \frac{\text{supernatant sugar, TN, TOC}}{\text{diet input}_{\text{dry mass}} \text{ (g DM)}} \qquad (16)$$

The diet digestibility (as % DM) was calculated analogous to *in vivo* waste reduction (Gold et al., 2020a) using equation (17) based on the dry mass of the pellet following centrifugation:

$$\text{Diet digestibility} = \left(1 - \frac{\text{pellet}_{\text{dry mass}} \text{ (g DM)}}{\text{feed input}_{\text{dry mass}} \text{ (g DM)}}\right) \times 100 \qquad (17)$$

In vivo feeding experiments

In vivo feeding experiments were executed with the same artificial diets and biowastes used in *in vitro* digestions. Waste reduction, larval weight and bioconversion rate was calculated as described in Gold et al. (2020a). For each artificial diet, 2-3 replicates with 40 BSFL with an initial weight of 5.4 mg DM were placed in plastic containers (5.5 cm diameter, 7.2 cm height) and fed every three days at a feeding rate of 30 mg DM/(larva × day) for a total of six days. Experiments were replicated three times. Feeding experiment results with biowastes were taken from Gold et al. (2020a). For each biowaste, 4-5 replicates with 80 BSFL with an initial weight of 3.8 mg DM were placed in plastic containers (7.5 cm diameter, 11 cm height) and fed every three days at a feeding rate of 27 mg DM/(larva x day) for a total of nine days. Experiments were conducted once. All experiments were conducted without light in a climate chamber (HPP 266, Memmert GmbH, Germany, 28°C, 70% relative humidity).

Comparison of in vivo and in vitro results

Results from *in vitro* digestions and *in vivo* feeding experiments were compared to assess whether the *in vitro* model can be indicative of feeding experiment results. Since *in vivo* feeding experiment designs typically vary between studies (Bosch et al., 2020) and static *in vitro* models are oversimplified (Bohn et al., 2018), we regarded a ranking method to be more meaningful than comparison of absolute results. For example, absolute diet digestibility can be expected to be much lower than waste reduction considering it is calculated following one digestion, whereas waste reduction is calculated following several digestions.

Ordinal ranking (e.g., 1, 2, 3) was applied separately to artificial diets and biowastes as per the mean results of the *in vitro* digestion products and *in vivo* performance metrics, considering the range of results for each parameter. Fractional ranking (e.g., 1, 2-3, 4) was applied when the mean value ($\pm$ standard deviation for n > 2, $\pm$ differences between repetition for n = 2) overlapped among diets. Lower ranks were given to diets with higher *in vitro* digestion products and higher *in vivo* performance metrics.

The ranking of diets based on *in vitro* digestibility was compared to *in vivo* waste reduction. A mean rank of the three *in vitro* supernatant nutrient parameters was calculated and compared to larval weight. The *in vitro* model was deemed indicative of *in vivo* feeding experiments results if diets received the same rank. Comparison between *in vitro* digestion products and *in vivo* feeding experiment metrics is justified considering that diet digestibility and waste reduction are calculated in a similar way (Gold et al., 2020a, Equation (17)), and that previous studies concluded that diet NFC, protein and organic matter can increase larval weight (Barragán-Fonseca et al. 2018b, Barragán-Fonseca et al. 2018c; Beniers and Graham 2019; Gold et al., 2020a; Lalander et al. 2019).

6.4   Results and Discussion

Diet midgut residence times

The present study determined, for the first time, diet midgut residence times and diet intake using artificial diets varying in protein and NFC content. Cephalic capsule width measurements identified BSFL used for residence time determination as late fifth instar/beginning sixth instar in all experiments (Table 6.3). Despite significant differences in wet larval weight between experiments, the length of the different midgut regions and total midgut length was not significantly different between experiments (Table 6.3). Therefore, to increase the quality of the residence time estimations, results of the foremost position of the dye in the midgut for the three experiments were pooled for modeling the midgut residence time distributions.

The probability density functions of the foremost colored artificial diet position over time in the three midgut regions are shown in Figure 6.1. The shape of the distributions demonstrates considerable variability in residence times between larvae grown on the same diet. This has also been previously reported for drone fly larvae (Eristalis tenax) (Waterhouse, 1954) and could be attributed to differences between larvae during development (e.g., due to differences in nutrient provision in previous life stages), physiological condition (e.g., molting), and the corresponding individual nutritional demand (Chapman, 2013; Edgecomb et al., 1994), or length of midgut regions and total midgut length (see standard deviation of midgut region lengths between larvae in Table 6.3).

Table 6.3 Mean morphometric parameters of BSFL used for determining diet midgut residence times.

	Unit	n*	Experiment 1	Experiment 2	Experiment 3
Cephalic capsule width	mm	10	1.03 (0.08)	1.00 (0.06)	1.05 (0.07)
Larval weight	mg	75	219 (28)	177 (36)	186 (31)
AMG length	cm	10	2.5 (0.2)	2.5 (0.2)	2.5 (0.2)
MMG length	cm	10	1.5 (0.2)	1.5 (0.2)	1.7 (0.2)
PMG length	cm	10	4.2 (0.8)	4.2 (0.4)	4.6 (0.5)
Midgut length	cm	10	8.2 (1.1)	8.2 (0.6)	8.8 (0.7)

* n= number of larvae used per morphometric parameter
In parenthesis: standard deviation; AMG: Anterior midgut; MMG: Middle midgut; PMG: Posterior midgut

As static *in vitro* models do not consider residence time distributions, descriptive statistics (i.e., mean, median, mode) of the probability density functions were calculated and are included in the Supplementary Material. In order to neither under- nor overestimate the diet residence time when modelling the BSFL midgut, the median (Table 6.4), which is more robust to outliers than the mean, was selected as the most meaningful parameter. In the following text, the median residence time is presented together with the mean ± standard deviation in parenthesis. As the distributions do not always follow a bell-shaped curve, residence time estimates depended on the descriptive statistic used. However, the overall trends in diet residence times between midgut regions and artificial diets presented in the following section also hold true for the other descriptive statistics. The diet residence time estimates are considered to be more reliable for AMG to PMG1 as they were based on the foremost dye position, whereas the PMG2 residence time was interpolated based on midgut length. Therefore, in the following presentation of PMG diet residence time results, PMG1 and PMG2 are given separately; the residence time estimate for PMG2 is supported by the collected experimental data.

Table 6.4 Median of probability density function of the BSFL midgut residence time distributions (min) with three artificial diets varying in protein and NFC content. See the Supplementary Material for the probability density functions and for more descriptive statistics.

Diets	n*	AMG	MMG	PMG 1	PMG 2	Total
P^{13}NFC^{8}	233	14	37	62	36	154
P^{13}NFC^{47}	218	17	46	77	44	191
P^{7}NFC^{47}	176	41	32	65	37	195

* n=total number of larvae dissected per diet
AMG: Anterior midgut; MMG: Middle midgut; PMG: Posterior midgut; n: number of larvae

Despite the variability between larvae, the results of this study demonstrate that diet residence time in the different midgut regions of BSFL is influenced by the protein and NFC content in the diet. As the NFC content correlates negatively with the cellulose and caloric content in the diet (Table 6.1), these parameters are also likely decisive for the diet residence time in BSFL. In addition, the diet intake in BSFL was measured to propose explanations for the observed residence times between diets. Diet intake and midgut residence time are likely connected; the more diet is ingested, the faster the gut content is pushed forward, reducing the diet midgut residence time (Karasov et al., 2011).

For the three artificial diets, the overall midgut residence time was 154 min (156 ± 31 min) for P^{13}NFC^{8}, 191 min (197 ± 38 min) for P^{13}NFC^{47}, and 195 min (202 ± 64 min) for P^{7}NFC^{47}. These results indicate that residence times in the BSFL midgut were shorter than those in the guts of other dipteran larvae, although this difference could also be due to different diets across studies. In fruit fly larvae (*Drosophila melanogaster*), which are much smaller, the diet takes approximately 30 min to reach the PMG.

Mumcuoglu et al. (2001) and Waterhouse (1954) reported total gut residence times of 60-90 min for green bottle fly larvae (*Lucilia sericata*) and 85-120 min for drone fly larvae. Considering the midgut length, this corresponds to a passage rate of 50-75 mm/h for drone fly larvae in comparison to 29-36 mm/h for BSFL (AMG to PMG2).
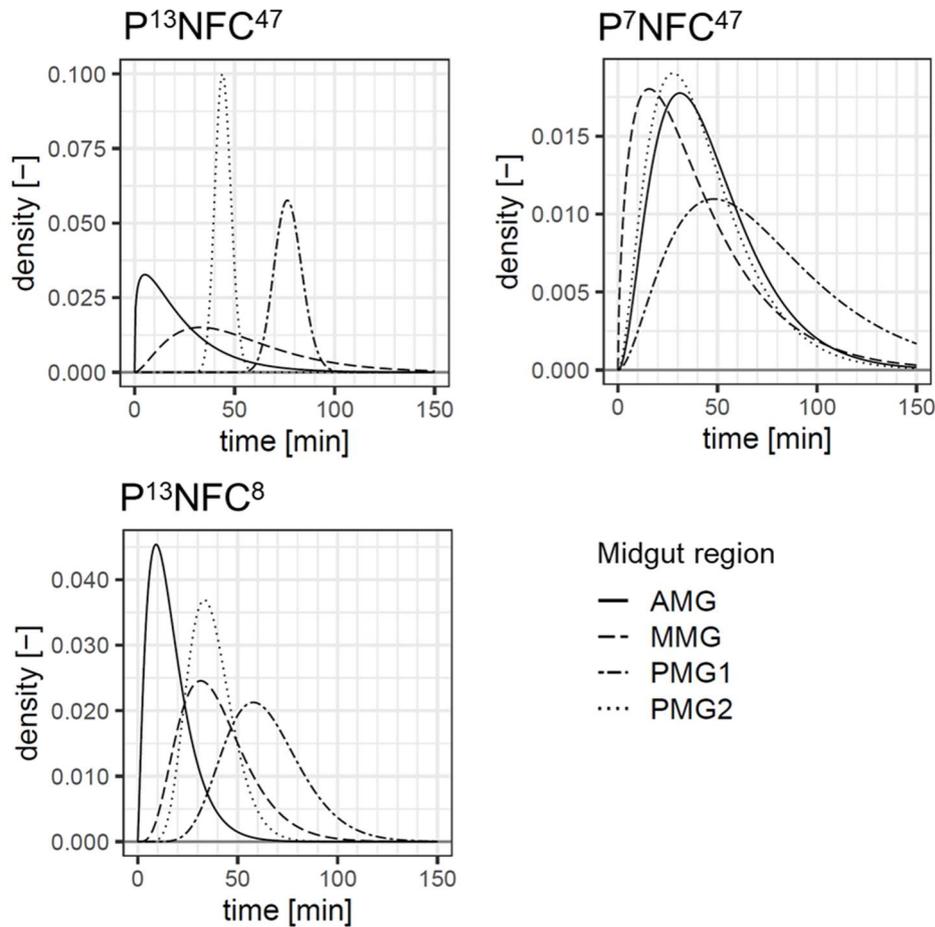
Figure 6.1 Probability density function of the BSFL midgut residence time for the three artificial diets. AMG: Anterior midgut, MMG: Middle midgut, PMG: Posterior midgut. See Table 6.4 for the medians of probability density functions.

