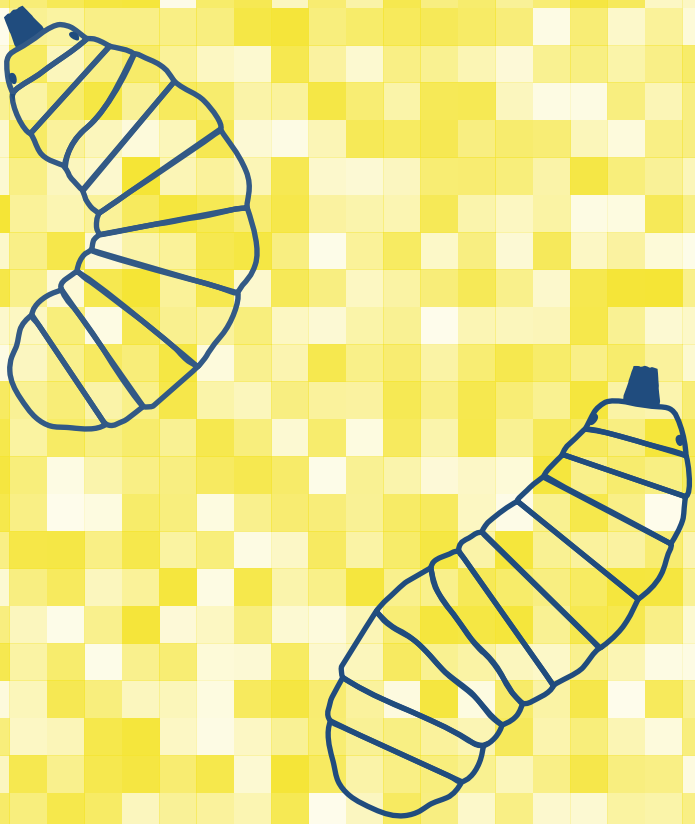
Of maggots and microbes



Stijn J.J. Schreven

Propositions

1. Microbes are essential for high performance of black soldier fly larvae. (this thesis)
2. The black soldier fly lacks a core microbiota. (this thesis)
3. The giant panda is preadapted to the Anthropocene.
4. Wild pollinator conservation requires restrictions on beekeeping.
5. Innovative science benefits as much from failures as from successes.
6. A restoration narrative best encourages the public for climate action.
7. Human rights advocacy is the most effective tool to protect nature.
8. Hands-on gardening is the best mix of creativity, sports, and meditation.

Propositions belonging to the thesis entitled:

"Of maggots and microbes"

Stijn J.J. Schreven

Wageningen, 4 June 2021

Of maggots and microbes

Stijn J. J. Schreven

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Of maggots and microbes

Stijn J. J. Schreven

Thesis

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Glossary

This glossary includes a number of central concepts used in this thesis, for which I judged it necessary to indicate which definition is used here because several circulate in literature, and which terms I regard as synonyms.

functional core microbiome: a subset of microbial functions (*i.e.* genes) essential to the host, that are associated with a given host across habitats (Lemanceau *et al.* 2017; Risely 2020).

microbiome: a characteristic microbial community occupying a reasonably well-defined habitat which has distinct physicochemical properties. (Berg *et al.* 2020; Whipps *et al.* 1988). The microbiome encompasses:

- **microbiota:** the assembly of microorganisms belonging to different kingdoms, *i.e.* the living members of the community (Berg *et al.* 2020);
- and their **theatre of activity:** microbial structures, metabolites, mobile genetic elements (*e.g.*, transposons and viruses), and relic DNA embedded in the environmental conditions of the habitat (Berg *et al.* 2020).

organic waste: all organic resources that are lost, wasted, or under-utilized in the food production system. Throughout this thesis, the term is considered interchangeable with organic residues, organic side streams, and organic by-products.

taxonomic core microbiome: a subset of microbial taxa associated with a given host across habitats, based on high prevalence across the host population or species (Lemanceau *et al.* 2017; Risely 2020).

Chapter 1

General introduction

The global food problem

By 2050, the global population is projected to grow from 7.7 billion people today to 9.7 billion (United Nations 2019). Simultaneously, per capita meat consumption is expected to increase because of an increase in income levels (FAO 2009). As a consequence, food production needs to increase by 70-110% to meet the projected global demand of 2050, with a rise in annual cereal production by almost 1 billion tons and in meat production by 200 million tons (Alexandratos & Bruinsma 2012; FAO 2009; Tilman *et al.* 2011). This likely leads to more land clearing in biodiverse regions such as the Amazon, as well as a further increase in freshwater consumption and greenhouse gas emissions (Alexandratos & Bruinsma 2012; Godfray *et al.* 2018; Ranganathan *et al.* 2016; Steinfeld *et al.* 2006; Tilman *et al.* 2011). Livestock production is accompanied by large emissions of greenhouse gases and ammonia, with manure accounting for the majority of anthropogenic nitrous oxide emissions, a compound with 296 times higher global warming potential than CO_2 (O'Mara 2011; Steinfeld *et al.* 2006).

Feed for livestock and fish often includes soymeal or fishmeal as a protein source. Both of these ingredients, however, come at environmental costs in the form of biodiversity loss and carbon emissions (Alder *et al.* 2008; FAO 2016; Gasparri *et al.* 2013; Gasparri *et al.* 2016; He *et al.* 2019; Smith *et al.* 2011; Taherzadeh & Caro 2019). Furthermore, the price of fishmeal is rising (FAO 2016; Tacon & Metian 2008), and soymeal production is limited by the area of cultivated land available whereas demand is growing (Masuda & Goldsmith 2009a, b). This urges livestock and fish farmers to look for alternative feed ingredients. Moreover, both soybeans and forage fish can directly be consumed by humans.

Novel alternative protein sources could contribute to a more sustainable food production (Parodi *et al.* 2018), aside from other possible strategies such as improving crop yields (Ray *et al.* 2013; Tilman *et al.* 2011) and addressing global population growth and consumption patterns (Berners-Lee *et al.* 2018; Ganivet 2019; Ranganathan *et al.* 2016). Edible insects may provide a sustainable alternative protein source for food and feed (Dicke 2018; Van Huis & Oonincx 2017; Van Huis *et al.* 2013; Veldkamp *et al.* 2012). Over 2000 insect species are consumed by people around the globe (Jongema 2017), either wild-collected or reared in small to industrial farms (Hanboonsong *et al.* 2013; Van Huis 2013). Compared to conventional livestock, insects contain comparable

nutritional value but use less land, freshwater, and feed (Van Huis 2013), and emit lower amounts of greenhouse gases (Oonincx *et al.* 2010; Parodi *et al.* 2020a). In comparison to soymeal or fishmeal, insects can be competitive in environmental impact when reared on organic waste (Alexander *et al.* 2017a; Bava *et al.* 2019; Smetana *et al.* 2016; Smetana *et al.* 2019; Van Huis & Oonincx 2017). Global food waste exceeds 1 billion tons per year, and its use via conversion to insect protein may both improve food security and reduce carbon emissions (Alexander *et al.* 2017b; Chen *et al.* 2020; FAO 2011).

Black soldier fly life cycle and nutritional value

The black soldier fly (BSF), *Hermetia illucens* (Linnaeus, 1758), is one of the most promising insect species for the conversion of organic waste into edible insect biomass (Barragán-Fonseca *et al.* 2017; Pastor *et al.* 2015; Sheppard *et al.* 1994). This (sub)tropical member of the Stratiomyidae family likely originated from the Americas, but at present has a near-cosmopolitan distribution (Khamis *et al.* 2020; Marshall *et al.* 2015; Ståhls *et al.* 2020). The larvae of BSF consume a wide range of organic waste substrates, *e.g.* human faeces, livestock manure, fruits and vegetables, sewage sludge, meat waste, fish offal, and seafood waste (Banks *et al.* 2014; Diener *et al.* 2009; Lalander *et al.* 2019; Nguyen *et al.* 2015; Nguyen *et al.* 2013; Villazana & Alyokhin 2019). Depending on the type of organic waste used to rear BSF, they can yield a product that has a similar protein content and quality as soymeal and fishmeal (Barragán-Fonseca *et al.* 2017; Gasco *et al.* 2019; Surendra *et al.* 2020).

The total life cycle of BSF takes about 40 days at 30 °C but can take shorter or longer depending predominantly on temperature and larval diet (Figure 1; Chia *et al.* 2018; Harnden & Tomberlin 2016). BSF adult females deposit their eggs onto or near larval food sources (Booth & Sheppard 1984), after which the eggs take about 3 days to eclose (Chia *et al.* 2018). Upon hatching, the larvae start foraging and pass through six larval instars by moulting before reaching the prepupal stage (Barros *et al.* 2019; Bruno *et al.* 2020; Gligorescu *et al.* 2019; Gobbi 2012; Kim *et al.* 2010). With each moult, the mouth morphology gains in complexity (Bruno *et al.* 2020) and larvae forage more voraciously (Gligorescu *et al.* 2019; Liu *et al.* 2017). The prepupa, which is the final, seventh instar, has a darkened skin and a complete and sclerotized cuticular plate that covers the mouthparts and disables feeding (Barros *et al.* 2019; Gligorescu *et al.* 2019; Schremmer 1986). The prepupal stage is also called the "wandering stage" as in this

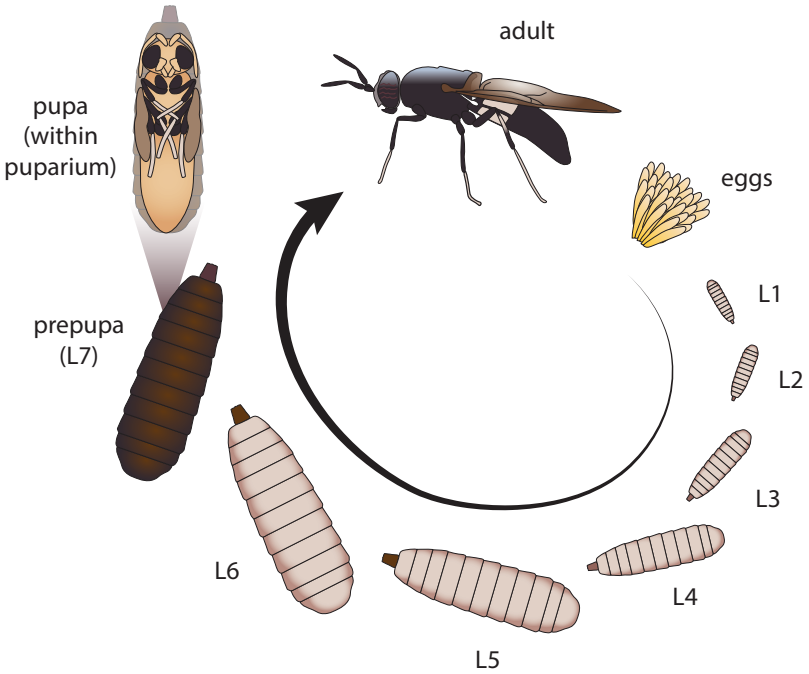

Figure 1. Life cycle of the black soldier fly, *Hermetia illucens* (L.) (Diptera: Stratiomyidae). L1-7 = first to seventh larval instars. After hatching, larvae develop through seven instars, of which the first six are foraging, the seventh – prepupal stage – is non-feeding. Within the skin of the prepupa (the puparium), the larva pupates and undergoes metamorphosis, eventually eclosing as the adult fly. Sizes of life stages are not proportional. Illustration by the author, based on Barros-Cordeiro *et al.* (2014) and Gligorescu *et al.* (2019).

stage the larvae often leave the substrate in search of a dry and sheltered place to pupate (Schremmer 1986). Pupation and metamorphosis take place inside the skin of this last instar, the puparium (Barros-Cordeiro *et al.* 2014; Gligorescu *et al.* 2019; Li *et al.* 2016). Once the adults emerge, their lifespan can be prolonged if they are provided with water, sugar, or a protein source (Bertinetti *et al.* 2019; Lupi *et al.* 2019).

The nutritional value of BSF larvae makes them suitable for partial replacement of soymeal and fishmeal in animal feed (Chia *et al.* 2019b; Dörper *et al.* in press; Gasco *et al.* 2019). BSF larvae can have a high protein and fat content depending on the diet (Barragán-Fonseca *et al.* 2017). Protein content decreases as larvae develop, whereas fat content increases (Liu *et al.* 2017; Zhu *et al.* 2019). Unlike the amino acid composition, BSF larval fatty acid composition is highly flexible and strongly influenced by the diet

(Barragán-Fonseca *et al.* 2017; Oonincx *et al.* 2015). BSF larvae can store energy by *de novo* synthesis of lauric acid (Zhu *et al.* 2019) as well as convert dietary fatty acids to lauric acid when the dietary fat content is low (Oonincx *et al.* 2015). This high lauric acid content is unique among dipterans (Oonincx *et al.* 2015). When dietary fat content is high, dietary fatty acids are incorporated directly in the larval body (Oonincx *et al.* 2015). Pigs, fish, and poultry fed BSF larvae generally have a performance and quality comparable to animals fed soymeal or fishmeal, although with a lower content of n-3 fatty acids (Chia *et al.* 2019b; Dörper *et al.* in press; Gasco *et al.* 2019; Henry *et al.* 2015; Moula & Detilleux 2019). However, BSF larvae can be enriched in n-3 fatty acids via their diet (Oonincx *et al.* 2020; St-Hilaire *et al.* 2007). In addition, livestock, poultry, and fish can have improved immunological parameters when fed BSF larvae (Dörper *et al.* in press; Spranghers *et al.* 2018; Xiao *et al.* 2018b).

Variation in larval performance on organic waste

One of the main challenges for BSF production, is to control the variation in larval performance, *e.g.* larval survival, growth, development, and conversion efficiency of biowaste into larval biomass (Barragán-Fonseca *et al.* 2017; Bosch *et al.* 2019; De Smet *et al.* 2018; Lalander *et al.* 2019). Many studies have investigated the effects of diet macronutrient composition on larval performance and nutritional quality (Barragán-Fonseca *et al.* 2019; Barragán-Fonseca *et al.* 2021; Barragán-Fonseca *et al.* 2018b; Cammack & Tomberlin 2017; Gold *et al.* 2020b). In general, artificial diets with high protein and carbohydrate contents and a protein:carbohydrate ratio of 1:1 to 1:4 were favourable for larval and adult performance (Barragán-Fonseca *et al.* 2019; Barragán-Fonseca *et al.* 2021; Cammack & Tomberlin 2017). Larval protein content varied within a much narrower range than fat content (Barragán-Fonseca *et al.* 2019; Barragán-Fonseca *et al.* 2021; Barragán-Fonseca *et al.* 2018b). In diets composed of mixed biowastes, larval performance varied between diets despite a similar protein:carbohydrate formulation, which could be due to variation in nutritional quality of macronutrients (*i.e.* composition and digestibility), lipid and fibre contents, or microbial numbers and composition of the biowastes (Barragán-Fonseca *et al.* 2018b; Gold *et al.* 2020b).

Other attributes of the rearing system such as moisture content, temperature, pH, feeding regime, and larval density influence larval performance as well (Barragán-Fonseca *et al.* 2018a; Bosch *et al.* 2019; Cammack & Tomberlin 2017; Cheng *et al.* 2017; Chia *et al.*

2018; Diener *et al.* 2009; Ma *et al.* 2018; Parra Paz *et al.* 2015). Low moisture content can reduce larval growth rate and survival, but the exact relation appears to depend on the type of diet and experimental setup (Cammack & Tomberlin 2017; Cheng *et al.* 2017). Larvae successfully develop between 15-37°C with fastest development at 30-35°C depending on the diet (Chia *et al.* 2018). Initial substrate pH of 4 or lower reduces larval performance, but larval performance is relatively constant at pH 6-10, with an optimum at pH 8 (Ma *et al.* 2018). Both high larval densities and low feeding rations can lead to intraspecific competition and low larval performance (Barragán-Fonseca *et al.* 2018a; Diener *et al.* 2009; Parra Paz *et al.* 2015). The optimum feeding ration, however, depends on the interaction between feeding regime, larval density, and diet nutrient concentration (Barragán-Fonseca *et al.* 2018a; Parra Paz *et al.* 2015).

The role of microorganisms in BSF larval performance

Organic waste harbours a rich microbial community of decomposers that interact with the BSF larvae (Benbow *et al.* 2019; Crippen *et al.* 2016). These microorganisms, including mainly bacteria and fungi, may provide essential nutrients, aid in macromolecule digestion, as well as compete for nutrients in the substrate (Burkepile *et al.* 2006; Engel & Moran 2013). Thus, the microbiome, *i.e.* the microbial community and its activity in a defined environment (see **Glossary**; Berg *et al.* 2020), of the substrate and larval gut likely introduces additional variation in larval performance (De Smet *et al.* 2018; Gold *et al.* 2018).

Microorganisms can influence BSF performance in different life stages. Adult females respond to microbial volatiles in their oviposition behaviour (Yang *et al.* 2017; Zheng *et al.* 2013b) and egg emergence can increase when eggs are inoculated with specific egg-associated bacteria (Yang *et al.* 2018). Larval performance can be improved by inoculation of the substrate with certain bacteria, fungi, or yeasts (Callegari *et al.* 2020; Isibika *et al.* 2019; Kooienga *et al.* 2020; Mazza *et al.* 2020; Richard *et al.* 2019; Wong *et al.* 2020; Xiao *et al.* 2018a; Yu *et al.* 2011). Bacterial inoculation of chicken manure can increase larval weight by 29% and decrease development time by 5 days compared to control (Mazza *et al.* 2020; Xiao *et al.* 2018a; Yu *et al.* 2011). The effects of inocula, however, vary among bacterial species: some species have no effect, a negative effect, or improve growth but delay development (Callegari *et al.* 2020; Kooienga *et al.* 2020; Mazza *et al.* 2020). Mixed bacterial cultures can enhance the positive effects of single-species inocula on larval

performance but can also negate the benefits or even reduce performance, depending on the composition of the mixture (Mazza *et al.* 2020). The addition of commercial yeasts to the feed substrate can increase larval weight and accelerate development (Richard *et al.* 2019; Wong *et al.* 2020). Isibika *et al.* (2019) showed that two fungal species and BSF gut bacteria improve banana peel conversion efficiency into larval biomass. Besides, such microbial treatments can alter larval nutritional composition (Mazza *et al.* 2020; Richard *et al.* 2019; Wong *et al.* 2020).

Microbiological and chemical safety

Edible insects like BSF larvae encompass a number of chemical and microbiological safety risks when used for human food and livestock feed, even more so when they are reared on organic waste (EFSA 2015; Van der Fels-Klerx *et al.* 2020; Wynants *et al.* 2019). Organic residues, such as food waste, agricultural side streams, and manure, can be contaminated with pathogens, toxins, pesticides, heavy metals, and pharmaceuticals (Bicudo & Goyal 2003; Jones & Martin 2003; Thompson & Darwish 2019; Wohde *et al.* 2016). These contaminants may affect BSF larval performance or jeopardize its safety as a feed component.

BSF larvae are able to degrade some of the microbial contaminants in organic waste, but others may persist in the larval gut. As mentioned before, the larvae produce antimicrobial peptides and lysozymes (Vogel *et al.* 2018), and in fact have one of the largest gene repertoires encoding immune compounds (Zhan *et al.* 2020). They reduce counts of pathogenic *Salmonella* spp., *Escherichia coli*, and viruses spiked in manure or aquaculture waste, but leave *Enterococcus* spp. populations unaffected (Lalander *et al.* 2013; Lalander *et al.* 2015; Lopes *et al.* 2020). Wynants *et al.* (2019) report pathogens such as *Salmonella* spp. and endospores of mainly *Bacillus cereus* from BSF larvae and/or residues, and indicate that decontamination is required prior to using larvae as feed. Bruno *et al.* (2019b) found that larvae fed protein-rich diet (fish) performed worse than those reared on standard or vegetable diets, and their gut microbiota were dominated by bacteria of the genus *Providencia*. *Providencia* is likely vertically transmitted (Zheng *et al.* 2013a). Whether pathogenic to BSF or not, Bruno *et al.* (2019b) and Wynants *et al.* (2019) show that potential human/animal pathogens can persist in the BSF gut. Thus far, BSF production has been spared from any major disease outbreaks (Joosten *et al.* 2020), but insect pathogens such as *Beauveria bassiana* pose potential threats to BSF adult health (Lecocq *et al.* 2021).

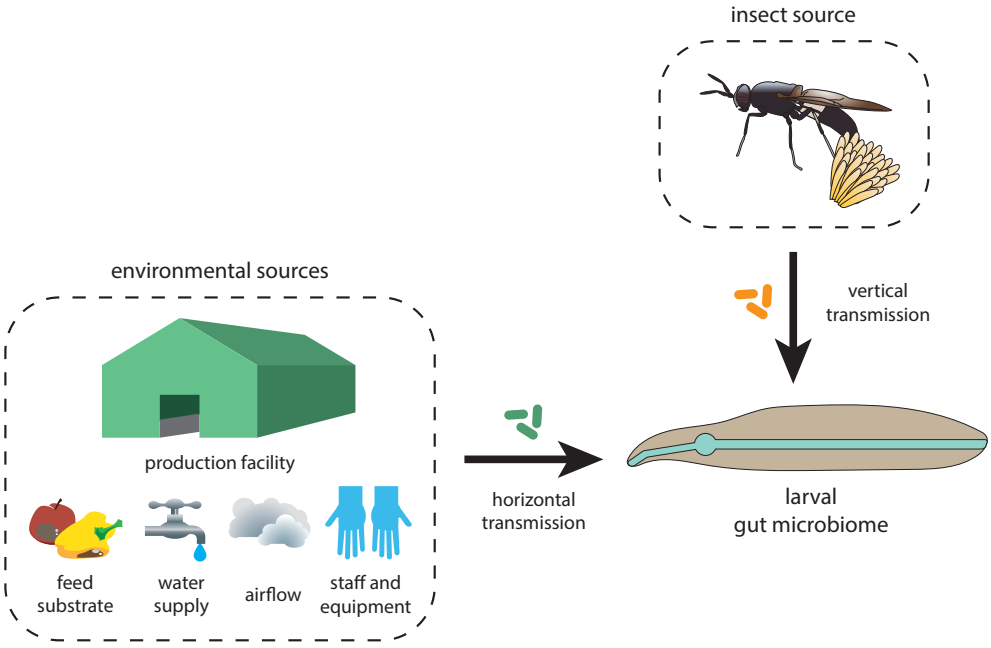

Figure 2. Community assembly of the larval gut microbiota. Microorganisms can colonize the neonate larval gut from different sources. Via horizontal transmission, the larva can acquire microorganisms from environmental sources, i.e. the production facility including feed substrate(s), water supply, airflow, and company staff and equipment. Another source is the vertical transmission of microorganisms from the adult fly to the eggs. Illustration by the author.

Studies on chemical hazards associated with BSF larvae have not yet considered phytotoxins and antinutritional compounds, although the larvae may ingest and sequester them when fed agricultural waste streams (ANSES 2015; EFSA 2015; Van der Spiegel *et al.* 2013). The fate of other chemical contaminants is diverse in BSF larvae, as well as the effects of these chemicals on larval performance. Heavy metals such as cadmium can accumulate in BSF larvae and alter the larval gut microbiota, but do not affect larval performance (Van der Fels-Klerx *et al.* 2016; Van der Fels-Klerx *et al.* 2020; Wu *et al.* 2020). Food packaging chemicals can also accumulate in the larval body (Van der Fels-Klerx *et al.* 2020). In contrast, the larvae tolerate high concentrations of mycotoxins without accumulation (Bosch *et al.* 2017). In fact, they are able to excrete and/or metabolize mycotoxins (Camenzuli *et al.* 2018; Leni *et al.* 2019; Meijer *et al.* 2019). BSF larvae are also able to substantially reduce the half-life of pharmaceuticals and pesticides in organic residues (Cai *et al.* 2018b; Lalander *et al.* 2016; Liu *et al.* 2020),

although pesticides do reduce BSF larval performance (Alyokhin *et al.* 2019; Tomberlin *et al.* 2002).

Community assembly of the larval gut microbiota

Establishment of the larval gut microbiota is similar to the ecological process of community assembly, where microorganisms colonize the new environment of the neonate gut via a number of sources (after Christian *et al.* 2015). For BSF larvae, these sources are predominantly the rearing environment (including the feed substrate) and the insect eggs (Figure 2; Crippen *et al.* 2016; Wynants *et al.* 2019; Zheng *et al.* 2013a). The environmental sources in industrial rearing first of all include the feed substrate but also relate to company hygiene, *e.g.* microbes associated with equipment, water supply, personnel, and air circulation in the facility (Wynants *et al.* 2019). The feed substrate (diet) is a major determinant of BSF larval gut microbiota (Bruno *et al.* 2019b; Jeon *et al.* 2011). The insect eggs may harbour microorganisms that are transmitted vertically by the adult female during oviposition or originate from the oviposition substrate (Zheng *et al.* 2013a).

The BSF larval gut exerts strong selection pressures on the ingested and resident microorganisms (Bonelli *et al.* 2019; Bruno *et al.* 2019b; Engel & Moran 2013). The midgut is the main compartment for digestion and has an extreme gradient in luminal pH from 6 in the anterior, to 2 in the middle part, and 8-9 in the posterior midgut (Bonelli *et al.* 2020; Bonelli *et al.* 2019). Enzymes and immune compounds such as lysozymes and antimicrobial peptides are secreted into the gut lumen and break down microbial cells (Bonelli *et al.* 2019; Vogel *et al.* 2018). This selection results in a progressively smaller subset of the ingested microorganisms surviving to the posterior midgut (Bruno *et al.* 2019b).

The BSF larval secretions and gut microbiota eventually end up in the substrate via the larval frass (Jiang *et al.* 2019). Larvae forage in aggregations that enhance these effects and alter the substrate properties on another scale through increased aeration, local temperature, and pH (Jiang *et al.* 2019; Ma *et al.* 2018; Meneguz *et al.* 2018; Putman 1978). As a consequence, BSF larvae are able to alter microbial populations and metabolism. They can reduce population sizes of *Salmonella* spp. and *E. coli*, increase decomposition rate, and change the microbial volatile blend emitted from the substrate (Beskin *et al.*

2018; Erickson *et al.* 2004; Jiang *et al.* 2019; Lalander *et al.* 2013; Liu *et al.* 2008; Lopes *et al.* 2020).

The feed substrate and larvae influence each other's microbiota, but their reciprocal effects have not been investigated in combination to quantify their relative importance in microbial community dynamics. Similarly, the effects of egg-associated microorganisms on larval performance have been tested as inoculates of single or mixed bacterial strains (Mazza *et al.* 2020; Yu *et al.* 2011), but the overall contribution of egg-associated microorganisms to larval microbiota and performance, relative to the contribution of substrate microorganisms, remains to be established.

Objectives and outline of this thesis

In this thesis I aimed to study and understand the performance and microbial ecology of BSF larvae on different feed substrates. First, I assessed the suitability of specific oilseed by-products as potential feed substrates for BSF larvae (**Chapter 2**). Second, I investigated the effect of larval density on bacterial community dynamics of substrate and larvae fed three substrates (including a mixed diet containing oilseed by-products) (**Chapter 3**). Third, I quantified the relative contribution of egg-associated and substrate-associated microorganisms on larval performance and bacterial community composition, in larvae fed chicken feed or chicken manure (**Chapter 4**). Finally, I discuss the implications of these findings in the light of ecological theory and industrial applications (**Chapter 5**).

In **Chapter 2**, I assessed how oilseed by-products influence BSF larval performance parameters (survival, biomass, development) and larval fatty acid composition. I reared BSF larvae on diets partly substituted with oilseed by-products of *Camelina sativa* (L.) Crantz and *Crambe hispanica* subsp. *abyssinica* (Hochst. *ex* R.E.Fr.) Prina. These two brassicaceous crops contain special fatty acids useful for food/feed and chemical industry, but also contain secondary metabolites, glucosinolates, that are enzymatically converted into toxic isothiocyanates.

In **Chapter 3**, I assessed the effect of larval density relative to substrate type on microbiota dynamics over time in the larvae and substrate. For this I included the 50% camelina seed press cake diet as used in the experiment reported in Chapter 2, as well as

two other substrate types, *i.e.* chicken feed and chicken manure. I administered neonate BSF larvae to the substrate in three densities (50, 100, or 200 larvae per container), sampled bacterial DNA on days 0, 5, 10, and 15, and compared the data with a substrate without larvae. Larval performance parameters, substrate pH, and moisture content were also measured in order to explain bacterial community composition. Bacterial community composition was determined from 16S rRNA gene amplicon sequencing. I applied linear regression models and multivariate statistics to analyse bacterial community composition and assess the relative importance of larval density and substrate type in explaining the microbiota variation.