Except for the diet lower in protein content, the residence time was shortest in the AMG and the longest in the PMG (Table 6.4, Figure 6.1). Diets also had the longest residence time in the PMG of housefly larvae (*Musca domestica*) (Espinoza-Fuentes and Terra, 1987) and different species of blow fly larvae (Waterhouse, 1954). The high digestive capability (i.e., high enzymatic activity and diet residence time) and the morphological features of the fly larvae PMG suggests that this midgut region plays a fundamental role in the decomposition of nutrients to monomers and in their absorption (Bonelli et al., 2019; Gold et al. 2018a). In addition, as the growth of bacteria is time-dependent, the higher diet residence time may be a contributor to the bacterial count in the PMG, which is higher than that in the AMG and MMG (Bruno et al., 2019).

Diet AMG residence times decreased with the diet protein content. As illustrated in Figure 6.1, P7NFC47 had an AMG residence time of 41 min (46 ± 26 min) in comparison to 14 (16 ± 11 min) and 17 min

(23 ± 20 min) for $P^{13}NFC^8$ and $P^{13}NFC^{47}$, respectively. As BSFL are unable to select their nutritional environment, the increase in the AMG residence time could be a post-ingestive mechanism to balance nutrient intake. Following ingestion, insects and their associated microbiota may balance nutrient decomposition and absorption. For example, locusts (Locusta migratoria) regulate macronutrient digestion and absorption, among others, through the amount of enzymes secreted into the gut, secreting fewer enzymes for nutrients in excess and more enzymes for nutrients in deficit (Clissold et al. 2010; Zanotto et al., 1993). Similar mechanisms were found in the common fruit fly by Miguel-Aliaga et al. (2018). In BSFL, Bonelli et al. (2020) observed protease activity in the AMG for low-protein diets while proteases were almost absent in this region in larvae reared on diets with excess protein. In addition to adjusting enzyme activities, prolonging the AMG diet residence time could be an additional post-ingestive mechanism of BSFL to improve protein digestion. As shown in Figure 6.2, protein ingestion was lowest for P^7NFC^{47}. Thus, as nutrient extraction efficiency is positively correlated with diet residence time (Karasov et al., 2011), BSFL could have increased access to free amino acids (which are indispensable for fly larval development) by increasing AMG diet residence time (Colombani et al., 2003; Danielsen et al., 2013; de Carvalho and Mirth, 2017; Lalander et al., 2019). This hypothesis could be tested in future research by measuring both midgut lumen enzyme activities and residence times between diets varying in protein content.

Diet NFC, and thereby cellulose and energy content, also appeared to have a significant influence on both diet ingestion and overall midgut residence time. For the diets higher in protein ($P^{13}NFC^8$ and $P^{13}NFC^{47}$), MMG and PMG diet residence times increased with the diet NFC content and decreased with the diet cellulose content. MMG and PMG residence times were 46 and 77 min for $P^{13}NFC^{47}$ in comparison to 37 and 62 min for $P^{13}NFC^8$, respectively. As shown in Figure 6.2, this decrease in MMG and PMG residence time meant that larvae ingested significantly more of the $P^{13}NFC^8$ diet than the other two diets. Lowering of the diet midgut residence time could be an indication of compensatory feeding in BSFL due to nutrient restrictions (i.e. low digestible nutrient and caloric content). Compensatory feeding has been reported for several other Dipteran species (Carvalho et al., 2005; Gelperin and Dethier, 1967; Simpson et al., 1989) and indicates that BSFL increase their diet intake and thereby lower the diet residence time to compensate for the low caloric content of $P^{13}NFC^8$. However, as shown in Figure 6.2, despite the increased diet ingestion, larvae ingested significantly less digestible energy by feeding on $P^{13}NFC^8$ than of the other two diets that are higher in NFC.
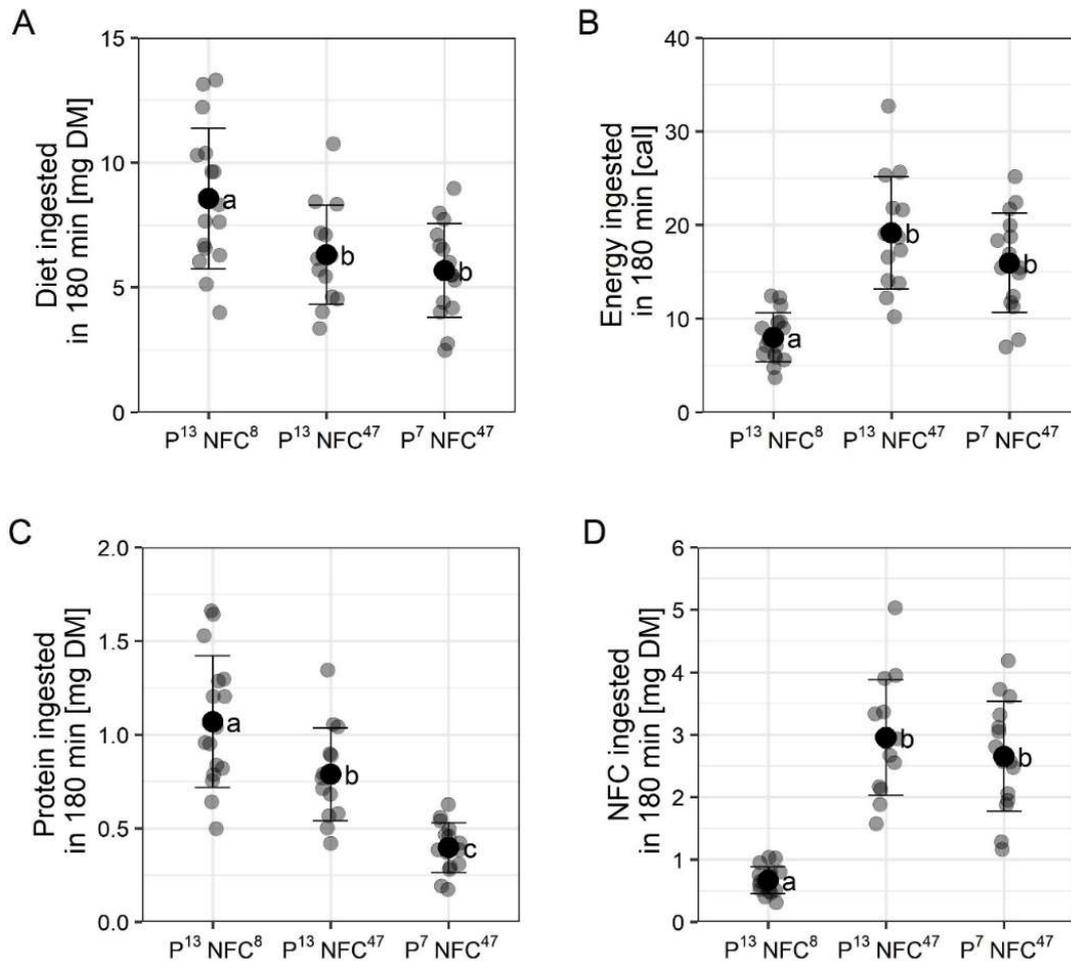
Figure 6.2 Mean diet (mg DM) (A), energy (cal) (B), protein (mg DM) (C), and NFC (mg DM) (D) ingested by BSFL in 180 min on the three artificial diets. Means, standard deviations, and results per larvae are displayed for all three experiments.

In summary, our results demonstrate that total and midgut region residence time in BSFL is influenced by the diet nutrient content. This influence is often neglected in *in vitro* models of humans and farmed animals. Depending on the diet nutrient content, *in vitro* models may over- or under-estimate *in vivo* performance based on the soluble nutrients in the supernatant. For example, as enzyme activity is the amount of substrate (e.g., starch) catalyzed per minute, the supernatant nutrient content as measured in this study is proportional to the residence time when substrates are available in excess and no inhibitors are present. However, not mimicking the influence of diet nutrients, and other parameters such as sex, instar, body size, and age, which have all been shown to influence diet residence time, is also one of the strengths of the model as it allows for cost-effective implementation. Diet residence times of 15 min in the AMG, 45 min in the MMG, and 90 min in the PMG were used in subsequent *in vitro* simulations of the BSFL midgut.