In **Chapter 4**, I quantified the relative contribution of egg-associated and substrate-associated microorganisms to larval performance and microbiota. Results were compared in two different substrate types, chicken feed and chicken manure. To assess the individual contributions of the different microbial sources (eggs or substrate), BSF eggs were sterilized via a disinfection protocol and substrates were autoclaved. Larval performance parameters were measured and compared among treatment groups. Bacterial abundance and community composition were determined via 16S rRNA gene-targeted qPCR and amplicon sequencing, respectively, and linear regression models and multivariate analysis were used to assess the effect of treatments on microbiota.

In **Chapter 5**, I discuss the findings of the previous chapters in an ecological context, treating the microbiota as an ecological community in interaction with the BSF larval host. I emphasize important aspects of microbial ecology in the BSF production system, such as nursery diets, diet shifts, and contaminants, in the light of ecological disturbance and resilience of microbial (meta)communities. I close with a discussion of potential future applications of the field of microbial ecology to the commercial BSF production for animal feed.

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Chapter 2

Life on a piece of cake: performance and fatty acid profiles of black soldier fly larvae fed oilseed by-products

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Abstract

The oilseed crops *Crambe hispanica* subsp. *abyssinica* and *Camelina sativa* produce oils rich in erucic acid and n-3 polyunsaturated fatty acids (PUFA), respectively. After pressing the oil, a seed cake remains as a protein-rich by-product. Edible insects may convert this seed press cake and the defatted seed meal produced from it into insect biomass suitable for animal feed. Black soldier fly larvae (BSF, *Hermetia illucens*) can grow on a wide range of organic waste types, but may be hindered by excess protein or the plant toxins characteristic for these two oilseed crops, *i.e.* glucosinolates and their breakdown products. We tested the effects of 25%, 50% and 100% oilseed by-product inclusion in the diet on survival, development, biomass production and fatty acid composition of BSF larvae.

Larval performance on diets with up to 50% camelina by-product or 25% crambe by-product was similar to performance on control diet (chicken feed), and decreased with higher inclusion percentages. Larval fatty acid profiles differed significantly among diets, with larvae fed press cake more distinct from control than larvae fed seed meal. Larvae fed camelina press cake had more α-linolenic acid, whereas larvae fed crambe contained most oleic acid. The n-6 : n-3 PUFA ratio decreased with increasing proportion of by-product, especially on camelina diets. Lauric acid content was highest in larvae fed 100% camelina meal or 50% crambe meal.

These results indicate that BSF larvae can be successfully grown on diets with camelina or crambe oilseed by-products, and that the resulting larval n-6 : n-3 PUFA ratio is favourable for animal feed. However, the fate of glucosinolates and their derivatives remains to be determined, to guarantee chemical safety of camelina- or crambe-fed BSF larvae for animal feed.

Keywords

Hermetia illucens, *Crambe hispanica* subsp. *abyssinica*, *Camelina sativa*, n-3 PUFA, glucosinolates

Introduction

The oilseed crops *Camelina sativa* (L.) Crantz and *Crambe hispanica* subsp. *abyssinica* (Hochst. *ex* R.E.Fr.) Prina can be cultivated on marginal arable lands within Europe, reducing the need to import tropical vegetable oils, such as palm kernel oil and coconut oil (Righini *et al.* 2016). Camelina oil contains high levels of linoleic acid (C18:2 n-6), α-linolenic acid (C18:3 n-3) and eicosenoic acid (C20:1), and may be used in fish feed, as industrial feedstock or for biodiesel (Righini *et al.* 2016). Crambe oil is especially rich in erucic acid (C22:1 *cis*-13), which serves as an important industrial feedstock for plastics and lubricants (Beaudoin *et al.* 2014). Due to high concentrations of antinutritional compounds the by-products of the seed oil extraction process cannot be fed to livestock animals, but may be converted by edible insects into proteins and lipids suitable for animal feed (Righini *et al.* 2016).

When fed organic waste streams (*e.g.* municipal waste, cattle manure) or by-products (*e.g.* distilled grains), insects provide a protein source that can be more sustainable than soymeal or fishmeal (Smetana *et al.* 2016), and can partially replace these ingredients in animal feed (Chia *et al.* 2019b; Gasco *et al.* 2019). Thus, edible insects can improve the sustainability of the agricultural sector, contributing to several of the United Nations' Sustainable Development Goals (Chia *et al.* 2019a; Dicke 2018; United Nations 2015).

Larvae of the black soldier fly *Hermetia illucens* (L.) (BSF; Diptera: Stratiomyidae) can convert a wide range of organic waste streams (*e.g.* livestock manure (Miranda *et al.* 2019), human faeces, food waste, abattoir waste, fruits and vegetables (Lalander *et al.* 2019), mushroom waste (Cai *et al.* 2017), brown algae (Liland *et al.* 2017), and seafood waste (Ewald *et al.* 2020; Villazana & Alyokhin 2019)) into insect biomass with a protein content of 34-63% and fat content of 7-58% on dry matter basis, suitable for fish, poultry and pig feed (Barragán-Fonseca *et al.* 2017; Ewald *et al.* 2020; Liland *et al.* 2017). The nutrient composition of the organic waste influences BSF performance. Barragán-Fonseca and colleagues (2019) showed that performance was high on substrates containing 10-15% dietary protein content and 10-60% carbohydrate content, whereas an excess of protein (in this case more than 37% of dry matter) increased larval mortality. Similarly, Lalander *et al.* (2019) related performance differences to protein content of feed substrates. The optimal proportions of proteins and carbohydrates for BSF also depend on their nutritional quality, *e.g.* the amino acid composition of proteins and the energy density of carbohydrates (Barragán-Fonseca *et al.* 2018b).

The by-products of crambe and camelina seed oil extraction, *i.e.* press cake and seed meal, contain 30-50% protein (Frame *et al.* 2007; Liu *et al.* 1994). Press cake results from mechanical oil extraction and still contains a considerable portion of residual oil; seed meal results from subsequent chemical solvent extraction and contains very little oil. Feeding such substrates to BSF larvae can provide added value to the insect product, since the diet influences BSF larval fatty acid composition (Barragán-Fonseca *et al.* 2017). BSF generally contains a high lauric acid (C12:0) content – up to 63% of total fatty acids (Danieli *et al.* 2019) – that is exceptional compared to other edible insects, and appears to convert longer-chain fatty acids to lauric acid in diets with low fat content (Oonincx *et al.* 2015). This fatty acid can be a useful livestock feed additive because of its antimicrobial properties (Spranghers *et al.* 2018). With higher dietary fat content, BSF larval fat contains more diverse dietary fatty acids (Oonincx *et al.* 2015).

The oilseed by-products, however, also contain several compounds that have antinutritional effects on livestock animals (EFSA 2008; Liu *et al.* 1994) and perhaps on BSF larvae as well. Both camelina and crambe belong to the plant family of Brassicaceae and contain glucosinolates, secondary metabolites that defend the plant against herbivory (Winde & Wittstock 2011). In intact plant tissue, glucosinolates and myrosinases (the enzymes that hydrolyse glucosinolates resulting in toxic products such as isothiocyanates) are stored in separate cells (Winde & Wittstock 2011). The dominant glucosinolates in camelina seeds are glucocamelinin, glucoarabin, and 11-methylsulfinylundecyl glucosinolate (Berhow *et al.* 2013); in crambe seeds 2-(*S*)-hydroxyl-3-butenyl glucosinolate (*epi*-progoitrin) is the most abundant glucosinolate (Matthäus 1997). Upon contact with the myrosinase enzyme, *e.g.* due to plant tissue disruption such as insect herbivory, these glucosinolates are metabolized into their active counterparts: glucocamelinin to 10-methylsulphinyldecyl isothiocyanate (ITC), glucoarabin to 9-methylsulphinylnonyl ITC, 11-methylsulfinylundecyl glucosinolate to 11-methylsulfinylundecyl ITC (Amyot *et al.* 2019), and *epi*-progoitrin to 5-vinyl oxazolidine-2-thione (5-vinyl OZT, or goitrin) and 2-*(S)*-1-cyano-2-hydroxy-3-butene (SCHB) (Peterson *et al.* 2000). Crambe seed meal has insecticidal effects on housefly larvae and adults (*Musca domestica* L., Diptera: Muscidae), with SCHB rather than goitrin causing toxicity (Peterson *et al.* 2000; Peterson *et al.* 1998; Tsao *et al.* 1996). The non-volatile isothiocyanates of camelina have not been tested for insecticidal effects, but because of the longer side-chain, toxicity to livestock animals is assumed to be lower than analogous rapeseed ITCs (Matthäus & Zubr 2000). BSF larvae can tolerate high

levels of mycotoxins such as aflatoxin B1 in their diet without effects on survival or biomass (Bosch *et al.* 2017), but to the best of our knowledge nothing is known about BSF performance when exposed to plant secondary metabolites.

In this study, we investigated the effect of chicken feed diet substituted with different proportions of crambe or camelina press cake or seed meal on BSF larval performance parameters (survival, development, biomass), fat content and fatty acid composition.

Materials and Methods

Insects

Eggs of the black soldier fly, *Hermetia illucens*, were collected from the stock colony at the Laboratory of Entomology (Wageningen University and Research, Wageningen, The Netherlands). This colony has been established with source material from the United States in 2008. The colony was reared on chicken feed ("Kuikenopfokmeel 1" (no. 600320), Kasper Faunafood BV, Woerden, The Netherlands) in a climate chamber at 27 ± 2 °C, 70 ± 10 % relative humidity and a photoperiod of L14:D10. Eggs were collected in three bundled corrugated cardboard strips on a moist substrate of sawdust, mouse droppings, and larval frass. After 24 h, the cardboard strips were transferred to a white polypropylene container (170 x 120 x 64 mm) with damp tissue, covered with a transparent non-perforated lid and incubated in the same climate chamber. Neonate larvae (< 24 h after hatching) were used in the experiments.

Feeds

We used chicken feed (the same feed as used for colony maintenance) as standard feed. Seed meals and press cakes originated from the 2015 field harvest of the University of Warmia and Mazury (UWM), Olsztyn, Poland. Press cakes were produced by UWM and delivered in January 2016. Seed meals were produced by OLEAD, Pessac, France, and delivered in August 2016. Press cake of camelina consisted of a 1:1 mixture of the accessions Midas and Omega. Press cake of crambe consisted of a mixture of five equal proportions of four accessions (9704-71, 9104-100 (two seed batches harvest from sown seeds collected in 2002/3 and 2011), Galactica and Nebula). Seed meals came from camelina accession Omega and crambe accession Galactica. Seed meal was

provided in sealed aluminium bags, stored at 4°C. Press cakes were delivered in plastic woven bags, stored at 4°C. Seed meals and press cakes were ground using mortar and pestle before use. Glucosinolate concentrations in press cakes are given in Table 1.

Experimental design

We tested the performance of BSF larvae on diets of chicken feed substituted on a dry matter basis with different percentages (0, 25, 50 and 100% substitution) of press cake or seed meal from crambe or camelina, resulting in 13 treatments: two crops x two crop by-products x three substitution levels, and the control diet (100% chicken feed). Macronutrient composition and water retention capacity of the diets are given in Table 2. Each treatment was replicated six times, set up in two batches of three replicates each on consecutive days. A replicate consisted of a white polypropylene container (170 x 120 x 64 mm) with 18 g DM diet, 36 ml tap water and 100 neonate larvae. The transparent lid of the container was perforated with 60 holes (1-2 mm diameter) for ventilation. Containers were placed in six trays (a tray per replicate), and their positions within a tray were randomly changed each day.

Larval performance measurements

A replicate was harvested on the day on which the first prepupa was observed in that replicate. This date was recorded, as well as total fresh larval biomass (Ohaus Adventurer Pro AV313, d = 0.001 g, Ohaus Corp. USA), the number of larvae and the number of prepupae. Survival rate was calculated as the number of larvae (including prepupae) at time of harvest divided by the number of larvae at the start of the experiment. Larvae were counted, rinsed with lukewarm tap water, and dried using tissue prior to weighing. Larvae were frozen at -20°C and later dried at 70°C until stable weight, to record total dry larval biomass. Individual larval weight was calculated as the total dry larval biomass divided by the number of larvae at time of harvest.

Fatty acid composition of larvae and feeds

Lipid extraction – Triplicate samples of each feed type and four randomly selected insect samples from each treatment were analysed for fatty acid composition. Total lipids from the insects and insect feeds were extracted according to the Folch procedure (Folch *et*

Table 1. Glucosinolate concentrations in camelina and crambe press cakes, in µmol/g sample. Glucosinolate analysis was done on freeze-dried samples using high-pressure liquid chromatography (HPLC), according to Grosser & van Dam (2017). All glucosinolates have been validated based on mass spectrometry (without distinction between progoitrin and *epi*-progoitrin).

Glucosinolate	Camelina	Crambe
sinigrin		0.45
(*epi*)-progoitrin[1]		27.93
4-hydroxyglucobrassicin		1.98
glucoarabin	6.51	
glucocamelinin	17.85	
11-(methylsulfinyl)-undecyl GSL	2.97	
Total glucosinolates	**27.33**	**30.35**

[1] sum of progoitrin and epi-progoitrin.

Table 2. Macronutrient composition and water retention capacity of feeds. Explanation of diet codes: CF = chicken feed (control), CAC = camelina press cake, CAM = camelina seed meal, CRC = crambe press cake, CRM = crambe seed meal. The numbers 25, 50 or 100 in diet codes indicate the inclusion percentage of by-product. Proximate analyses (Weende) of 100% oilseed by-products were done in duplicate. Macronutrient data of CF are from Kasper Faunafood BV, Woerden, The Netherlands. Nutrient data for inclusion percentages 25 and 50% were calculated from the CF and 100% oilseed by-products. Water retention capacity was measured in triplicate for all 13 diets using the traditional centrifugation method, AACC International Method 56-11-02 (Jacobs *et al.* 2015).

Diet	Oilseed by-product inclusion	Chicken feed inclusion	DM content	Crude protein	Crude fibre	Crude fat	Crude ash	Water retention capacity
	(% DM)	(% DM)	(% FM)	(% DM)	(% DM)	(% DM)	(% DM)	(ml/g DM)
CF	0%	100%	88.0	22.7	5.1	5.1	6.7	1.7
CAC25	25%	75%		27.1	6.7	7.8	6.4	2.1
CAC50	50%	50%		31.5	8.3	10.4	6.0	3.0
CAC100	100%	0%	88.0	40.4	11.5	15.7	5.3	6.3
CAM25	25%	75%		28.9	6.9	4.2	6.6	2.4
CAM50	50%	50%		35.1	8.6	3.3	6.5	3.9
CAM100	100%	0%	91.7	47.6	12.2	1.6	6.3	8.1
CRC25	25%	75%		24.0	9.1	7.1	6.8	2.0
CRC50	50%	50%		25.3	13.0	9.0	7.0	2.5
CRC100	100%	0%	88.7	27.9	21.0	12.9	7.2	2.6
CRM25	25%	75%		29.8	5.6	4.2	7.0	2.3
CRM50	50%	50%		36.9	6.1	3.3	7.2	2.7
CRM100	100%	0%	93.4	51.0	7.1	1.5	7.8	2.4

al. 1957), adapted by Tzompa-Sosa *et al.* (2014). Oven-dried larval samples were ground using a Waring Blendor 34Bl99 (Conair Corporation, USA) and weighed into 100-mL glass tubes. The samples were then mixed with dichloromethane:methanol (both HPLC grade, purchased from Actu-All Chemicals, Oss, The Netherlands) (2:1, v/v) in a ratio of sample to solvent 1:20. The tubes were then sonicated (20 s) and shaken for 2 h. After this step, ultrapure water was added to the tubes to obtain a final mixture of dichloromethane:methanol:water ratio equal to 8:4:3 (v/v/v) by taking into account the original moisture content of the samples. The tubes were centrifuged at 2000 rpm for 20 min at 20°C (Heraeus Multifuge X3R, Thermo Fisher Scientific, Langenselbold, Germany). The upper aqueous layer was discarded by using a glass Pasteur pipette. The remaining lipid/solvent/pellet mixture was kept under a fume hood for 12 h. Then the mixture was filtered over a filter paper (Whatman 595 ½, ø185 mm, Whatman GmbH, Dassel, Germany) into a pre-weighed glass flask. The glass flasks containing the dichloromethane and the lipids were then dried by a rotary evaporator at 40°C (Büchi Rotavapor R-215, BÜCHI Labortechnik AG, Flawil, Switzerland). The flasks were flushed with N_2 in order to evaporate the remaining solvents and placed in a ventilated oven at 60°C for 2 h (Binder GmbH, Tuttlingen, Germany). Then the flasks were weighed in order to assess the lipid content (% DM), *i.e.* the weight of extracted lipids divided by the weight of ground-up larvae. The lipids were then stored under N_2 at -20°C for further analysis.

Determination of fatty acid composition – The fatty acid composition of the samples was analysed according to the ISO standard NEN-ISO 16958:2015(E). Fatty acid methyl esters (FAMEs) of the extracted lipids were prepared according to the ISO standard method ISO5509:2000(E). Around 50 mg lipids were methylated with 200 µl 1 M KOH at room temperature in order to obtain the respective FAMEs. The fatty acid composition was determined by means of gas chromatography with flame ionization detector (GC-FID) (Thermo Scientific Trace GC Ultra) using WCOT fused silica column (100 m × 0.25 mm i.d. x 0.2 µm f.t., Coating Select Fame, Varian, Houten, The Netherlands). The gas chromatograms were analysed with Chromeleon 7.0 (Thermo Fischer Scientific Inc, Langenselbold, Germany) and the absolute peak areas were determined for each fatty acid. The fatty acid composition was then expressed in mass fractions as g fatty acid/100 g in lipid (%) by using the relative peak areas.

Statistical analyses

Survival rate, development time, total larval biomass, individual larval weight, and larval fat content were analysed for differences among diets, with a random intercept for batch, using linear mixed model regression (LMM) (Zuur *et al.* 2009), using the lme function from the nlme package v.3.1-137 (Pinheiro *et al.* 2018). A variance structure was tested for Diet, and model selection was done based on the likelihood ratio test (Pinheiro & Bates 2000). Post-hoc pairwise comparisons were made using Estimated Marginal Means in the emmeans function from the emmeans package (version 1.3.4), with Tukey-adjustment of P-values (Lenth 2020).

Overall changes in fatty acid composition were analysed in a Constrained Correspondence Analysis (CCA) with Diet as a constraining variable, using the cca function from the vegan package version 2.5-4 (Oksanen *et al.* 2019). The effect of diet was tested using a permutation test for CCA, *i.e.* anova.cca, with 999 permutations (Legendre *et al.* 2011).

Dietary differences in fatty acid percentages were tested via Generalized Least Squares regression (GLS) with a variance structure for Diet, using the gls function from the nlme package, and post-hoc comparisons as mentioned above for performance parameters. Fatty acids with a group average below 0.05% of total fatty acids were regarded as "not detected" and excluded from analysis (*i.e.* the diet x fatty acid combination).

In all tests, significance level alpha was set at 0.05. All figures were created using the R package ggplot2 (Wickham 2016). All analyses were done in the statistical software R version 3.5.0 (R Core Team 2018).

Results

Larval performance and fat content

Diet significantly influenced all larval performance parameters studied ($P < 0.0001$). In general, larval performance was better (*i.e.* higher survival, total larval biomass and individual larval weight, shorter development time) on camelina than on crambe, on cake than on meal, and on diets with lower inclusion percentages of by-product. On all camelina diets except 100% camelina meal, survival was relatively high between 85-

96%. On crambe diets, survival was lower: no larvae survived on the 100% crambe meal diet, and significantly fewer larvae survived on diets with 50 or 100% crambe cake (71% and 64%, respectively) compared to control diet (94%; Figure 1A). Development of larvae to the prepupal stage took similar time on control diet, 25% crambe cake and all camelina diets (14 - 19 days) except the 100% meal and cake (21 days; Figure 1B). Total larval biomass was highest on control and camelina cake diets (2.4 - 2.8 g DM), and significantly lower than control on 100% camelina meal (1.6 g DM), 100% crambe cake (0.9 g DM) and 50% crambe meal (1.4 g DM; Figure 1C). This pattern is similar for individual larval weight: larvae on 100% crambe cake weighed significantly less (0.014 g DM) than on control diet (0.027 g DM); the weights of larvae on the other diets were similar (0.020 - 0.030 g DM; Figure 1D). Fat content was high (16 - 20% DM) in larvae fed cakes (except 100% crambe cake: 10% DM) and low in larvae fed camelina meal (10 - 11%), but most groups did not differ significantly due to large within-group variations (Figure 1E).

Fatty acids in feed

Considerable differences were found in the fatty acid composition of the feeds (Table 3). Camelina cake and meal were enriched in α-linolenic acid (C18:3 n-3; 23% and 21% of total fat, respectively) and gondoic acid (C20:1 *cis*-11; 8% and 6%, respectively) compared to the other feeds, whereas crambe meal and especially crambe cake were abundant in erucic acid (C22:1 *cis*-13; 10% and 43%, respectively). Palmitic acid (C16:0), stearic acid (C18:0) and linoleic acid (C18:2 n-6) occurred in all feeds but were most abundant in chicken feed (20%, 3% and 45%, respectively). Oleic acid (C18:1 *cis*-9) was most abundant in crambe meal (30%), followed by chicken feed (27%) and crambe cake (24%).

Fatty acids in larvae

Diet explained a significant part of the inertia in larval fatty acid profiles (R^2_{adj} = 0.82; permutation test on CCA under reduced model, with diet as constraining variable: χ^2= 0.241, $F_{df(11,36)}$ = 14.89, P = 0.001). Fatty acid profiles of larvae fed seed meals were more similar to profiles of larvae fed chicken feed, whereas major shifts occurred along the first CCA axis for larvae fed crambe cake, and along the second CCA axis for larvae fed camelina cake (Figure 2). The fatty acids with the largest relative contributions (at

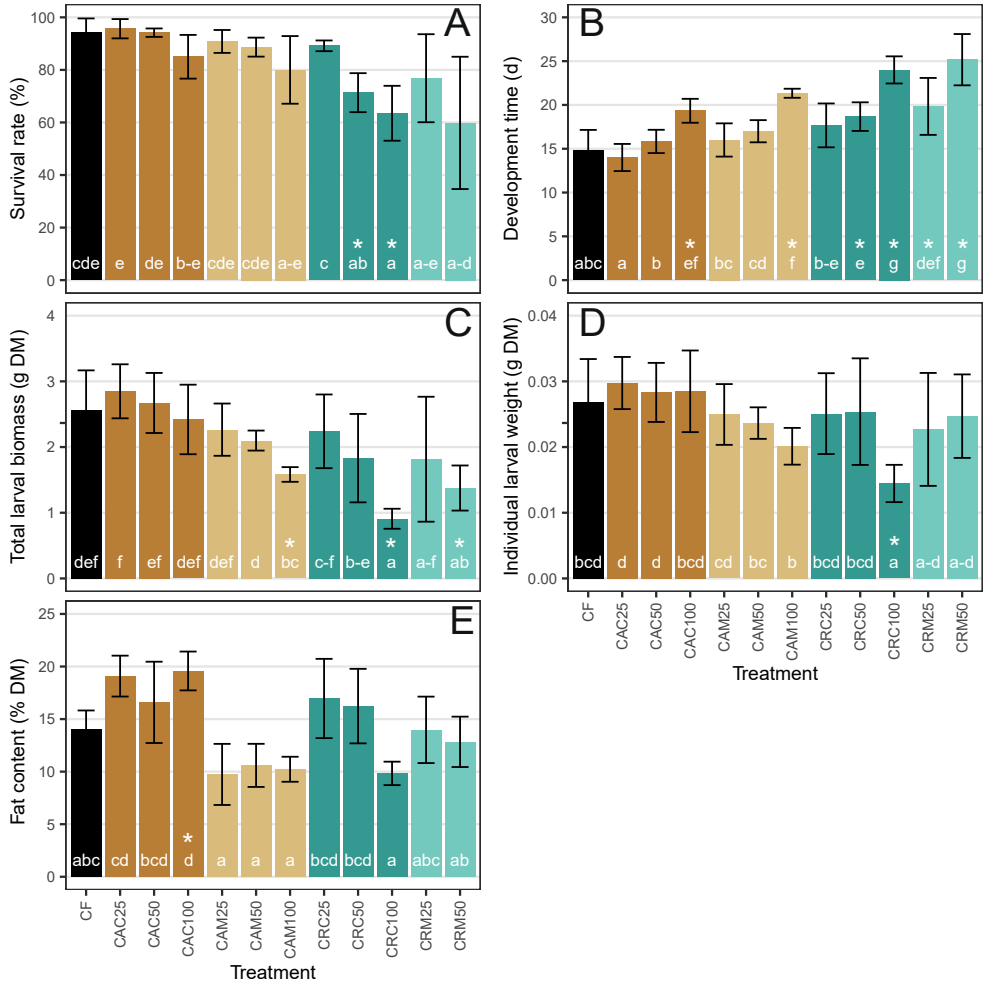

Figure 1. Performance parameters (mean ± SD) of BSF larvae fed different diets. A) survival rate of larvae, in %; B) development time to first prepupa, in days; C) total larval biomass, in gram dry matter; D) individual larval weight, in gram dry matter; E) fat content of larvae, in % of dry matter weight. For definition of diet codes, see Table 1. Means that share no letters are significantly different, and means with an asterisk differ significantly from control CF (Estimated Marginal Means with Tukey-adjusted P-values, $\alpha = 0.05$).

least 3%) to the constrained inertia were α-linolenic acid (C18:3 n-3; 22%), lauric acid (C12:0, 13%), oleic acid (C18:1 *cis*-9; 9%), *cis*-7 hexadecenoic acid (C16:1 *cis*-7) (7%), erucic acid (C22:1 *cis*-13; 6%), henicosanoic acid (C21:0, 6%), linoleic acid (C18:2 n-6; 5%), and palmitic acid (C16:0; 3%).

Table 3. Fatty acid composition (g fatty acid/ 100 g fat (%), mean ± SD) of feeds. Grey rows are only meant to improve readability.