Table 6.5 Mean digestibility of diets in *in vitro* simulations of the BSFL midgut and waste reduction in *in vivo* feeding experiments. The asterisks indicate diets for which the ranking based on *in vitro* results deviated from the ranking of the diets based on *in vivo* feeding experiments.

	in vitro		*in vivo*	
	Digestibility	Rank	Waste reduction	Rank
	% DM		% DM	
Artificial diets				
$P^{13}NFC^8$	15.0 (4.6)	4	31.7 (1.8)	4
$P^{13}NFC^{47}$	30.0 (2.8)	2	48.9 (3.9)	1-2
P^7NFC^{78} *	37.2 (2.5)	1	41.0 (1.4)	3
P^7NFC^{47} *	26.5	3	47.0 (3.4)	1-2
Biowastes				
Cow manure	11.4 (10.6)	4-5	12.7 (0.9)	5
Mill by-products *	19.0 (3.6)	4-5	56.4 (1.2)	1-2
Canteen waste	45.0 (14.6)	1-3	37.9 (3.8)	3
Poultry slaughterhouse waste *	36.9 (1.4)	1-3	30.7 (4.7)	4
Vegetable canteen waste	49.9 (5.6)	1-3	58.4 (1.4)	1-2

In parenthesis: standard deviation for samples where n ≥ 3 and differences between analyses where n = 2
In vivo results for biowastes were taken from Gold et al. (2020a)

In vitro simulation of midgut digestion

The residence time estimates were used to mimic the BSFL midgut *in vitro* for the first time. As validation of the *in vitro* digestion model, the study assessed whether ranking of the diets based on *in vitro* digestion products is similar to the ranking of diets based on waste reduction and larval weight recorded in *in vivo* feeding experiments.

Based on the *in vitro* simulations, P7NFC78 and P13NFC47, and canteen and slaughterhouse wastes were expected to have the highest waste reduction and larval weight in *in vivo* feeding experiments. Diet digestibility (Table 6.5) and supernatant nutrient content (Table 6.6) were highest for these diets, likely due to their highly digestible nutrient content (i.e. protein, NFC, and lipids, see Table 6.1). Accordingly, P7NFC47 and P13NFC8, which are lower in protein and/or NFC, and cow manure and mill by-products, which are richer in fiber and/or have lower organic matter content, received a higher ranking, indicating that a lower *in vivo* waste reduction and larval weight can be expected. The correlation between *in vitro* digestion product results and the diet nutrient composition indicates that the *in vitro* model could also complement and/or replace conventional diet gross nutrient composition analyses (see parameters in Table 6.1) frequently completed for BSFL diets.

Comparison of the ranking based on *in vitro* results and the ranking based on the *in vivo* performance shows that *in vitro* simulations can predict *in vivo* outcomes. The approach was able to broadly distinguish between the best and the worst performing diets (Table 6.4, Table 6.5, Table 6.6). For example, P13NFC8 and P7NFC47, and vegetable canteen waste and cow manure received the same ranking based on *in vitro* simulations and *in vivo* feeding experiments. This confirms that the ranking of biowastes based on results of digestion products from *in vitro* digestion could be a valuable tool for screening of different biowastes based on BSFL treatment performance.

Table 6.6 Mean supernatant nutrient content following *in vitro* simulation of the BSFL midgut and larval weight and bioconversion rate results of *in vivo* feeding experiments. The asterisks indicate diets for which the ranking based on *in vitro* results deviated from the ranking of the diets based on *in vivo* feeding experiments.

	in vitro									*in vivo*		
	Glucose	Rank	TN	Rank	Total AA	Rank	TOC	Rank	Mean rank	Larval weight	Bioconversion rate	Rank
	g/(L x g DM)	-	g/(L x g DM)	-	g/L	-	g/(L x g DM)	-	-	mg DM	% DM	-
Artificial diets												
$P^{13}NFC^8$	0.6 (0.1)	4	3.3 (0.7)	1-2	-	-	17.5 (2.6)	4	3/3-4	21.0 (2.1)	6.5 (0.9)	4
$P^{13}NFC^{47}$	3.8 (0.6)	2-3	3.7 (0.6)	1-2	-	-	30.9 (3.6)	1-2	2/1-2	47.5 (2.5)	17.5 (1.3)	1
P^7NFC^{78} *	6.8 (0.2)	1	2.7 (0.2)	3	-	-	38.6 (1.8)	1-2	2/1-2	32.7 (2.2)	10.9 (1.4)	3
P^7NFC^{47} *	3.6 (0.5)	2-3	1.8 (0.3)	4	-	-	23.8 (0.9)	3	3/3-4	41.5 (0.2)	14.9 (0.3)	2
Biowastes												
Cow manure	0 (0)	4-5	1.7 (0.3)	-	0.5 (0.1)	5	10.7 (1.0)	5	5/5	14.3 (0.4)	3.8 (0.2)	5
Mill by-products	1.7 (0.3)	2-3	3.5 (0.1)	-	1.8 (0.1)	3-4	24.6 (0.5)	4	3/3-4	41.7 (0.9)	14.9 (0.3)	3
Canteen waste	1.5 (0.3)	2-3	9.1 (2.2)	-	5.5 (0.7)	2	47.7 (7.2)	1-3	2/1-2	44.2 (5.9	15.3 (2.1)	2
Poultry slaughterhouse waste	0 (0)	4-5	12.4 (0.7)	-	9.9 (2.8)	1	44.4 (2.4)	1-3	3/3-4	39.4 (0.7)	13.4 (0.5)	4
Vegetable canteen waste	3.6 (0.3)	1	3.3 (0.1)	-	1.9 (0.3)	3-4	49.3 (0.3)	1-3	2/1-2	59.1 (2.6)	22.7 (1.1)	1

In parenthesis: standard deviation for samples where n ≥ 3 and differences between analyses where n = 2

In vivo results for biowastes were taken from Gold et al. (2020a)

Ranking: Diets received a rank based on the mean diet digestibility, glucose, TN, and TOC content considering the range of results for each parameter. When the mean value (± standard deviation for n > 2 *in vitro* repetitions and ± differences between repetition where n = 2) for these parameters overlapped for different diets, they received the same rank. Mean rank of the three nutrient parameters was calculated for each diet. The number of ranks was equal to the number of diets per group (n = 4 for artificial diets, n = 5 for biowastes).

Simulation limitations

Despite these promising results, several differences between the *in vitro* and *in vivo* ranking (Table 6.5 and Table 6.6) highlighted the limitations of the laboratory-based simulation, digestion product analyses, and/or the applied ranking procedure. In particular, the ranking based on *in vitro* digestion products was i) unable to predict the differences in *in vivo* performance between vegetable canteen waste, canteen waste, and poultry slaughterhouse waste; ii) overestimated the *in vivo* performance of P7NFC78 and poultry slaughterhouse waste; and iii) under-estimated the *in vivo* performance of mill by products.

These discrepancies are to be expected as static *in vitro* digestion models are oversimplified and do not include all complex and dynamic digestion processes (Bohn et al., 2018). For example, dynamic changes in temperature during BSFL treatment (Bloukounon-Goubalan et al., 2020), multiple digestion of the diet by BSFL, differences in properties of enzymes in the midgut lumen and commercial enzymes, dynamic changes in enzyme activities in relation to the nutritional environment (Benkel and Hickey, 1986; Clissold et al., 2010), external digestion (Sakaguchi and Suzuki, 2013), and lipases, lysozymes, and chitinases (Bonelli et al., 2019; Terra and Ferreira, 2012) in the fly larvae midgut were not considered in this study. An additional explanation for the mismatch between the *in vitro* and *in vivo* ranking could be false assumptions regarding the effect of supernatant nutrients on larval weight. The current study did not estimate nutrient bioavailability and/or bioactivity to BSFL (McClements et al., 2009), but instead assumed that all nutrients released from the diet during *in vitro* simulation are absorbed by the larvae into the hemolymph, metabolized, and contribute to larval growth (Oomen et al., 2002, 2003). In addition, the procedure of this study assumed that all nutrients measured in the supernatant have the same linear effect on larval weight. This might be an oversimplification as previous work on BSFL and common fruit fly larvae indicate that protein can be more important than NFC in determining larval weight and development time (Danielsen et al., 2013; de Carvalho and Mirth, 2017; Lalander et al., 2019). In addition, insufficiency or excess of nutrients or poor nutrient ratios (e.g., protein to NFC) affect larval development (Barragán-Fonseca et al. 2018b; Gold et al., 2020a).

The lack of detailed knowledge on BSFL biology currently limits mimicking all these processes in an *in vitro* model. This would also be impractical considering the costs involved (e.g., enzyme costs). However, based on this validation of the simulated midgut, future studies should assess whether a stronger correlation can be established between *in vitro* and *in vivo* results by considering the nutrient requirements of BSFL. Fly larvae adjust their diet intake and digestion based on their nutritional environment and nutritional needs. For example, fly larvae control diet intake based on protein, and over- or under-consuming other nutrients such as sugars (de Carvalho and Mirth, 2017). In addition, digestion of excess nutrients, such as sugars, can result in metabolic costs, decreasing larval weight and increasing development time (Matzkin et al., 2011; Musselman et al., 2011). This mechanism could explain the overestimation of the *in vivo* performance based on *in vitro* results for P7NFC78 and slaughterhouse waste. These diets are unbalanced in terms of nutrient content and nutrient ratios (Table 6.1), compared to nutritionally balanced diets for BSFL (e.g., protein to NFC ratios of 2:1 – 1:4, NFC content < 40%) (Barragán-Fonseca et al. 2018b; Gold et al., 2020a). P7NFC78 was high in NFC, while the slaughterhouse waste was high in protein. An additional explanation for their lower performance *in vivo* than *in vitro* could be suboptimal diet palatability (Cohen, 2005). In contrast to all other diets, the slaughterhouse and canteen wastes were more digestible *in vitro* than *in vivo*, supporting the hypothesis

that BSFL do not digest all of these nutrients *in vivo*, owing to adjustments in diet intake and digestion that are, in turn, governed by poor nutrient contents and/or ratios or suboptimal diet palatability.