			Diet [3,4,5]				
Fatty acid [1]	**Fatty acid name**	**P value** [2]	**CF**	**CAC**	**CAM**	**CRC**	**CRM**
C10:0	capric	9.1E-02	n.d.	0.10 ± 0.01	0.12 ± 0.09	n.d.	0.40 ± 0.25
C12:0	lauric	4.7E-10	0.05 ± 0.01a	n.d.	n.d.	0.20 ± 0.04b	2.92 ± 0.07c
C14:0	myristic	4.1E-04	0.25 ± 0.02b	0.11 ± 0.07a	0.09 ± 0.00a	0.06 ± 0.02a	0.10 ± 0.02a
C15:0	pentadecanoic	5.7E-03	n.d.	n.d.	0.08 ± 0.00b	n.d.	0.07 ± 0.00a
C16:0	palmitic	7.6E-12	19.51 ± 0.70d	4.85 ± 0.34a	11.03 ± 0.26b	3.93 ± 0.52a	12.80 ± 0.28c
C16:1 cis-7	cis-7 hexadecenoic	6.7E-06	n.d.	0.12 ± 0.00a	0.13 ± 0.01a	0.13 ± 0.00a	0.18 ± 0.01b
C16:1 cis-9	palmitoleic	2.2E-15	0.13 ± 0.01a	0.33 ± 0.02c	0.28 ± 0.00b	0.49 ± 0.01d	1.02 ± 0.01e
C17:0	margaric	2.3E-04	0.09 ± 0.00b	0.06 ± 0.01a	0.08 ± 0.00b	n.d.	0.10 ± 0.00c
C18:0	stearic	1.3E-12	3.13 ± 0.04d	1.50 ± 0.08b	2.28 ± 0.03c	0.86 ± 0.07a	1.46 ± 0.02b
C18:1 cis-9	oleic	1.7E-11	26.61 ± 0.47d	21.52 ± 0.24b	17.49 ± 0.25a	24.32 ± 0.18c	29.51 ± 0.51e
C18:1 cis-11	cis-11 octadecenoic	3.1E-06	0.90 ± 0.08b	1.10 ± 0.20bc	1.50 ± 0.01d	0.54 ± 0.01a	1.25 ± 0.02cd
C18:2 n-6	linoleic (LA)	1.4E-12	44.97 ± 1.36d	32.43 ± 0.25c	30.93 ± 0.28c	14.32 ± 0.28a	27.87 ± 0.28b
C18:3 n-3	α-linolenic (ALA)	<2.2E-16	3.13 ± 0.14a	22.61 ± 0.18e	21.13 ± 0.04d	4.74 ± 0.09b	7.41 ± 0.14c
C20:0	arachidic	3.2E-04	0.32 ± 0.00a	0.62 ± 0.21b	0.84 ± 0.07b	0.60 ± 0.01b	0.33 ± 0.02a
C20:1 cis-11	gondoic	4.1E-14	0.26 ± 0.01a	7.81 ± 0.14e	6.17 ± 0.27d	1.76 ± 0.02b	2.22 ± 0.02c
C20:1 trans-11	trans-11 eicosenoic	2.6E-01	n.d.	0.07 ± 0.01	0.08 ± 0.00	n.d.	n.d.
C20:2 n-6	cis-11,14 eicosadienoic	2.3E-12	n.d.	1.39 ± 0.02c	1.42 ± 0.04c	0.16 ± 0.01a	0.34 ± 0.01b
C20:3 n-3	eicosatrienoic (ETE)	1.2E-08	0.06 ± 0.01a	0.66 ± 0.00b	0.6.0 ± 0.03b	1.13 ± 0.04d	0.89 ± 0.12c
C20:4 n-6	arachidonic (ARA)	6.0E-06	n.d.	0.34 ± 0.02b	0.05 ± 0.00a	n.d.	n.d.
C22:0	behenic	4.6E-13	0.18 ± 0.01a	0.24 ± 0.02a	0.26 ± 0.01a	1.84 ± 0.07c	0.43 ± 0.05b
C22:1 cis-13	erucic	5.4E-13	n.d.	3.08 ± 0.34a	4.21 ± 0.21a	43.33 ± 0.62c	10.18 ± 0.75b
C24:0	lignoceric	6.5E-12	0.12 ± 0.00a	0.20 ± 0.01c	0.31 ± 0.01b	0.60 ± 0.01d	0.18 ± 0.02b
C24:1 cis-15	nervonic	1.2E-08	n.d.	0.62 ± 0.03b	0.86 ± 0.03c	0.81 ± 0.03c	0.26 ± 0.01a

[1] Fatty acids that were detected, but with all means < 0.05%: C11:0, C13:1, C20:1 n-6, C21:0, C21:1 n-9, C22:3 n-3, C22:5 n-6, CLA. [2] P value gives the outcome of the one-way ANOVA test for differences among diets. [3] Explanation of diet codes: CF = chicken feed (control), CAC = camelina press cake, CAM = camelina seed meal, CRC = crambe press cake, CRM = crambe seed meal. [4] Means of the same fatty acid that share no letters, are significantly different (Estimated Marginal Means with Tukey-adjusted P-values, α= 0.05). [5] n.d. = not detected (or mean < 0.05%).

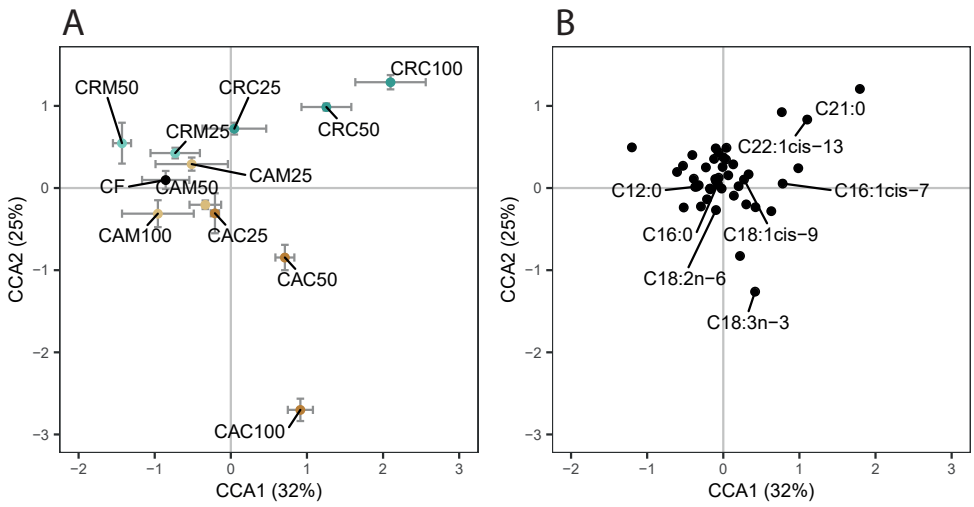

Figure 2. Fatty acid composition of larvae fed different diets (Constrained Correspondence Analysis, with Diet as constraining variable). A) display of samples along the 1st and 2nd CCA axes (mean CCA scores ± SD for each diet). B) display of fatty acids along the same axes as in (A), with fatty acids labelled that contributed more than 3% to the constrained inertia. For definition of diet codes, see Table 1; for full names of fatty acids, see Table S1. The percentage explained inertia of each CCA axis is in parentheses.

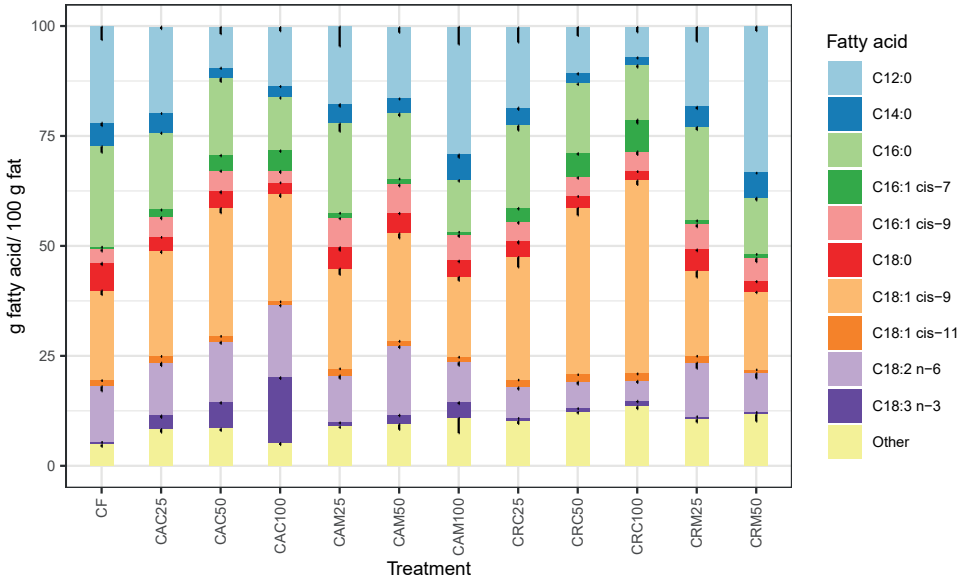

Figure 3. The ten most abundant fatty acids in larvae fed different diets (mean ± SD, g fatty acid/ 100 g fat (%)). For definition of diet codes, see Table 1; for full names of fatty acids, see Table S1. Statistical test output can be found in Table S1.

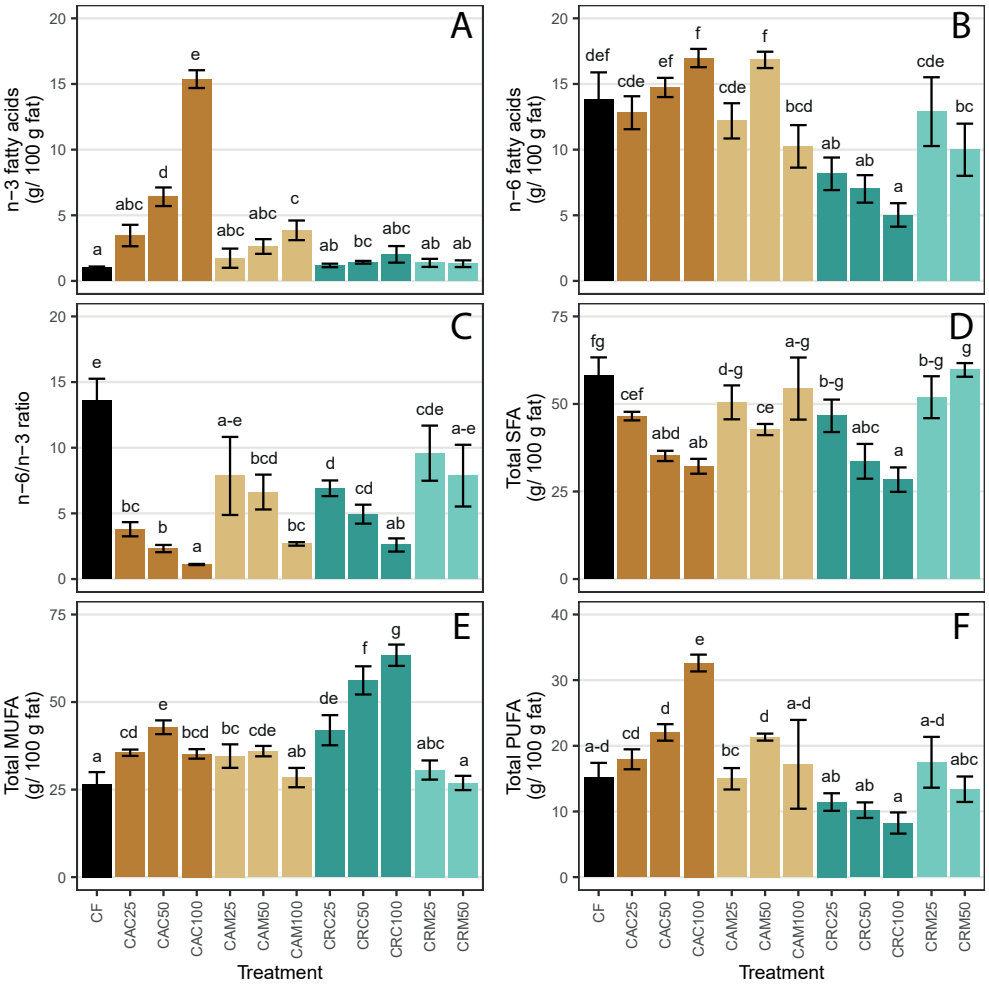

Figure 4. Fatty acid classes in larvae fed different diets (mean ± SD, g fatty acid / 100 g fat (%)). A) n-3 poly-unsaturated fatty acids (PUFA); B) n-6 PUFA; C) n-6 / n-3 ratio; D) total saturated fatty acids (SFA); E) total mono-unsaturated fatty acids (MUFA); F) total PUFA. For explanation of diet codes, see Table 1. Means that share no letters, are significantly different (Estimated Marginal Means with Tukey-adjusted P-values, $\alpha = 0.05$).

Most individual fatty acids of larvae differed significantly in contents among diets (Figures 3 and 4, Table S1). Larvae fed 100% camelina seed meal or 50% crambe seed meal had the highest lauric acid content of all groups, *i.e.* 29% and 33%, respectively. The larvae fed cake of either crop species showed most differences in fatty acids compared to control. When fed on diets with increasing proportions of camelina cake, larvae showed a clear increase in polyunsaturated fatty acids (PUFA; from 18% to 33% of total fat), mainly α-linolenic acid, and a decrease in saturated fatty acids (SFA; from 47% to 32%), mainly lauric acid, myristic acid and palmitic acid. Larvae fed crambe cake showed a strong increase in mono-unsaturated fatty acids (MUFA; from 42% to 63%), especially oleic acid, as well as a decrease in SFA (from 47% to 28%), with increasing proportion of cake in the diet. Erucic acid was present at much lower levels (1-3%) in the larvae than in the crambe cake (43%) they were fed (Tables 3 and S1).

Within the PUFA fraction of the larvae, n-3 fatty acids increased with the addition of cake to the diet, especially for camelina (from 3% to 15% of total fat; Figure 4A). On the other hand, n-6 fatty acids decreased markedly in larvae fed crambe cake (from 8% to 5%; Figure 4B). This resulted in lower ratios of n-6 : n-3 PUFA in larvae fed either cake (camelina: 1.1 - 3.8, crambe: 2.6 - 6.9; Figure 4C). Larvae fed seed meal showed similar but smaller changes in n-3 and n-6 fatty acids.

Discussion

This study shows that BSF larval performance was similar to control diet when reared on chicken feed replaced with up to 50% camelina seed oil by-product or up to 25% with crambe by-product, and that larval fatty acid profiles shifted especially with an increasing percentage of cake of either crop species in the diet, decreasing the ratio of n-6 : n-3 PUFA.

Effects of dietary secondary plant compounds on BSF larval performance

Previous studies have shown that edible insects can perform similarly on control diets and diets partially replaced by oilseed by-products, although dependent on the type of oilseed crop and the inclusion percentage, also negative effects have been reported. On diets with 10% soymeal or 10-20% rapeseed meal or press cake, yellow mealworms (*Tenebrio molitor* L.; Coleoptera: Tenebrionidae) reached a biomass similar to control

diet, but biomass was lower on diets including 20% soymeal or 10-20% linseed meal (Nielsen 2016). On the other hand, BSF larvae showed no change in development time and increased survival and biomass with increasing percentage (from 10 to 30%) of rapeseed *Brassica napus* L. double-zero cultivar (low in erucic acid and glucosinolates) press cake in the diet, but when fed with 100% rapeseed cake, larval development was delayed (unpublished data).

In the present study, the higher larval performance on camelina than on crambe by-products may be caused by a lower toxicity of the glucosinolates (Table 1) and their enzymatic breakdown products in camelina than in crambe. In contrast to previous studies (Matthäus 1997), total glucosinolate concentrations in press cakes of both crops were similar in our study. Performance differences may therefore be caused by qualitative rather than quantitative differences in glucosinolates. The toxicity of glucosinolates of camelina relative to those of crambe is unknown, but the main glucosinolates of camelina hydrolyse into non-volatile isothiocyanates (ITCs) and are expected to be less toxic (Matthäus & Zubr 2000). However, camelina ITCs and seed meal extract do cause some cytotoxicity in mouse cells (Das *et al.* 2014). Detrimental effects of secondary plant compounds of crambe and camelina have not been tested on BSF so far, but effects of crambe seed meal and glucosinolates have been studied in dipteran insects. For instance, defatted crambe seed meal was found to be toxic to aquatic mosquito larvae (*Aedes aegypti* (L.); Diptera: Culicidae) and maggots and adults of the housefly (Peterson *et al.* 2000; Peterson *et al.* 1998; Tsao *et al.* 1996). The nitrile 2-(*S*)-1-cyano-2-hydroxy-3-butene (SCHB) appeared to be the main active component, rather than goitrin (Peterson *et al.* 2000; Peterson *et al.* 1998).

On the other hand, some dipteran species appear to be more or less resistant to ITCs, likely dependent on the degree of dietary specialisation on brassicaceous plants. The larvae of the cabbage root fly *Delia radicum* (L.) (Diptera: Anthomyiidae), a specialist herbivore of Brassicaceae, house gut bacteria that degrade aromatic ITCs (Welte *et al.* 2016b). Other dipterans may metabolize ITCs via more general detoxification enzymes, *i.e.* glutathione-S-transferases (GST) and cytochrome P450 monooxygenases. Larvae of hoverfly species (Diptera: Syrphidae) preying on *Brassica*-feeding aphids have higher *in vitro* GST activity than saprophagous and coprophagous species (Vanhaelen *et al.* 2001). The recently sequenced BSF genome reveals an expansion of the repertoire of cytochrome P450 and GST gene families compared to genomes of other dipterans (Zhan

et al. 2020), suggesting that BSF may be able to detoxify a wider array of xenobiotics. However, there is no conclusive evidence yet on the role of GST specifically in ITC detoxification *in vivo* (Winde & Wittstock 2011) and both enzyme families comprise many different enzymes that may have low affinity to ITCs. In addition, it is unknown if the high pressure applied to expel the seed oil, resulting in a brief pulse of temperatures of 60-70 °C, and in the case of seed meal, the extraction of oil remaining in the seed cake by extraction using apolar solvents, affect the activity of myrosinase. If these seed treatments result in (partial) denaturation of myrosinase, the formation of ITCs and other toxic products may be reduced.

Whether BSF or its gut bacteria are able to detoxify glucosinolates and their derivatives is unknown, but considering its generalist detritivorous feeding habits this seems unlikely. These secondary metabolites may therefore be a major component causing the observed differences in performance. Nonetheless, BSF larvae are able to tolerate novel selection pressures, whether or not aided by their gut bacteria. BSF larval gut bacteria can rapidly degrade the antibiotic tetracycline in chicken manure (Cai *et al.* 2018b) and BSF larvae themselves are able to tolerate high levels of mycotoxins (Bosch *et al.* 2017; Camenzuli *et al.* 2018). Rather than accumulating the mycotoxins, the larvae catabolize and/or excrete them (Camenzuli *et al.* 2018; Meijer *et al.* 2019).

Effects of dietary macronutrient levels on BSF larval performance

Larvae performed better (*i.e.* higher survival, faster development, larger biomass; Figure 1) on press cakes than on seed meals. This may be caused by the higher fat content and lower protein content in the press cake diets compared to the seed meal diets. The press cakes contained 13-16% fat and 28-40% protein, compared to 1-2% fat and 48-51% protein in the seed meals (Table 2). Formulating the diets with 25, 50, or 100% of seed meal resulted in higher protein content and lower fat content than in press cake diets of the same inclusion percentage (Table 2). Several studies suggest that there is an optimum dietary protein content for BSF larvae, though this optimum value depends on the total protein and carbohydrate contents, the ratio between them and the protein quality, *i.e.* protein digestibility and amino acid composition (Barragán-Fonseca *et al.* 2019; Barragán-Fonseca *et al.* 2018b; Cammack & Tomberlin 2017). Larvae feeding on excessive protein may suffer from higher concentrations of toxic nitrogenous waste, *i.e.* via excretion of uric acid and its breakdown into subsequently allantoin, urea and

finally ammonia (Green & Popa 2012), resulting in increased larval mortality (Barragán-Fonseca *et al.* 2019). Besides the detrimental effects of excess protein, a low diet fat content can also prolong larval development time compared to diet with high fat content (Oonincx *et al.* 2015).

Other diet properties affecting larval performance

Differences in physical and microbiological properties of the diets may also have affected larval performance. Diets differed in water retention capacity, with chicken feed and crambe diets having lowest capacity, and camelina diets retaining most water (Table 2). So although we added 2 ml water per gram DM of diet, some diets appeared drier than others. This may have affected the rate of water evaporation from the diet and consequently may have caused differences in substrate moisture content over time. Effects of moisture content on BSF larval performance can even be larger than the effect of diet macronutrient composition (Cammack & Tomberlin 2017), and differences in moisture content can underlie shifts in the microbial community of the substrate (Cammack *et al.* 2018).

Oilseed by-products changed larval fatty acid profiles and reduced n-6 : n-3 ratios

Since the fatty acid compositions of the oilseed by-products and control chicken feed were very different (Table 3), and the fatty acid profile of BSF larvae is known to depend on the diet (Danieli *et al.* 2019; Liland *et al.* 2017; Moula *et al.* 2018; Oonincx *et al.* 2020; Oonincx *et al.* 2015; Spranghers *et al.* 2017), differences in larval fatty acid profiles were expected among the tested diets.

In the larvae fed press cakes, long chain fatty acids were more abundant, of which some originated directly from the diet (*e.g.* linoleic acid and α-linolenic acid in camelina). However, erucic acid, the most abundant fatty acid (43%) in crambe press cake, was hardly found (1-3%) in larvae fed crambe cake; in contrast, these larvae contained significantly more oleic acid (44%) than larvae from other diets, and almost twice as much as the oleic acid content (24%) of the feed. This may suggest that BSF larvae were able to convert erucic acid via partial β-oxidation (chain-shortening) to oleic acid – a pathway that, to the best of our knowledge, is unknown in insects so far, but has been observed in rats (Golovko & Murphy 2006).

In our study, larvae fed chicken feed, seed meal, or 25% press cake contained more lauric acid than those fed 50-100% press cake (Figure 3; Table S1). BSF larvae may convert dietary fatty acids into lauric acid when dietary fat is limited (Oonincx *et al.* 2015) and can accumulate fat by *de novo* synthesis of lauric acid (Zhu *et al.* 2019).

The inclusion of camelina by-product in diets led to a reduced n-6 : n-3 ratio in the larvae (Figure 4C; Table S1), even when fed camelina seed meal containing only 1.5% oil (Table 2). This mainly happened through an increase in α-linolenic acid content in the larvae. Stearidonic acid (C18:4 n-3) and docosahexaenoic acid (C22:6n-3, DHA) were also present in the larvae, but no intermediate n-3 PUFA derived from α-linolenic acid. This suggests that BSF larvae are able to synthesise stearidonic acid from α-linolenic acid, but lack the enzymes to synthesise longer-chain n-3 PUFA; DHA may be produced via an unknown pathway. Similarly, it has been shown that supplementing the diet with 1% flaxseed oil caused BSF larval n-6 : n-3 ratio to drop below 5, because of higher α-linolenic acid content but no other n-3 PUFA (Oonincx *et al.* 2020). Enrichment of longer-chain n-3 PUFA in BSF larvae did occur when these fatty acids were present in the diet, *e.g.* fish waste (Barroso *et al.* 2019; St-Hilaire *et al.* 2007) and mussels (Ewald *et al.* 2020).

In larvae fed crambe cake, the n-6 : n-3 ratio was also reduced with increased proportions of crambe cake in the diet, mainly due to a slight decrease in linoleic acid (from 7.0% to 4.6%) and an increase in α-linolenic acid (from 0.6% to 1.3%; Figure 3; Table S1). Compared to the fatty acid compositions of the feeds, *i.e.* 45% linoleic acid and 3% α-linolenic acid in chicken feed and 14% and 5% in crambe cake (Table 3), respectively, the changes in the larvae appear to be very subtle.

Although larval fat content on control diet (14% DM) was within the range reported for BSF on chicken feed (13 - 25% DM) (Bosch *et al.* 2014; Oonincx *et al.* 2015), the lauric acid content in our study was lower than in comparable studies. Lauric acid is often the dominant fatty acid found in BSF larvae, accounting for 21-63% of total lipids (Barragán-Fonseca *et al.* 2017; Danieli *et al.* 2019; Liland *et al.* 2017; Moula *et al.* 2018; Oonincx *et al.* 2015; Spranghers *et al.* 2017). Our control chicken feed resulted in 22% lauric acid in larval fat, whereas Oonincx *et al.* (2015) reported 48% lauric acid in larvae of the same colony on similar diet. On the other hand, we detected more palmitic acid (22.85% vs. 12.7%), stearic acid (6.3% vs. 2.1%), oleic acid (20.44% vs. 10.2%) and linoleic acid (12.67% vs. 9.4%) than Oonincx *et al.* (2015).

This difference could be due to methodological differences between the studies, regarding the preservation, extraction, and detection of fatty acids. First, the killing and storage method may have significantly influenced the fatty acid profiles, since lipases in the insect tissue remain active even at -20°C, as opposed to blanching, which stops lipolysis (Caligiani *et al.* 2019; Larouche *et al.* 2019). However, in both studies the larval samples were killed and stored at -20°C, and the only difference may be in storage time. We dried samples after eight months of storage, whereas Oonincx *et al.* (2015) dried their samples prior to storage. Second, Oonincx *et al.* (2015) used the chloroform : methanol extraction (Folch *et al.* 1957), whereas we replaced chloroform with the less toxic dichloromethane, according to Tzompa-Sosa *et al.* (2014). Nevertheless, dichloromethane would rather lead to a similar or more efficient fat extraction from animal tissues than chloroform (Cequier-Sánchez *et al.* 2008). Lastly, the sensitivity and resolution of the GC method may have influenced the elution of fatty acids therefore leading to detection of minor fatty acids. In this way, more peaks are detected and annotated as fatty acids and the relative abundance of individual fatty acids will drop.

BSF larvae fed crambe or camelina as animal feed: pros and cons

BSF larvae can partially replace soymeal or fishmeal in feed for pigs, poultry and fish, without significant changes in animal performance (Chia *et al.* 2019b; Gasco *et al.* 2019). Product quality can however be affected, for example the n-3 PUFA content in meat decreased when animals were fed insect-based diets (Gasco *et al.* 2019). Increasing n-3 PUFA content in BSF larvae like in our study, could alleviate such a drawback, and yield a n-6 : n-3 ratio recommended for human health, *i.e.* lower than 5 and ideally 2:1 or 1:1 (Simopoulos 2010). Additional health benefits of BSF larvae to livestock animals could come from the antimicrobial properties of lauric acid against Gram-positive bacteria (Spranghers *et al.* 2018).

Erucic acid is only allowed at maximally 0.4% of total fatty acids in food for new-born infants (European Commission 2019), and can cause adverse effects in poultry at an intake rate of 20 mg/kg body weight per day (EFSA 2016). It causes myocardial lipidosis due to poor β-oxidation in the mitochondria, reducing the contractile force of the heart muscle (EFSA 2016). Although crambe oil contained high levels of erucic acid, levels were more than 15-fold lower in resulting larvae, at only 1% erucic acid of total fatty acids for larvae fed 25% crambe cake. In this regard, BSF larvae fed crambe

cake may be suitable as animal feed, whereas the levels in crambe cake are too high to allow it as animal feed.

The most pressing question regarding the suitability of BSF larvae fed camelina or crambe for animal feed, however, is what happens to the glucosinolates from either crop. The fate of glucosinolates was not determined in the larvae or residues, and to our knowledge no study to date covers the topic of BSF and glucosinolates or any other secondary plant metabolites. This is an important area for future research (Van der Spiegel *et al.* 2013), since organic waste streams can contain a diversity of such plant compounds that could end up in a BSF-based bioconversion system and jeopardize product safety as animal feed.

Conclusion

BSF larvae can be successfully grown on chicken feed with partial replacement by oilseed by-products, up to 50% for camelina and 25% for crambe. Larval performance at these inclusion percentages was similar to that of control. Besides, larval fatty acid profiles had a more favourable n-6 : n-3 PUFA ratio (2.3 – 9.6) than control (13.6), and low erucic acid content (1%) despite high levels of this fatty acid in crambe cake (43%). Thus, BSF larvae may be of better quality for feeding livestock than the oilseed by-products. However, knowledge on the fate of glucosinolates in the larvae is crucial before use as animal feed.

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Author contributions

JvL acquired funding for the study. SS, JvL, and MD planned and designed the study. SS conducted the insect experiment. SS prepared the samples for fatty acid analysis in collaboration with SY and HvV. SS analysed the data statistically and interpreted the fatty acid analysis together with SY and HvV. SS wrote the first version of the manuscript and processed comments from all authors. All authors read and approved the final manuscript.

Supplementary information

Table S1. Fatty acid composition (g / 100 g total fat (%), mean ± SD) of larvae fed different diets. For definition of diet codes, see caption Table 1. "n.d" = not detected (or mean < 0.05%). Test indicates the statistical test (LM = one-way ANOVA, or GLS = Generalized Least Squares regression) for differences among diets, and P value gives the outcome of this test. Means of the same fatty acid that share no letters, are significantly different (Estimated Marginal Means with Tukey-adjusted P-values, alpha = 0.05). Grey rows are only meant to improve readability of the table. One fatty acid, C18:3 trans isomers, was excluded from all analyses, since it was only detected in one sample of CAM50 (0.54%) and one sample of CAM100 (11.77%), and these values were considered outliers.