Further research could also consider simulating the midgut microbiota. In BSFL treatment, microbes in the biowaste and/or midgut are able to use diet constituents as nutrient sources and thereby likely contribute to waste reduction. Microbes produce digestible biomass and metabolites (e.g., short chain fatty acids) for BSFL that can contribute to larval growth (Douglas, 2010, 2009; Lam et al., 2009a; Romero et al., 2006; Wong et al., 2014). Microbial-mediated processes can be especially valuable on nutrient poor diets and/or on diets that are constituted by nutrients that are indigestible by BSFL, such as hemicelluloses (Storelli et al., 2011; Zhao et al., 2017). Similar to intestinal digestion in humans, microbial enzymes (e.g., β-glucanases, xylanases, and pectinases) (Terra and Ferreira, 2012) in the biowaste and/or the midgut may bring about partial digestion of hemicelluloses in BSFL treatment (Bruno et al., 2019; De Smet et al., 2018; Gold et al., 2020a, 2018a; Jeon et al., 2011; Lee et al., 2014; Rehman et al., 2017). This was not considered in this study and could explain why mill by-products, which are mainly plant cell walls consisting of hemicelluloses (such as xylans or β glucans) (Merali et al., 2015), received a higher performance ranking based on the *in vitro* simulation than measured in *in vivo* feeding experiments (Table 6.5 and Table 6.6). As an initial step of microbial simulation in the BSFL midgut, PMG could serve as the primary area of focus, owing to the longest diet residence time and the highest bacterial load (Bruno et al., 2019) in this region. Microbial fermentation could be considered by the addition of enzymes, specific microbes isolated from the PMG, or the entire PMG content (Wahlgren et al., 2019).

6.5 Conclusions

The present study is the first to evaluate *in vitro* simulation of the BSFL midgut. Despite not completely mimicking the complexity of the BSFL digestive system, such as differences in midgut residence times with diet nutrient content, *in vitro* simulations were able to predict the outcomes of *in vivo* feeding experiments. Digesting diets in an *in vitro* model and subsequent analyses of the formed digestion products indicated *in vivo* BSFL waste reduction and larval weight, and diet nutrient composition. For several diets, the performance estimated based on *in vitro* results did not match with the results in the feeding experiments. More reliable estimates of *in vivo* feeding experiment outcomes could presumably be provided by considering the nutrient requirements of BSFL, hemicellulose digestion, and the diet and gut microbiota. However, the main advantages of *in vitro* simulations are their simplicity and low cost. Thus, the benefits of simulations resulting in more accurate mimicking of digestive processes needs to be balanced with the added resources required for their implementation (e.g., laboratory equipment, consumables, labor).

7. Chapter – Novel experimental methods for the investigation of *Hermetia illucens* L. (Diptera: Stratiomyidae) larvae

Moritz Gold[1,2,†] Melanie Binggeli[1,†] Fabienne Kurt[3], Tomas de Wouters[3], Markus Reichlin[3], Christian Zurbrügg[2], Alexander Mathys[1] and Michael Kreuzer[4,5]

[1]ETH Zurich: Swiss Federal Institute of Technology Zurich, Laboratory of Sustainable Food Processing, Schmelzbergstrasse 9, 8092 Zurich, Switzerland
[2]Eawag: Swiss Federal Institute of Aquatic Science and Technology, Department Sanitation, Water and Solid Waste for Development (Sandec), Überlandstrasse 133, 8600 Dübendorf, Switzerland
[3]PharmaBiome AG, Schmelzbergstrasse 7, 8092 Zurich, Switzerland
[4]ETH Zurich, Institute of Agricultural Sciences, Universitätstrasse 2, 8092 Zurich, Switzerland

†These two authors contributed equally

7.1 Abstract

Large-scale insect rearing for food and feed production can be improved by understanding diet - digestion and host-microbe interactions. To examine these processes in black soldier fly (*Hermetia illucens* L.; Diptera: Stratiomyidae) larvae (BSFL), two protocols were developed. Protocol 1 describes a method to produce viable, sterile BSFL and a gentle method for diet sterilisation. Sterile BSFL can be used to study the diverse role of microbes in larval development. Nutrient requirements of sterile BSFL are met only through diet. Viable sterile BSFL were consistently generated using a four-step treatment with alternating immersions of eggs for 2 min each in ethanol (70%) and sodium hypochlorite (0.6%), over two cycles. A non-thermal method of diet sterilisation, namely high-energy electron beam (HEEB) treatment, was introduced. Subsequently, growth of sterile BSFL was observed on the HEEB-treated diet but not on an autoclaved diet. In Protocol 2, we propose a novel method to collect frass from individual larvae. We then measured the metabolites in frass, using high-pressure liquid chromatography. This method is a promising tool to estimate the diet and nutrient requirements of sterile BSFL, thus, increasing the performance and reliability of BSFL rearing. We discuss in detail, the possible applications and limitations of our methods in BSFL research.

7.2 Introduction

Upcycling of organic wastes (e.g. animal manure, food waste) and side streams (e.g. food industry by-products) by insects (Zurbrügg et al. 2018) for sustainable food and feed production, requires efficient large-scale rearing facilities (Smetana et al. 2019). Currently, larvae of the black soldier fly (BSF; *Hermetia illucens* L. (Diptera: Stratiomyidae)) are among the most promising insects reared as animal feed (Bosch et al. 2020, Pinotti et al. 2019) and food (Barroso et al. 2017). Extensive research has been conducted on the influence of dietary nutrients on the growth of BSF larvae (BSFL) (Lalander et al. 2013, Nguyen et al. 2013, Barragán-Fonseca et al. 2017 and 2018c, Cammack and Tomberlin 2017, Spranghers et al. 2017, Gold et al. 2020a). Process reliability is a major challenge in large-scale rearing of insects for animal feed markets. Current process performance (e.g. larval growth, bioconversion rate) is often low or varies between diets (Lalander et al. 2018, Nyakeri et al. 2019, Gold et al. 2020a), thus, affecting day-to-day operations and sustainability (Smetana et al. 2019). It is therefore necessary to determine the specific nutrient requirements of BSFL as well as their nutrient utilisation.

In order to thrive, insects depend on their gut microbiota, as these microbes degrade food constituents to products that can be absorbed in the gut (Douglas 2010, Engel and Moran 2013). In addition, they produce compounds and metabolites, that are either essential for the organism's metabolism (Shin et al. 2011, Storelli et al. 2011 and 2018), or play a role in immune response (Broderick and Lemaitre 2012), and detoxification of contaminants (e.g. secondary plant metabolites, mycotoxins) (Fusetto et al. 2017). BSFL harbour a rich and diverse microbial community (Bruno et al. 2019). The addition of bacteria to diets has further increased rearing performance (Somroo et al. 2019). However, the role of microbiota in BSFL and their application in mass-rearing of this insect is yet poorly understood (De Smet et al. 2018); although several similarities may be derived from extensive research on the *Drosophila melanogaster* model (Gold et al. 2018a, Shin et al. 2011, Storelli et al. 2011 and 2018). These processes have been examined across several disciplines, in sterile organisms. Numerous attempts have been made to sterilise insect eggs (Table 7.1). Sodium hypochlorite is an especially promising chemical that induces egg sterilisation by removing the embryonic egg chorion (Broderick and Lemaitre 2012). Egg sterilisation is well established in several insect species, especially *Drosophila* spp. However, in existing

literature (Table 7.1) a large variation is observed in the concentration and combinations of chemicals used, such as ethanol and formaldehyde, as well as the number of egg immersion cycles. In addition, no papers have been published on successful rearing of both sterile and growing BSFL that can possibly be used as a research model system. It is difficult to achieve maximal hatching rates at complete egg and larval sterility, since both sterility success and mortality increase with increasing concentration of the sterilizing chemical and duration of exposure. Moreover, since BSF eggs are oviposited in clusters of several hundred eggs, it is necessary to separate them, to allow the sterilizing chemicals to treat the entire surface area of the egg without destroying the inner layer that carries the larva. Results of our preliminary experiments as described in the first protocol below indicate that currently established methods for production of sterile *Drosophila* spp. are unable to produce sterile and growing BSFL.

Table 7.1 Established sterilisation methods for the production of sterile insects, as described in literature.

Insect species	Method	Reference
Dacus dorsalis	Immersion in a solution with 10% formaldehyde and 0.2% hydrochloric acid for 10 min, followed by four washing cycles with sterile, double distilled water	Tamashiro et al. 1990
Drosophila spp.	Immersion in 2.7% sodium hypochlorite for 2 min, two washing cycles with 70% ethanol, followed by two washing cycles with sterile, distilled water	Brummel et al. 2004
Drosophila melanogaster	Immersion in 10% sodium hypochlorite for 5 min, rinsed three times with sterile water	Ridley et al. 2012
Drosophila melanogaster	Three washing cycles with 0.6% sodium hypochlorite, followed by three washing cycles with sterile water	Newell and Douglas 2014
Drosophila melanogaster	Immersion in 2.7% sodium hypochlorite for 2–3 min, followed by two washing cycles with 70% ethanol and three washing cycles with sterile water for 10 min	Sabat et al. 2015
Drosophila melanogaster	Two washing cycles with 0.6% sodium hypochlorite for 2.5 min each, followed by three washing cycles with sterile water	Koyle et al. 2016
Drosophila melanogaster	Three washing cycles with 0.6% sodium hypochlorite solution, followed by three rinsing cycles with sterile water	Kim et al. 2018
Drosophila melanogaster	Immersion in 1% active chlorine (50% bleach) for 5 min, two washing cycles with 70% ethanol for 1 min and sterile water for 1 min	Kietz et al. 2018
Lucilia sericata	Immersion in 0.05% sodium hypochlorite for 5 min, followed by immersion in 5% formaldehyde for 5 min, washed with sterile distilled water	Mumcuoglu et al. 2001
Musca domestica	Immersion in 0.26% sodium hypochlorite for 25 min, followed by three washing cycles with sterile water	Schmidtmann and Martin 1992
Hermetia illucens	Three washing cycles of 70% ethanol followed by three washing cycles with sterile water	Wynants et al. 2019

As in livestock, dietary nutrients (e.g. protein, carbohydrates) are essential for development in insects (Danielsen et al. 2013). Information on both nutrient composition and digestibility of the diet is required to determine diet quality. This information also facilitates calculation of both the protein/amino acid and energy requirements of BSFL according to their developmental stage (i.e., hatchlings vs. late-instar larvae), environmenttal (e.g. temperature), and operating conditions (e.g. feeding depth affecting oxygen supply). With this knowledge, diets with an optimal nutrient composition can be provided in the ideal amounts. Digestibility measurements determined by mass balance calculations between nutrients in food/feed and animal/human/animal faeces, using the nutritional value of individual foods/feeds described in databases, is the current standard in human and livestock nutrition. Similar methods may be implemented to obtain information on the digestibility of

BSFL diets. When combined with growth assessment methods, they can provide information on nutrient requirements of BSFL. However, to the best of our knowledge, there has not yet not been any method, describing the collection of frass from BSFL. As these larvae naturally live within their diet, they consume from and excrete into the same environment. The resulting residue, is therefore a mixture of unconsumed diet and frass, and cannot be completely separated into its constituent fractions. Few methods of insect frass collection have been reported, so far. Hagley (1962) constructed a collection cage and Sharma (1973) elucidated a method in which whole insects were fixed in wax. In both methods, the insects were immobilised, which likely affected their behaviour and diet intake. Pathak et al. (1982) developed a parafilm sachet method covering insects living on a plant, where frass can be clearly distinguished from dietary plant material. This is not possible with BSFL. Therefore, frass collection in BSFL is currently limited to indirect methods such as measurement of waste reduction, and larval growth and composition (Gold et al. 2020a). Waste reduction underestimates the amount of diet ingested, since diet residue and frass are analysed together. Data on larval growth and composition aggregates several digestive processes. It, therefore, hampers identification of the effect of individual nutrients, and whether growth was limited by other nutritional (e.g. imbalanced protein-energy ratio) or non-nutritional factors (e.g. nutrient supply exceeds the genetically pre-determined growth potential). Other approaches, such as collecting entire gut contents from individual larvae, includes both digested as well as undigested contents, thus, making it impossible to accurately measure the effectively digested diet portion.

The objective of this study was to develop protocols that will help researchers to better understand the role of gut microbiota and diet nutrient requirements in BSFL. First, we established a method that successfully generated sterile larvae in Protocol 1. Subsequently, we hypothesized that microbes provide nutrients that are essential for larval development. Protocol 1 also describes a method to sterilise eggs and enable larval development, even in the absence of diet- and larva-associated microbes. In Protocol 2, we constructed and tested a device to collect frass from individual larvae. The frass collected using our device is free from diet residues.

7.3 Protocol 1: Establishment of Sterile, Viable, and Growing BSFL

Experimental Design and Procedures

Production of Sterile BSFL

Test treatments varied in type, composition, and concentration of chemicals, and duration of exposure (Table 7.2). Series 1 consisted of five treatments (one replicate each) that have successfully achieved insect sterilisation in the past (as listed in Table 7.1). Series 2 consisted of four treatments (three replicates each) that were designed based on the results of Series 1. Likewise, Series 3 consisted of three treatments (three replicates each) that were designed based on the results of Series 2. The experiments were conducted once. All procedures were performed using sterile techniques under a laminar flow cabinet.

Table 7.2 Sterilisation treatments tested to generate sterile BSF eggs (Protocol 1)[a].

Series	Treatment	Chemicals[b] used for immersion	Concentrations (% v/v)	Duration (min)
1	1	(a) 3× NaClO	(a) 0.6	(a) 2+2+2
	2	(a) 1× NaClO (b) 2× C_2H_5OH	(a) 2.7 (b) 70	(a) 2 (b) 1+1
	3	(a) 1× NaClO	(a) 10	(a) 5
	4	(a) 1× NaClO (b) 1× C_2H_5OH	(a) 10 (b) 70	(a) 5 (b) 5
	5	(a) 3× C_2H_5OH	(a) 70	(a) 5, 1+1
2	6	(a) 3× NaClO	(a) 0.6	(a) 2+2+2
	7	(a) 1× NaClO (b) 2× C_2H_5OH	(a) 2.7 (b) 70	(a) 2 (b) 1+1
	8	(a) 1× NaClO (b) 1× C_2H_5OH	(a) 10 (b) 70	(a) 5 (b) 5
	9	(a) 1× C_2H_5OH (b) 1× NaClO	(a) 70 (b) 0.6	(a) 1 (b) 1
3	10	(a) 1× NaClO (b) 2× C_2H_5OH	(a) 2.7 (b) 70	(a) 2 (b) 1+1
	11	(a) 1× C_2H_5OH (b) 1× NaClO (c) 1× C_2H_5OH (d) 1× NaClO	(a, c) 70 (b, d) 0.6	(a, c) 2 (b, d) 2
	12	(a) 2× C_2H_5OH (b) 1× NaClO:C_2H_5OH = 1:1	(a) 70 (b) 10, 70	(a) 2 (b) 2

[a](a), (b), (c), (d) = subsequent steps; all eggs were washed three times with sterile water after NaClO and/or C_2H_5OH treatments.
[b]NaClO = sodium hypochlorite. C_2H_5OH = ethanol.

Eggs were obtained from a BSF colony maintained by Eawag (Dübendorf, Switzerland), according to Dortmans et al. (2017). Then, 0.1 g of eggs were transferred to a 2 mL Eppendorf tube using a metal spatula and separated with gentle stirring. They were sterilised using the treatments listed in Table 7.2. After each immersion in NaClO and/or C_2H_5OH, we discarded the supernatant before adding the next round of chemicals. After completion of all chemical washing cycles, we washed the eggs thrice with sterile water.

We incubated the eggs at 30°C for 2 weeks in two different media in an incubator. One medium was thioglycollate broth (VWR, Dietikon, Switzerland) with a pH of 7.1 ± 0.2; 3 mL was dispensed into 15 mL falcon tubes. It consisted (g/L) of pancreatic digest of casein, 15.0; yeast extract, 5.0; dextrose, 5.5; sodium chloride, 2.5; agar, 0.75; L-cystine, 0.5; sodium thioglycollate, 0.5; and resazurin sodium, 0.001. Another medium was Columbia blood agar (VWR, Dietikon, Switzerland) distributed in petri dishes (diameter: 90 mm). It consisted (g/L) of defibrinated sheep blood, 50; agar, 15; pancreatic digest of

casein, 10; meat peptic digest, 5; heart pancreatic digest, 3; yeast extract, 5; sodium chloride, 5; and starch, 1.

Egg sterility, larval hatching, and growth were determined as absent/present every four days by visual inspection of falcon tubes and agar plates. Falcon tubes were observed for colour change while agar plates were observed for possible visible colonies and larval crawling lines. Replicates were considered to be non sterile, if they appeared turbid or did not resemble eggs, egg debris, larvae, their crawling lines, or frass. Hatching was defined as the stage where larvae could be seen outside the eggs. Growth was defined as the stage when larvae had increased in size, such that they were larger than newly-hatched larvae. In each series, treatments were compared with two control groups; untreated eggs (three replicates each) and eggs exposed to three washing cycles with sterile water (three replicates each) (Brummel et al. 2004, Ridley et al. 2012).

Data was analysed in R version 3.6.2 (R Core Team, 2017). Absent/present data for egg sterility, larval hatching, and growth was normalised by the number of replicates. The mean and standard deviation were calculated for per treatment and control. All procedures were performed using sterile techniques under a laminar flow cabinet.

Optimisation of Nutrient Supply for Sterile BSFL

Even though we succeeded at egg sterilisation and larval hatching (Table 7.2, Treatment 11), the hatched larvae did not grow in either thioglycollate broth or on Columbia blood agar. We hypothesized that, this was due to a lack of essential nutrients that are usually provided by larva associated microbes. Given the widespread applications of sterile insects, we further assumed that larval growth could be sustained by feeding them with a diet containing all essential nutrients, including those that are provided by microbes. Therefore, we tested the effect of four different diets on larval growth. Diets were prepared in glass bottles (1 to 3 L), mixed with sterile water, and autoclaved (FOB5, Fedegari Group, Albuzzano PV, Italy) at 121°C for 15 min before distribution in petri dishes (diameter: 90 mm). Diet 1 consisted (mg/g) of yeast (Sigma Aldrich, Buchs, Switzerland), 500; D-glucose (Sigma Aldrich, Buchs, Switzerland), 100; and standard agar (VWR, Dietikon, Switzerland), 15. Diet 2 consisted (mg/g) of yeast, 250; D-glucose, 250; and standard agar, 15. Diet 3 consisted (mg/g) of yeast, 250; D-glucose, 250; and meat liver agar (Sigma Aldrich, Buchs, Switzerland), 34. Diet 4 consisted (mg/g) of yeast, 250; D-glucose, 250; and Columbia agar, 42. Diets were topped up with Ssterile water was added to bring up to 1 g/g in all four dietsto 100%.

However, the autoclaved diets did not promote larval growth. Therefore, we then sterilised the diets using a non-thermal treatment, namely the high-energy electron beam (HEEB) technique. Prior to HEEB treatment, diets were sealed in air-tight and water-tight polyamide-polyethylene bags up to a maximum fill line of 4 cm and then transported on ice to a commercial HEEB operator (Leoni Studer AG, Däniken, Switzerland). Diets were irradiated with 10 MeV electron beam at a dose of 32 kGy in accordance with the ISO 11137-3:2017 standard (ISO 2017), and subsequently, surface sterilised for 30 min on both sides, using the UV sterilisation program of a laminar flow cabinet. Finally, diets were distributed on sterile petri dishes (diameter: 90 mm) in triplicate. Sterility of the HEEB-treated diets was confirmed in triplicate after incubation at 30°C for 7 days on Columbia blood agar plates and in thioglycollate broth. Three different diets that could possibly provide all essential nutrients were treated with HEEB. The first diet was poultry feed (UFA 625, UFA AG, Switzerland) where sterile water had

been added, resulting in a moisture content of 600 mg/g. Poultry feed has been used as a positive control in several BSFL growth studies (Cammack and Tomberlin 2017, Diener et al. 2009 and 2011a, Nguyen et al. 2013, Spranghers et al. 2017). The second diet was a liver pie (Le Parfait, Nestlé, Vevey, Switzerland). According to the manufacturer's statement, the product contains pig liver, yeast, palm fat, maltodextrin, potato starch, salt, sunflower oil, corn starch, and extracts of flowers, herbs, and spices. Liver-based diets have also been used by Mumcuoglu et al. (2001). Liver is a rich source of B vitamins such as riboflavin that are known to promote insect growth In addition, *Lactobacillus plantarum* that produces B vitamins such as riboflavin (Li and Gu 2016), is known to promote insect growth (Li and Gu 2016, Storelli et al. 2011, Téfit et al. 2017). Liver is a rich source of B vitamins. The third diet was an artificial diet previously used by Cammack and Tomberlin (2017) consisting (per g) of 440 mg cellulose, 126 mg casein, 42 mg peptone, 42 mg albumen, 105 mg sucrose, 105 mg dextrin, 5.5 µL linoleic acid, 0.55 mg cholesterol, 0.275 mg ascorbate, 0.180 mg vitamin mixture, 0.125 µL ethanol, and 25 mg Wesson's salt mixture (all Sigma Aldrich, Buchs, Switzerland).