	C10:0	C11:0	C12:0	C13:0	C13:1	iso-C14:0	C14:0	C14:1
Diet	**capric**	**undecylic**	**lauric**	**tridecanoic**	**cis-9 tridecenoic**	**isomyristic**	**myristic**	**myristoleic**
CF	0.52 ± 0.06b	0.06 ± 0.04	21.86 ± 6.01abcd	n.d.	0.06 ± 0.04a	n.d.	5.36 ± 1.43abcd	0.06 ± 0.05ab
CAC25	0.48 ± 0.07ab	0.06 ± 0.05	19.71 ± 1.06c	n.d.	n.d.	n.d.	4.41 ± 0.16c	0.10 ± 0.07abc
CAC50	0.35 ± 0.07ab	0.09 ± 0.02	9.3 ± 3.26abc	n.d.	n.d.	0.07 ± 0.03a	2.39 ± 0.44ab	n.d.
CAC100	0.34 ± 0.05ab	0.06 ± 0.01	13.42 ± 1.80ab	n.d.	n.d.	n.d.	2.57 ± 0.33ab	n.d.
CAM25	0.52 ± 0.13b	0.06 ± 0.01	17.65 ± 9.01abcd	0.08 ± 0.03a	0.06 ± 0.03a	0.12 ± 0.02a	4.43 ± 1.11abcd	0.15 ± 0.02bc
CAM50	0.54 ± 0.10b	0.06 ± 0.02	16.18 ± 2.35bc	n.d.	n.d.	0.31 ± 0.06b	3.43 ± 0.34bc	0.13 ± 0.03abc
CAM100	0.84 ± 0.13c	0.07 ± 0.01	29.07 ± 8.04abcd	0.14 ± 0.02b	n.d.	0.37 ± 0.18ab	5.82 ± 1.37abcd	0.24 ± 0.04c
CRC25	0.53 ± 0.17b	n.d.	18.22 ± 6.76abcd	n.d.	n.d.	n.d.	4.09 ± 0.95abcd	0.09 ± 0.03ab
CRC50	0.28 ± 0.09ab	n.d.	10.51 ± 4.01abc	n.d.	0.07 ± 0.01ab	0.12 ± 0.06a	2.36 ± 0.58ab	n.d.
CRC100	0.22 ± 0.07a	n.d.	6.94 ± 2.39a	0.06 ± 0.01a	0.06 ± 0.04a	0.46 ± 0.11b	1.79 ± 0.44a	0.05 ± 0.02a
CRM25	0.54 ± 0.20b	n.d.	18.16 ± 6.62abcd	n.d.	0.14 ± 0.04b	0.39 ± 0.39ab	4.59 ± 0.95abcd	0.13 ± 0.05abc
CRM50	0.90 ± 0.15c	0.12 ± 0.04	33.28 ± 2.04d	0.19 ± 0.03b	n.d.	1.15 ± 0.63ab	5.68 ± 0.20d	0.32 ± 0.14abc
Test	LM	GLS	GLS	LM	LM	GLS	GLS	GLS
P value	<0.0001	0.0659	<0.0001	<0.0001	0.0159	<0.0001	<0.0001	<0.0001

Table S1. (continued)

	iso-C15:0	C15:0	C16:0	C16:1 cis-7	C16:1 cis-9	C16:1 trans-9	C17:0	C17:1 cis-10
Diet	**iso-pentadecanoic**	**pentadecanoic**	**palmitic**	**cis-7 hexadecenoic**	**palmitoleic**	**trans-9 hexadecenoic**	**margaric**	**cis-10 heptadecenoic**
CF	0.06 ± 0.07a	0.44 ± 0.16ab	22.85 ± 2.82cd	0.56 ± 0.19a	3.06 ± 0.93ab	0.19 ± 0.11ab	0.50 ± 0.12bcd	0.37 ± 0.13abc
CAC25	0.16 ± 0.03ab	0.36 ± 0.13ab	17.48 ± 0.44cd	1.71 ± 0.32bc	4.54 ± 0.87abc	0.40 ± 0.24ab	0.32 ± 0.05ab	0.42 ± 0.11abc
CAC50	0.38 ± 0.04c	0.39 ± 0.06b	17.44 ± 1.62bcd	3.56 ± 0.56de	4.70 ± 0.52abc	0.30 ± 0.08b	0.39 ± 0.09abc	0.63 ± 0.06bc
CAC100	0.26 ± 0.05abc	0.18 ± 0.03a	12.06 ± 0.46a	4.66 ± 0.54de	2.63 ± 0.87a	0.05 ± 0.05a	0.24 ± 0.04a	0.74 ± 0.06c
CAM25	0.47 ± 0.11bc	0.69 ± 0.15b	20.31 ± 3.60abcd	1.17 ± 0.22ab	6.65 ± 0.42c	0.52 ± 0.14b	0.63 ± 0.20cd	0.67 ± 0.06c
CAM50	1.05 ± 0.08d	0.45 ± 0.09ab	14.82 ± 1.07abc	1.31 ± 0.17bc	6.47 ± 0.75c	0.39 ± 0.07b	0.67 ± 0.07d	0.53 ± 0.20abc
CAM100	1.29 ± 0.51abcd	0.42 ± 0.06b	11.78 ± 0.56a	0.64 ± 0.11a	5.90 ± 0.92c	0.25 ± 0.08ab	0.59 ± 0.08cd	0.35 ± 0.06a
CRC25	0.19 ± 0.03ab	0.39 ± 0.07ab	18.73 ± 2.20abcd	3.19 ± 0.60cd	4.38 ± 0.71abc	0.66 ± 0.30ab	0.41 ± 0.07abc	0.39 ± 0.04a
CRC50	0.42 ± 0.11bc	0.35 ± 0.04b	15.84 ± 0.58bc	5.46 ± 0.66e	4.34 ± 0.59abc	0.55 ± 0.19ab	0.41 ± 0.04abc	0.76 ± 0.30abc
CRC100	1.25 ± 0.22d	0.44 ± 0.06b	12.36 ± 0.96a	7.23 ± 1.70cde	4.52 ± 1.51abc	0.54 ± 0.14b	0.48 ± 0.03bcd	0.46 ± 0.05ab
CRM25	0.80 ± 0.63abcd	0.35 ± 0.08ab	21.25 ± 1.32d	0.92 ± 0.39ab	5.69 ± 1.16c	0.54 ± 0.37ab	0.55 ± 0.13bcd	0.74 ± 0.19abc
CRM50	2.17 ± 0.75abcd	0.33 ± 0.03b	12.82 ± 1.11ab	0.93 ± 0.09ab	5.20 ± 1.68bc	0.46 ± 0.35ab	0.42 ± 0.11abc	0.26 ± 0.07a
Test	GLS	GLS	GLS	GLS	LM	GLS	LM	GLS
P value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

Table S1. (continued)

	C18:0	C18:1 cis-9	C18:1 trans-9	C18:1 cis-11	C18:2 n-6	C18:2 trans	C18:2 cis-9, trans-12
Diet	**stearic**	**oleic**	**elaidic**	**cis-11 octadecenoic**	**linoleic (LA)**	**octadecenoic trans isomers**	**cis-9, trans-12 octadecadienoic**
CF	6.30 ± 0.99f	20.44 ± 1.98abc	0.16 ± 0.12ab	1.34 ± 0.18cdef	12.67 ± 2.21efg	n.d.	n.d.
CAC25	3.22 ± 0.15bde	23.85 ± 0.79bcde	n.d.	1.51 ± 0.18cdef	12.00 ± 1.43defg	n.d.	n.d.
CAC50	3.84 ± 0.85abcdef	29.10 ± 2.21e	0.24 ± 0.11ab	1.28 ± 0.09cde	13.74 ± 0.87fgh	n.d.	n.d.
CAC100	2.68 ± 0.23abcd	24.42 ± 1.13cde	n.d.	0.74 ± 0.06a	16.44 ± 0.56h	n.d.	n.d.
CAM25	4.86 ± 1.46abcdef	22.73 ± 2.72abcd	0.43 ± 0.28ab	1.56 ± 0.12cdef	10.67 ± 1.37cdef	n.d.	n.d.
CAM50	4.65 ± 0.46ef	24.44 ± 2.16cde	0.07 ± 0.03a	1.18 ± 0.28bc	15.63 ± 0.81gh	n.d.	n.d.
CAM100	3.68 ± 0.62abcdef	18.19 ± 1.70ab	0.24 ± 0.32ab	1.22 ± 0.21cd	9.07 ± 1.50bcde	n.d.	0.09 ± 0.02
CRC25	3.60 ± 1.00abcdef	27.91 ± 4.61de	1.14 ± 0.78ab	1.73 ± 0.10f	7.01 ± 0.88abc	n.d.	n.d.
CRC50	2.64 ± 0.10ac	37.90 ± 3.32f	0.57 ± 0.50ab	1.62 ± 0.07def	6.06 ± 1.23ab	n.d.	n.d.
CRC100	2.07 ± 0.19a	43.91 ± 1.97g	0.18 ± 0.01b	1.66 ± 0.28ef	4.55 ± 0.90a	n.d.	0.08 ± 0.04
CRM25	4.92 ± 0.69cdef	19.29 ± 2.82abc	0.78 ± 0.66ab	1.54 ± 0.11cdef	12.23 ± 2.60defg	0.26 ± 0.13a	n.d.
CRM50	2.43 ± 0.37ab	17.72 ± 0.49a	0.29 ± 0.23ab	0.80 ± 0.06ab	8.87 ± 2.29bcd	0.55 ± 0.12b	n.d.
Test	GLS	LM	GLS	LM	LM	LM	LM
P value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0193	0.8806

Table S1. (continued)

	C18:2 trans-9, cis-12	C18:3 n-3	C18:3 n-6	CLA1 [1]	CLA2 [1]	C18:4 n-3	C19:1 cis-10	C19:1 trans-10
Diet	**trans-9, cis-12 octadecadienoic**	**α-linolenic (ALA)**	**γ-linolenic (GLA)**			**stearidonic**	**cis-10 nonadecenoic**	**trans-10 nonadecenoic**
CF	0.06 ± 0.01a	0.58 ± 0.12a	0.37 ± 0.17c	0.29 ± 0.18a	n.d.	n.d.	0.30 ± 0.04b	n.d.
CAC25	0.07 ± 0.02ab	3.09 ± 0.96ab	0.16 ± 0.07abc	1.59 ± 0.64ab	n.d.	0.19 ± 0.13ab	0.08 ± 0.02a	0.56 ± 0.4
CAC50	0.08 ± 0.03ab	6.01 ± 0.61c	0.08 ± 0.09a	0.81 ± 0.52ab	n.d.	0.16 ± 0.08ab	n.d.	0.28 ± 0.18
CAC100	0.06 ± 0.01a	14.95 ± 0.70d	0.11 ± 0.01ab	0.20 ± 0.19a	n.d.	0.06 ± 0.06a	n.d.	n.d.
CAM25	0.09 ± 0.03ab	0.93 ± 0.38a	0.30 ± 0.13abc	0.91 ± 0.38ab	n.d.	0.29 ± 0.12abc	0.10 ± 0.02a	0.11 ± 0.06
CAM50	1.30 ± 1.39ab	2.16 ± 0.53ab	0.34 ± 0.11bc	0.39 ± 0.14a	n.d.	0.08 ± 0.07ab	n.d.	0.78 ± 0.84
CAM100	n.d.	3.60 ± 0.81bc	0.64 ± 0.15d	n.d.	n.d.	n.d.	n.d.	0.26 ± 0.46
CRC25	0.08 ± 0.01ab	0.63 ± 0.09a	0.07 ± 0.09a	1.96 ± 1.08ab	n.d.	0.29 ± 0.06abc	0.27 ± 0.08ab	0.31 ± 0.18
CRC50	0.13 ± 0.04ab	0.80 ± 0.15a	n.d.	1.60 ± 0.20b	n.d.	0.38 ± 0.10bcd	0.12 ± 0.02a	0.28 ± 0.22
CRC100	0.11 ± 0.01b	1.28 ± 0.58ab	n.d.	0.75 ± 0.09a	0.25 ± 0.19	0.58 ± 0.09cd	0.14 ± 0.03a	0.09 ± 0.02
CRM25	0.17 ± 0.05ab	0.63 ± 0.12a	n.d.	2.95 ± 1.29ab	0.09 ± 0.03	0.67 ± 0.19d	0.18 ± 0.13ab	0.09 ± 0.07
CRM50	0.12 ± 0.09ab	0.45 ± 0.11a	n.d.	1.89 ± 1.23ab	0.08 ± 0.06	0.68 ± 0.26d	0.11 ± 0.04a	0.05 ± 0.06
Test	GLS	GLS	LM	GLS	GLS	LM	GLS	GLS
P value	<0.0001	<0.0001	<0.0001	<0.0001	0.2643	<0.0001	<0.0001	0.0218

[1] CLA1 and CLA2 are conjugated linoleic acid isomers, i.e. cis/trans isomers of Δ9,11-octadecadienoic acid.