Larval hatching and growth on diets was assessed according to the methods described above by inoculating diets with 0.1 g of BSF eggs (five replicates per diet) treated with Treatment 11 (Table 7.2). Egg sterility was assessed (five replicates per diet) according to the methods described above with thioglycollate broth and Columbia blood agar. Egg sterility, larval hatching, and growth were determined as absent/present following incubation for 10 days at 30°C. Data was analysed as described above for the production of sterile BSFL.

Larval sterility, hatching, and growth were assessed according to the methods described above. 0.1 g of sterile BSF eggs (five replicates per diet) were incubated for 10 days at 30°C.

Results and Discussion

Protocol 1 describes the production of sterile, viable, and growing BSFL. Only Treatment 11 listed in Table 7.2 resulted in eggs that successfully hatched into viable larvae (Table 7.3). These larvae grew on all three HEEB-treated diets (poultry feed, liver paste, and artificial diet), but not on the autoclaved diets (Table 7.4).

Our protocol differs from the established methods for *Drosophila* spp. (Table 7.1). Unlike *Drosophila* spp., the immersion of BSF eggs in sodium hypochlorite for several minutes did not result in sterilisation. The successful treatment involves two cycles, each starting with ethanol (2 min), followed by sodium hypochlorite (2 min). These differences could be due to species differences in egg size, physiology, and/or microbial load and composition.

Table 7.3 Effect of sterilisation treatments (Protocol 1) on egg sterility, larval hatching, and growth determined by inoculation of eggs in thioglycollate broth and on Columbia blood agar.

Series	n	Treatment	Egg sterility % of replicates	Larval hatching % of replicates	Larval growth % of replicates
1	1	1	0	n.d.	100
	1	2	0	n.d.	100
	1	3	0	100	100
	1	4	0	100	100
	1	5	0	100	100
	1	Untreated	0	100	100
	1	H$_2$O control	0	100	100
2	3	6	0	100	100
	3	7	33	100	33 (non-sterile replicates)
	3	8	33	100	66 (non-sterile replicates)
	3	9	0	100	100
	3	Untreated	0	100	100
	3	H$_2$O control	0	100	100
3	3	10	0	100	66
	3	11	100	100	0
	3	12	0	100	66
	3	Untreated	0	100	100
	3	H$_2$O control	0	100	100

n = number of replicates; n.d. = not detected.

Our results also suggest that microbes are important for growth of BSFL. In contrast to *Drosophila* spp., sterile BSFL did not grow on autoclaved diets (Table 7.4), while non-sterile BSFL did (Table 7.3). Growth of sterile BSFL was observed in 40% of the replicates of HEEB-treated poultry feed and artificial diet, and in 60% of the replicates of HEEB-treated liver pie diet. The eggs used for these tests had been subjected to Treatment 11 (Table 7.2). They were found to be sterile based on visual assessment following incubation in thioglycollate broth and on Columbia agar. However, the lack of larval growth in some of the replicates likely indicates that even Treatment 11 may have been damaging to some extent. Although we cannot rule out the possibility that some essential nutrients had been lacking in the four autoclaved artificial diets; results of our growth tests more likely suggest that sterile BSFL need nutrients that are inactivated during autoclaving. Essential heat-sensitive compounds such as bioactive peptides or vitamins might be better preserved by HEEB treatment rather than autoclaving. Unlike *Drosophila* larvae, that grow well under axenic conditions on several autoclaved diets (Brummel et al. 2004, Kim et al. 2018, Koyle et al. 2016, Newell and Douglas 2014, Ridley et al. 2012), other insect species such as *Musca domestica* (Schmidtmann and Martin 1992, Watson et al. 1993) and *Musca autumnalis* (Hollis et al. 1985) were found to be strongly dependent on the presence of microbes for their growth. Cohen (2015) suggested that larva-associated microbes synthesize essential nutrients (e.g. vitamins, amino acids, signalling molecules, and sterols) that may otherwise be lacking in insect diets. Therefore, these insect species are unable to grow when sterilised.

Table 7.4 Effect of various diets in combination with diet sterilisation treatments on larval sterility, hatching, and growth. Eggs used were sterilised with Treatment 11 (listed in Table 7.2) (Protocol 1).

Diet	Sterilisation treatment	n	Larval sterility % of replicates	Larval hatching % of replicates	Larval growth % of replicates
Artificial diet 1	Autoclave	5	100	n.d.	0
Artificial diet 2	Autoclave	5	100	n.d.	0
Artificial diet 3	Autoclave	5	100	n.d.	0
Artificial diet 4	Autoclave	5	100	n.d.	0
Poultry feed	HEEB	5	100	100	40
Liver paste	HEEB	5	100	100	60
Artificial diet	HEEB	5	100	100	40
Untreated	Untreated	5	0	100	100
H₂O control	Untreated	5	0	100	100

n=number of replicates; n.d. = not detected; HEEB = high-energy electron beam.

Proof of Concept and Limitations

We demonstrated that a four-step treatment with alternating immersions of ethanol and sodium hypochlorite can produce sterile and viable BSFL that can possibly be used as a research model system. In this protocol, we developed a method to produce sterile viable and growing BSFL. Our HEEB-treated diets then facilitated the growth of sterile larvae, while our autoclaved diets did not. These results indicate that there are still unknown micronutrients that affect larval growth. Also, certain metabolites that may be normally synthesised by microbes are essential for larval growth. However, the survival and hatching of sterilised BSF eggs may not depend on these factors.

Several limitations should be considered when interpreting our results and their applications in future research:

- Tests were conducted with few biological replicates (i.e. 3-5) in one experiment.
- Compared to visual identification of microbial growth in the thioglycollate broth and Columbia agar plates, microbial gene expression by RNA sequencing (Brummel et al. 2004, Ridley et al. 2012) would have provided more conclusive evidence that sterile insects were free of microbial contamination.
- In future, all test diets should be autoclaved at the same time as HEEB treatment, to unambiguously identify whether it's the sterilisation process or differences among diets that primarily affects the growth of sterile BSFL.
- Growth of sterile BSFL on HEEB-treated diets was not reliable. Unintentional microbial contamination may have contributed to the growth of sterile BSFL. Therefore, mixing antibiotics in their diets may prevent this problem (Sharon et al. 2010, Heys et al. 2018).
- Growth of sterile larvae was much slower compared to non-sterile ones. However, this was determined on the basis of visual observations, which can be subjective. Therefore, in future experiments, increasing the experimental duration will allow determination of larval weight and thus more conclusive results.

7.4   Protocol 2: Frass Collection to Study BSFL Digestion

Experimental Design and Procedures

We developed a novel device to collect BSFL frass and assessed its impact on larval behaviour and reliability of frass collection. Squares with a side length of approximately 1.5 times the diameter of an individual larva were cut from thin plastic bags (Aromata, Lidl, Switzerland) (Figure 7.1A). Ultra Gel glue (Pattex, Henkel & Cie. AG, Pratteln, Switzerland) was applied to two adjoining edges of the plastic square (Figure 7.1B). The larva was then placed onto the square such that the top edge of the square was aligned horizontally with the middle of the second and third bottom segments (Figure 7.1C). The plastic sheet was then rolled up and sealed (Figure 7.1D). Finally, glue was applied to the bottom edge of the plastic sheet to close the device (Figure 7.1E). We tested the device on 15 to 18 day old larvae from the colony and the poultry feed (both mentioned in Protocol 1). After separating the residue, we attached the frass collection device to 25 larvae. Larval weight was then recorded with a precision laboratory scale. We used a light microscope to confirm that the frass collection device was fitted correctly. Another 25 larvae without a frass collection device, served as the control group. Larvae were then placed in a plastic container with the poultry feed moistened to 600 mg water/g of feed. Feed was provided at a rate of 250 mg wet weight/larva per day which likely exceeded the *ad libitum* intake. After feeding for 24 h in a climate chamber (HPP 260, Memmert GmbH, Germany) at 28°C and 75% relative humidity, larvae were separated from the remaining feed residue (Figure 7.1F), washed with tap water, and dried with paper towels. Next, the collection devices attached to larvae were opened at the bottom and, frass was gently removed using a small metallic spatula and, transferred to a tared 2 mL Eppendorf tube, and weighed with a precision balance (BM-65, Phoenix instrument, Garbsen, Germany). Finally, larvae were weighed with their empty collection device. Larval weight (mg wet weight) was calculated by subtracting the weight of the empty collection device from the larval weight with the empty collection device. Diet reduction (mg wet weight/day) was calculated by subtracting the initial diet weight from the remaining feed residue weight and dividing it by the feeding duration of one day.

Samples of the diet (poultry feed), frass collected by the developed device, and larval gut homogenate from the control group (Bonelli et al. 2019) were analysed (after removing the chitin) for metabolites associated with insect and microbial digestion (Cohen 2015, Kim et al. 2018). Similar to the method used for BSFL by Bonelli et al. (2019) the larval gut homogenate sample was prepared using dissecting scissors by removal of the chitin, fat body and trachea surrounding the gut from three larvae. We analysed glucose, galactose, citrate, succinate, lactate, formate, acetate, propionate, and ethanol using high-performance liquid chromatography (HPLC). Before injection into the HPLC system, 100 mg of the sample was transferred into a 2 mL Eppendorf tube. Then, 300 μL of 10 mM sulphuric acid and a glass bead were added. Samples were vortexed until homogenous and centrifuged at 16,000 g at 4°C for 10 min, and then 150 μL of the supernatant was filtered through a 0.45 μm nylon membrane (Infochroma AG, Zug, Switzerland) into a conical flask insert. The HPLC system consisted of a VWR Hitachi Chromaster 5450 RI-Detector using a Rezex ROA-Organic Acid (4 %) precolumn connected to a Rezex ROA-Organic Acid (8 %) column, equipped with a Security Guard Carbo-H cartridge (4 × 3.0 mm). Sample volumes of 40 μL were injected into the HPLC with a flow rate of 0.6 mL/min at a constant column temperature of 80 °C using a mixture of sulfuric acid (10 mM) and sodium azide (0.05 g/L) as eluent. Concentrations were determined using external standards via comparison of the retention time. Peaks were integrated using the EZChromElite software. (Version V3.3.2 SP2, Hitachi High-Tech Science Corporation). Limit of detection was defined as > 0.8 mM.
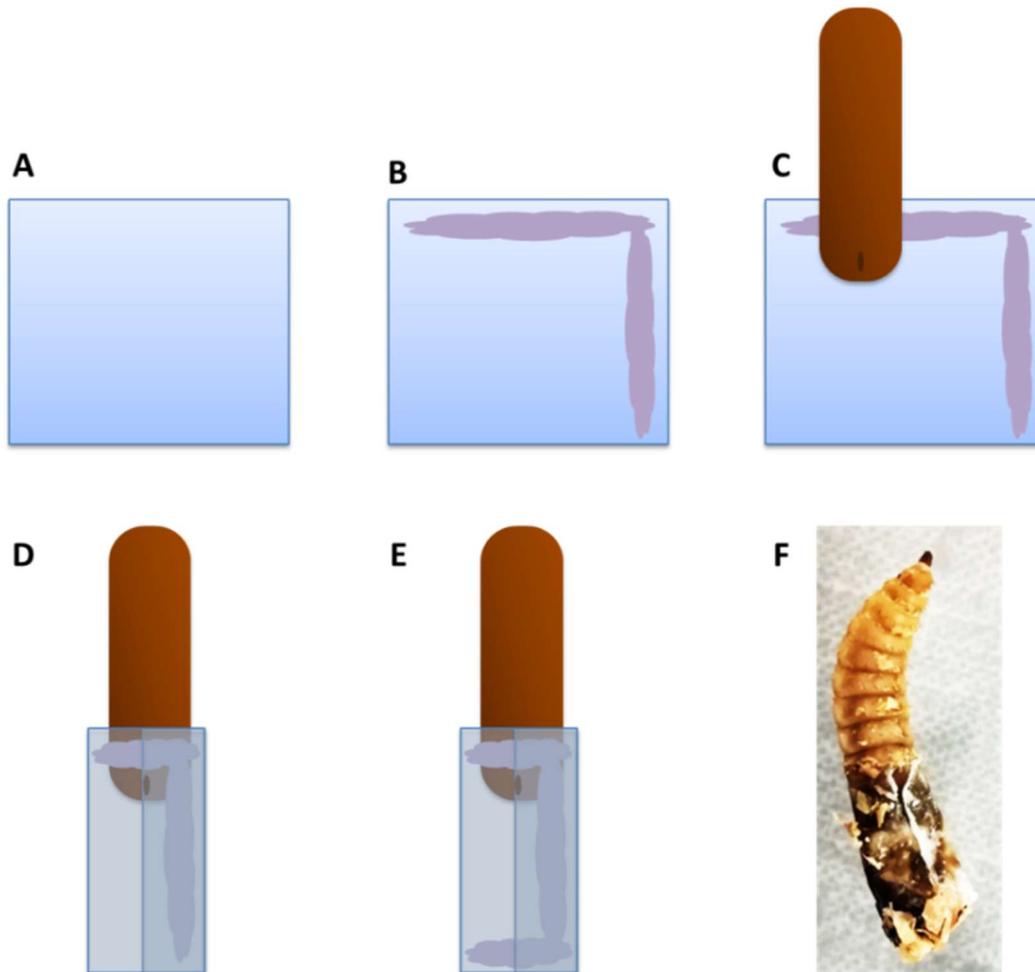
Figure 7.1 Method to attach frass collection device to black soldier fly larvae: (A) Square cut from plastic sheet, side length approximately 1.5 times the larval diameter; (B) Application of instant glue; (C) Attaching to larval rear, as low as possible to allow movement, yet high enough to enable frass excretion; (D) Plastic sheet rolled around larvae and sealed; (E) Sealing bottom end of plastic sheet with glue; (F) BSFL larva with collected frass, following feeding for 24 h on poultry feed and separation from the residue.

Data was analysed in R version 3.6.2 (R Core Team, 2017). The mean and standard deviation of larval weight and diet reduction was calculated.

Results and Discussion

This is the first study to develop a device to collect frass from BSFL, and analyse it for the presence of metabolites that are produced during microbial fermentation. The frass collection device did not impede larval development. Mean ± standard deviation larval weight for the group with the collection device was 234 mg ± 15 at the beginning of the experiment and 259 mg ± 26 at the end of the experiment (n=24, one larva with a weight gain of 101 mg was considered as an outlier). Likewise, for the control group (n=25) larval weight at the beginning was 239 mg ± 16 and at the end was 248 mg ± 20. Diet reduction (in wet weight) was 66 mg/day per larva in the group with the collection device and 56 mg/day per larva in the control group. During the 24 h period of larval feeding, 194 mg frass from 25 larvae was collected using the device, resulting in an average of 7.8 mg frass per larva. Some

amount of frass could not be collected, as it was stuck to the plastic material, and removing it with water would have interfered with the determination of total frass mass and downstream analyses of metabolites.

Our novel collection method facilitated the analysis of BSFL frass and comparison of the nutrient and metabolite composition of degraded diet to that of larval diet and larval guts. We detected a concentration gradient for the different metabolites (Table 7.5). The concentration of metabolites associated with the diet nutrients (glucose and galactose) was generally higher in the diet compared to larval guts, indicating towards suggesting nutrient digestion in the larval digestive tract. Interestingly, all metabolites associated with microbial fermentation (succinate, lactate, formate, acetate, propionate, and ethanol) were more concentrated in frass than in either diet or guts. A higher concentration of these metabolites in frass could be explained by catabolic activity of enzymes either in the larval digestive tract or by microbes. However, since the concentrations of the commonly known carbohydrate degradation pathway metabolites lactate and acetate were higher in frass compared to larval gut content, microbial activity appears more plausible. Bacterial strains producing lactate and acetate, namely *Lactobacillus* and *Acetobacter* respectively, are known to occur in gut microbiota of *Drosophila* (Storelli et al. 2011, Téfit et al. 2017). Further, several *Lactobacillus* strains are also prevalent in BSFL microbiota (Bruno et al. 2019, Jeon et al. 2011).

Table 7.5 Metabolites (μmol/g) detected in poultry feed, larval gut homogenate (composite of three larvae guts), and larval frass (composite from 25 larvae) (Protocol 2).

Compound	Poultry feed	Larval gut homogenate	Larval frass
Glucose	30.7	12.4	182.3
Galactose	20.3	1.0	6.4
Citrate	8.3	0.8	0
Succinate	1.1	1.6	6.5
Lactate	3.2	3.2	183.1
Formate	11.6	3.9	18.0
Acetate	1.8	2.7	65.6
Propionate	2.2	2.7	4.7
Ethanol	0	3.9	18.8

Proof of Concept and Limitations

We developed a method to collect frass from individual BSFL and determined the composition of nutrients and metabolites in BSFL frass for the first time. In large-scale BSFL rearing, such a device will help determine the digestibility of various organic wastes and side streams, using mass balance calculations between dietary nutrients and frass. It will also help identify the specific nutrient requirements of BSFL. This knowledge could increase the efficiency and reliability of rearing performance, which is a key determinant of the sustainability of insect larval biomass as raw material, in food and animal feed markets.

Although promising, this protocol has several limitations that should be considered in future research:

- Our frass collection device needs to be attached and removed manually, which is impractical for a large number of larvae. To determine insect nutrient requirements, a large sample size (e.g. > 1000) is needed. Otherwise, most nutrients cannot be analysed (one exception might be N content with micro-Kjeldahl).

- Probable sources of error include overestimation of larval weight due to incomplete emptying of the collection device. Also, water can accumulate between the plastic material and body of the larva, thus, possibly altering the larval moisture content.

- Frass underestimation is possible if the method is implemented as described, due to the incomplete emptying of collection devices. This can be overcome by flushing both larvae and the collection device with water, and drying them. Alternately, even partial frass samples can be used to determine digestibility, by mixing indigestible markers like alkanes (Mayes et al. 1986) into the diet. The proportion of diet that disappears (is digested) in the gut can be calculated from the increase in marker concentration between diet and frass.

- Considering the long storage time of frass in the collection device (24 h) and high temperature (28 °C), it is possible that the change in quantity and composition of nutrients, metabolites, and microbes after excretion, is partially due to decomposition of digestible nutrients outside of the larval gut.

- Alternatives to our frass collection device should also be considered. These include larval dissection and extraction of the midgut content as described by Bonelli et al. (2019). Posterior midgut or hindgut content is likely similar to frass. Additionally, Storelli et al. (2018) suggested feeding *Drosophila melanogaster* larvae with a blue dye, bathe them overnight in phosphate buffered saline (PBS), determine the frass quantity from the absorbance of the blue dye in PBS, and use the suspension for analyses of nutrients and metabolites.

- To verify whether bacterial metabolism is responsible for metabolite production in the frass, DNA or RNA sequencing can be conducted on the diet, gut content, and frass.

7.5 Conclusions

The present study describes novel methods for the generation of sterile BSFL, gently sterilised larval diets, and a device to collect BSFL frass. These methods could be used in research on host-microbe interactions and larval nutrient requirements. Results from such studies may possibly improve process performance and reliability in large-scale BSFL rearing. These methods are therefore valuable to the emerging insect industry, for the production of raw material for food and animal feed markets. Our methods need to be further refined and tested.

Conclusions and Perspectives

BSFL hold promise to contribute to waste management, and be a superior and more sustainable monogastric livestock feed (i.e. poultry, fish, pigs, pets). Producing BSFL with a lower environmental impact than current feed ingredients requires use of substrates for growth that are not used as food or feed. Examples include municipal organic solid waste, animal and human manures and agri-food wastes (e.g. vegetable and fruit pomace/peels). This work aimed to develop solutions to improve the frequently unreliable and low rearing performance of biowastes by optimizing larval nutrient supply and addition of beneficial microbes. Since research methods on BSFL are still poorly developed, this thesis further investigated sterilization of larvae, collection of frass from individual larval and *in vitro* simulation of BSFL digestion. Key findings and future research directions are summarized in the following graphical summary.
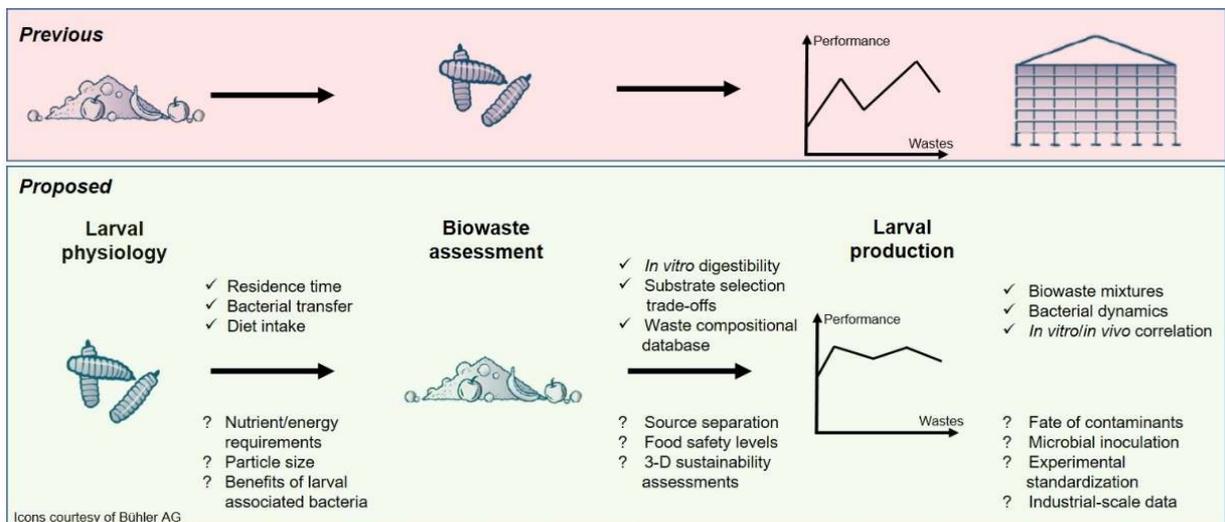
Formulation of biowaste mixtures based on protein and non-fibre carbohydrates is a promising approach for more efficient and predictable BSFL rearing using a range of biowastes. In comparison to individual wastes, biowaste formulations resulted in a higher rearing performance and lower variability. Biowaste formulation is especially beneficial since it integrates wastes with an individually low performance (e.g animal manures or slaughterhouse waste) into efficient BSFL substrates. In comparison to previous trial-and-error testing of biowaste mixtures, the proposed approach is systematic and considers the existing and growing knowledge on BSFL digestion. Rearing performance variability was higher than expected, but could be reduced by keeping other nutrients such as lipids and fibres within narrower limits. In parallel, determination of larval nutrient and energy requirements (e.g. by mass balance calculations between substrate and frass for different development stages) is needed to provide nutrients in ideal amounts. Such estimates are the standard in other efficient livestock systems. The developed frass collection method must be further refined, validated and more precise alternatives should be examined. It is possible that collection of frass by exctretion into sterile water and spectrometric quantification, similar to the method used for the determination of substrate midgut residence time, produces more accurate data. Despite balancing of the substrate nutrient content, a certain variability in the rearing performance is inevitable due to the intrinsic variability of different batches of the same waste type (e.g. different vegetable wastes) and among waste types (e.g. vegetable compared to municipal solid waste). Waste compositional data in the developed open access web

application (moritzgold.shinyapps.io/BSF_app/) and consideration of the waste origin (e.g. food waste = former human food, animal manure = feed digested by animals digestive system) are pragmatic approaches to overcome the lack of resources for an accurate determination of the nutrient composition. Even waste images can be helpful for an initial assessment of the moisture content and percentage of inorganic (e.g. plastic, glass) and fibrous (e.g. leaves, peel) materials.

Digesting substrates in an *in vitro* model, mimicking midgut digestion can also deliver valuable information for providing nutrients to larvae in ideal amounts. For example, digestion of substrates in the *in vitro* model was indicative of *in vivo* outcomes. In order to improve the model accuracy, it is essential to promote an understanding of the complex BSFL digestion process. BSFL adjusted the digestive residence time based on the substrate nutrient content, suggesting post-ingestive mechanisms and compensatory feeding. The reliability of digestion simulation could be improved by future research that considers substrate particle size and high-abundant gut bacteria (e.g. members of the genera *Morganella*, *Providencia*, *Enterococcus*, *Proteus* and *Dysgonomonas*). The advantages of *in vitro* simulations, however, are simplicity and low cost. Therefore, the benefits of more closely mimicking digestive processes need to be balanced with the additional resources required to implement them. It is also worth considering the developed *in vitro* model in order to study the influence of biowaste pre-processing (microbial, physical and chemical treatments) and storage, as well as the inactivation of pathogens and the decomposition of chemicals.

The addition of microbial inoculants, which were studied as a solution to increasing rearing performance by beneficial microbes, did not result in performance improvements. Incoulants were derived from the residue of a previous rearing cycle, typically abundant with intestinal bacteria (i.e. *Providencia*, *Dysgonomonas*, *Morganella*, and *Proteus*) considered beneficial for substrate decomposition. The lack of these genera in two agri-food waste residues highlighted that despite the growing knowledge for BSFL and the knowledge existing for other phylogenetically close fly species, the complex microbial processes involved in BSFL rearing are still poorly understood and require further research. Additional studies will be needed to elucidate the occurrence of intestinal bacteria in the residue, isolate members of these taxa and assess their true potential to influence larval growth and substrate reduction. Gently sterilized larvae and residues are a promising approach to research these processes. Whereas, determination of nutrient requirements of sterile BSFL appears challenging due to the extremely poor growth, sterile BSFL and substrates are useful to elucidate the complex interactions between larvae and microbes, for example by studying the effect of pure-culture and bacteria mixture addition to sterile BSFL and substrates. The use of the residue for inoculant production should not be completely rejected, since sterile bacteria that are a food for BSFL were used as a negative control. Because bacterial inoculants can also decrease larval growth, further investigations are required to better understand the mechanisms underlying the effects of some beneficial bacteria on rearing performance.

Despite the global abundance of waste, there are currently presumably insufficient quantities of safe, efficient and inexpensive substrates for BSFL production to have a dramatic impact on the sustainability of livestock feed production. Globally, large amounts of highly nutritious BSFL substrates are wasted due to mixing of organics and inorganics or prohibitively expensive collection and transport. This is an old challenge in waste management, but the high livestock feed price and aims to increase the amount of affordable locally produced livestock feed is a new opportunity to incentivise biowaste recycling according to circular economy principles. This should be actively pursued by stakeholders of

the emerging insect industry (e.g. insect associations and producers, municipalities, governments, livestock companies). However, even if the availability could be increased, serious concerns remain regarding the safe use of biowaste in BSFL rearing for the production of livestock feeds. Global wide-spread research is urgently needed on the fate of microbial and chemical contaminants in BSFL rearing substrates (e.g. human and animal manures, municipal solid waste) with high potential to produce more sustainable feeds. Examples include the transfer of pharmaceutical and agricultural residues, bacterial and viral pathogens (e.g. African Swine Fever) and misfolded prion proteins. This includes the development of appropriate policies and regulations, and their subsequent enforcement. Similar to the widespread use of food waste as livestock feed, it appears technically possible to use biowastes for insect production in a well-regulated system considering source control (e.g. heavy metals), heat treatment (e.g. pathogens), biosecurity (e.g. cross-contamination between substrates and feed) and traceability.

Better estimates on the sustainability of BSFL-based feed production and waste management are needed to inform meaningful actions by scientists, producers, food safety authorities, governments, funding agencies and consumers of BSFL-based products (e.g. pet owners, aquaculture and poultry farmers). In order to achieve a holistic benefit compared to the status quo, future assessments should take into account not only environmental impacts but also social and economic impacts. For example, of diverting unmanaged waste to BSFL production. In addition, impacts of existing feeds (e.g. soybean meal, fishmeal) such as biodiversity loss through deforestation and overfishing, and competition for land between humans and livestock should be measured. Altering of process conditions such as mechanical substrate disintegration, addition of bacterial inoculants and biowaste formulation are process-related scenarios. Ultimately, expansion of assessments to consider direct use of treated biowastes (e.g. food wastes) as livestock feed, feed affordability, nutritional quality, livestock behaviour and health, and non-food applications, can ensure broader benefits for the food system. Preconditions for such multi-dimensional sustainability assessments are reliable data on waste management and BSFL production. Overall, data in this space is decentralized with many gaps. In addition, existing industry-scale data is proprietary. Regarding BSFL production, data concerning emissions, use of energy, water and wastewater, and substrate production, collection and transport, for different process complexities, configurations, conditions, scales and substrates is needed. Considering global trends of population growth, waste production, food demand and supply, the described research and development would be especially impactful in low- and middle-income countries.

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