Table S1. (continued)

	C20:0	C20:1 cis-11	C20:2 n-6	C20:3 n-6	C20:3 n-3	C20:4 n-6	C20:4 n-3	C20:5 n-3
Diet	**arachidic**	**gondoic**	**cis-11,14 ecosadienoic**	**dihomo-γ-linolenic**	**eicosatrienoic (ETE)**	**arachidonic (ARA)**	**eicosatetraenoic (ETA)**	**eicosapentaenoic (EPA)**
CF	0.18 ± 0.06abcd	0.06 ± 0.02a	n.d.	n.d.	0.24 ± 0.06b	0.13 ± 0.02ab	n.d.	n.d.
CAC25	0.21 ± 0.04abcd	1.78 ± 0.37def	0.16 ± 0.05	n.d.	0.09 ± 0.04a	0.20 ± 0.03ab	n.d.	n.d.
CAC50	0.33 ± 0.08d	2.05 ± 0.24f	0.14 ± 0.03	0.08 ± 0.07	0.08 ± 0.03a	0.21 ± 0.06ab	n.d.	n.d.
CAC100	0.21 ± 0.05abcd	1.57 ± 0.38bcdef	0.17 ± 0.05	n.d.	0.12 ± 0.04a	0.20 ± 0.09ab	n.d.	n.d.
CAM25	0.32 ± 0.05cd	0.20 ± 0.13a	n.d.	n.d.	n.d.	0.45 ± 0.10b	n.d.	n.d.
CAM50	0.31 ± 0.11bcd	0.26 ± 0.10a	0.07 ± 0.07	n.d.	n.d.	0.54 ± 0.21ab	n.d.	n.d.
CAM100	0.16 ± 0.04abc	0.58 ± 0.28abc	0.11 ± 0.03	n.d.	n.d.	0.28 ± 0.15ab	n.d.	n.d.
CRC25	0.22 ± 0.08abcd	0.65 ± 0.10bd	n.d.	n.d.	n.d.	0.12 ± 0.04a	0.08 ± 0.07	n.d.
CRC50	0.32 ± 0.06cd	1.32 ± 0.14ce	n.d.	0.09 ± 0.05	0.12 ± 0.05a	0.17 ± 0.08ab	0.05 ± 0.04	n.d.
CRC100	0.30 ± 0.05bcd	1.53 ± 0.29bcdef	n.d.	0.11 ± 0.03	0.09 ± 0.03a	0.24 ± 0.04ab	n.d.	0.06 ± 0.08
CRM25	0.15 ± 0.07ab	0.12 ± 0.01a	n.d.	n.d.	n.d.	0.36 ± 0.21ab	n.d.	n.d.
CRM50	0.11 ± 0.04a	0.07 ± 0.03a	n.d.	n.d.	n.d.	1.03 ± 0.40ab	n.d.	n.d.
Test	LM	GLS	LM	LM	LM	GLS	LM	
P value	<0.0001	<0.0001	0.0666	0.7342	0.0006	<0.0001	0.487	

Table S1. (continued)

	C21:0	C21:1 cis-12	C22:0	C22:1 cis-13	C22:1 trans-13	C22:2 n-6	C22:5 n-3	C22:5 n-6
Diet	**henicosanoic**	**cis-12 henicosenoic**	**behenic**	**erucic**	**trans-13 docosenoic**	**docosadienoic**	**docosapentaenoic (DPA) n-3**	**docosapentaenoic (DPA) n-6**
CF	n.d.	n.d.	0.09 ± 0.04a	n.d.	n.d.	n.d.	n.d.	0.56 ± 0.19
CAC25	n.d.	0.45 ± 0.22	0.07 ± 0.04a	0.07 ± 0.03a	n.d.	n.d.	n.d.	0.26 ± 0.17
CAC50	n.d.	0.45 ± 0.29	0.15 ± 0.05abc	0.09 ± 0.01a	n.d.	n.d.	n.d.	0.45 ± 0.29
CAC100	n.d.	0.19 ± 0.19	0.10 ± 0.04ab	0.10 ± 0.03a	n.d.	n.d.	n.d.	n.d.
CAM25	n.d.	n.d.	0.23 ± 0.02bc	0.20 ± 0.40ab	n.d.	0.15 ± 0.08	n.d.	0.61 ± 0.28
CAM50	n.d.	n.d.	0.18 ± 0.10abc	0.31 ± 0.36ab	0.07 ± 0.15	n.d.	n.d.	0.21 ± 0.07
CAM100	n.d.	n.d.	0.11 ± 0.04ab	0.50 ± 0.53ab	n.d.	n.d.	n.d.	0.15 ± 0.04
CRC25	n.d.	0.15 ± 0.08	0.11 ± 0.07abc	1.04 ± 0.28b	n.d.	0.10 ± 0.06	0.05 ± 0.04	0.84 ± 0.50
CRC50	0.08 ± 0.08	0.17 ± 0.02	0.23 ± 0.06bc	2.97 ± 0.36c	n.d.	0.07 ± 0.03	n.d.	0.60 ± 0.27
CRC100	1.70 ± 0.92	0.17 ± 0.02	0.26 ± 0.07c	2.81 ± 0.96abc	n.d.	n.d.	n.d.	0.08 ± 0.04
CRM25	0.08 ± 0.05	0.13 ± 0.07	0.07 ± 0.06a	n.d.	n.d.	n.d.	n.d.	0.23 ± 0.20
CRM50	0.07 ± 0.07	0.09 ± 0.05	n.d.	n.d.	n.d.	n.d.	n.d.	0.07 ± 0.10
Test	GLS	GLS	LM	GLS		LM		GLS
P value	0.0315	0.0088	0.0001	<0.0001		0.1968		<0.0001

Table S1. (continued)

Diet	C22:6 n-3 docosahexaenoic (DHA)	C24:0 lignoceric	n-3 PUFA	n-6 PUFA	n-6 / n-3	SFA	MUFA	PUFA
CF	0.12 ± 0.08	n.d.	1.02 ± 0.08a	13.79 ± 2.10def	13.56 ± 1.69e	58.23 ± 5.07fg	26.61 ± 3.38a	15.17 ± 2.23abcd
CAC25	0.08 ± 0.09	n.d.	3.46 ± 0.82abc	12.81 ± 1.26cde	3.79 ± 0.54bc	46.53 ± 1.23cef	35.52 ± 0.91cd	17.95 ± 1.52cd
CAC50	0.15 ± 0.03	n.d.	6.41 ± 0.71d	14.73 ± 0.73ef	2.32 ± 0.28b	35.16 ± 1.46abd	42.80 ± 1.96e	22.04 ± 1.26d
CAC100	0.20 ± 0.12	n.d.	15.37 ± 0.68e	16.97 ± 0.70f	1.11 ± 0.04a	32.20 ± 2.12ab	35.19 ± 1.36bcd	32.61 ± 1.28e
CAM25	0.47 ± 0.36	0.09 ± 0.11	1.73 ± 0.73abc	12.19 ± 1.34cde	7.85 ± 2.97abcde	50.46 ± 4.84defg	34.57 ± 3.36bc	14.98 ± 1.63bc
CAM50	0.37 ± 0.17	0.05 ± 0.06	2.62 ± 0.56abc	16.84 ± 0.62f	6.62 ± 1.33bcd	42.69 ± 1.59ce	35.98 ± 1.48cde	21.33 ± 0.53d
CAM100	0.18 ± 0.11	0.06 ± 0.05	3.85 ± 0.75c	10.25 ± 1.62bcd	2.68 ± 0.13bc	54.40 ± 8.87abcdefg	28.42 ± 2.76ab	17.18 ± 6.76abcde
CRC25	0.09 ± 0.07	n.d.	1.18 ± 0.13ab	8.16 ± 1.24ab	6.91 ± 0.60d	46.61 ± 4.63bcdefg	41.96 ± 4.30de	11.44 ± 1.34ab
CRC50	n.d.	n.d.	1.42 ± 0.11bc	7.01 ± 1.05ab	4.94 ± 0.72cd	33.62 ± 4.97abc	56.18 ± 4.03f	10.20 ± 1.18ab
CRC100	n.d.	n.d.	2.02 ± 0.63abc	5.03 ± 0.89a	2.59 ± 0.50ab	28.39 ± 3.49a	63.37 ± 3.04g	8.24 ± 1.61a
CRM25	0.05 ± 0.11	n.d.	1.38 ± 0.31ab	12.89 ± 2.62cde	9.58 ± 2.10cde	51.93 ± 5.98bcdefg	30.58 ± 2.74abc	17.49 ± 3.87abcd
CRM50	0.18 ± 0.06	n.d.	1.31 ± 0.26ab	9.99 ± 1.99bc	7.87 ± 2.35abcde	59.72 ± 1.95g	26.89 ± 2.03a	13.39 ± 1.94abc
Test	GLS	LM	GLS	LM	GLS	GLS	LM	GLS
P value	0.0527	0.7461	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

Chapter 3

Power of the crowd: substrate-dependent impact of black soldier fly larvae on bacterial community composition in substrate and larval gut

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Submitted.

Abstract

Background: Black soldier fly larvae are used as a sustainable component of animal feed, because they can convert a wide range of organic waste types into insect biomass. In the decomposing substrate, they interact with a rich microbial community of bacteria and fungi, which strongly depends on the type of substrate. These microorganisms may impact larval performance, whereas the larvae themselves can alter substrate properties and bacterial communities – an impact that is enhanced as larvae aggregate. However, the relative importance of substrate type and larval density on bacterial community dynamics is unknown. We investigated four larval densities (0 (control), 50, 100, or 200 larvae per container (520 ml, Ø 75 mm)) and three feed substrates (chicken feed, chicken manure, and camelina oilseed press cake) and sampled bacterial communities of substrates and larvae at three time points over 15 days.

Results: We found that black soldier fly larvae altered bacterial community composition over time in all three feed substrates and that substrate type was the strongest driver of bacterial community composition. The impact of the larvae depended on substrate and larval density, which was possibly related to substrate nutritional value, foraging behaviour, and larval performance. Larval and substrate microbiota differed for chicken manure and camelina, whereas they overlapped in chicken feed.

Conclusion: These findings demonstrate the flexibility of the association between bacteria and black soldier fly larvae and support the substrate-dependent impact of black soldier fly larvae on bacteria both within the larvae and in the substrate. This study indicates that substrate composition and larval density can alter bacterial community composition and possibly be used to improve insect microbiological safety.

Keywords

16S rRNA gene, amplicon sequencing, *Hermetia illucens,* larval density, pH, manure, *Camelina sativa*, microbiota

Background

The saprophagous larvae of the black soldier fly, *Hermetia illucens* L. (Diptera: Stratiomyidae; BSF), are promising agents in the management of organic waste and its conversion into insect biomass for animal feed (Barragán-Fonseca *et al.* 2017). In most bioconversion systems, the larvae interact with a rich microbial community of bacteria (Jeon *et al.* 2011), fungi (Boccazzi *et al.* 2017), viruses (Chen *et al.* 2019), and possibly archaea and protists (Gurung *et al.* 2019). Bacteria produce volatiles that provide information on resource quality for ovipositing adult flies (Zheng *et al.* 2013b), they can increase egg emergence rates (Yang *et al.* 2018), and increase larval performance (Skaro 2018; Somroo *et al.* 2019; Xiao *et al.* 2018a; Yu *et al.* 2011).

At the same time, the BSF larvae impact substrate bacterial communities and physicochemical properties through digestion, immune defence, and larval aggregation, similar to several other detritivorous fly species (Gold *et al.* 2018). The larval gut poses a strong selection pressure on ingested bacteria, due to the production of a range of lysozymes and antimicrobial peptides (Vogel *et al.* 2018) and a drastic pH gradient going from pH 6 to pH 2 to pH 8 in respectively anterior, middle and posterior midgut (Bonelli *et al.* 2019). The majority of ingested bacteria are thus digested, and only a subset survives and may reproduce in the gut (Bruno *et al.* 2019b). Moreover, the foraging of BSF larvae in an aggregation, or maggot mass, changes substrate pH to 8-9 regardless of the initial pH (Ma *et al.* 2018; Meneguz *et al.* 2018), decreases manure moisture content and emission of microbial volatiles such as indole (Beskin *et al.* 2018), and reduces populations of *Escherichia coli* and *Salmonella* spp. (Erickson *et al.* 2004; Lalander *et al.* 2013; Liu *et al.* 2008). This impact of larvae on the substrate likely increases with larval age and size and is therefore time dependent (Wynants *et al.* 2019).

Although BSF larvae can impact the substrate and its microbiota, their role in shaping the composition of the bacterial community, relative to the type of feed substrate itself remains to be investigated. Elucidating the effect of BSF larvae on substrate microbiota is important in the waste management industry, for instance to determine whether human and animal pathogens and food spoilage bacteria can be controlled by manipulating the larval density in the system and the timing of harvest (De Smet *et al.* 2018; EFSA 2015). On the one hand, larval gut microbiota can significantly differ among larvae fed different substrates (Jeon *et al.* 2011; Zhan *et al.* 2020), and the gut

microbiota reflects a shrinking subset of substrate-associated bacteria as digestion progresses through the midgut (Bruno *et al.* 2019b). On the other hand, the microbiota of larvae can be very different to that of their feed substrates (Wynants *et al.* 2019), while bacterial taxa can be shared across larvae from different substrates (Jeon *et al.* 2011; Wynants *et al.* 2019; Zhan *et al.* 2020) and between different life stages (Zheng *et al.* 2013a). This suggests the existence of a BSF core microbiota (De Smet *et al.* 2018; Wynants *et al.* 2019; Zhan *et al.* 2020).

We aimed to elucidate the relative importance of larval density and substrate type in structuring bacterial community composition in substrate and larval gut. We tested four different larval densities (0 (control), 50, 100, or 200 larvae per container (520 ml, Ø 75 mm)) on three feed substrates (chicken feed, chicken manure, and camelina oilseed press cake) and sampled bacterial communities of substrates and larvae over time (day 0, *i.e.* the start of the experiment, day 5, 10 and 15) (Figure 1).

Results

Larval performance

Larvae performed differently on the three substrates, and individual larval weight decreased with higher larval density in two substrates (Figure 2). Survival rate differed among substrates, but not among larval densities (GLS, main effect substrate: $p < 0.001$; main effect density: $p = 0.296$; Figure 2a). More larvae survived on camelina substrate (88 – 92%) than on the other two substrates (chicken feed 60 – 66%, chicken manure 69 – 84%). In chicken feed, larvae were significantly more advanced into the prepupal stage (86 – 98%) than larvae fed camelina substrate (0 – 2%) or chicken manure (9 – 16%) (GLS, main effect substrate: $p < 0.001$; Figure 2b). Larval density negatively affected individual larval weight in chicken manure and chicken feed (GLM, main effect substrate: $p < 0.001$; main effect density: $p < 0.001$; Figure 2c). In chicken manure, individual larval weight differed between all larval densities (50 larvae per container: 0.070 g DM, 100 larvae per container: 0.044 g DM, 200 larvae per container: 0.024 g DM), whereas in chicken feed the larvae at the highest density (200 per container: 0.055 g DM) were smaller than at the lowest density (50 per container: 0.081 g DM).

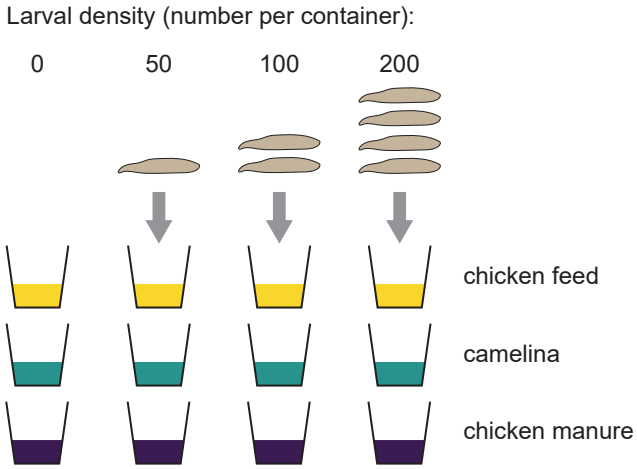

Figure 1. Experimental design of the study. We tested the effect of larval density (0, 50, 100, 200 larvae per container) on substrate and larval microbiota in three feed substrates (chicken feed, camelina, chicken manure).

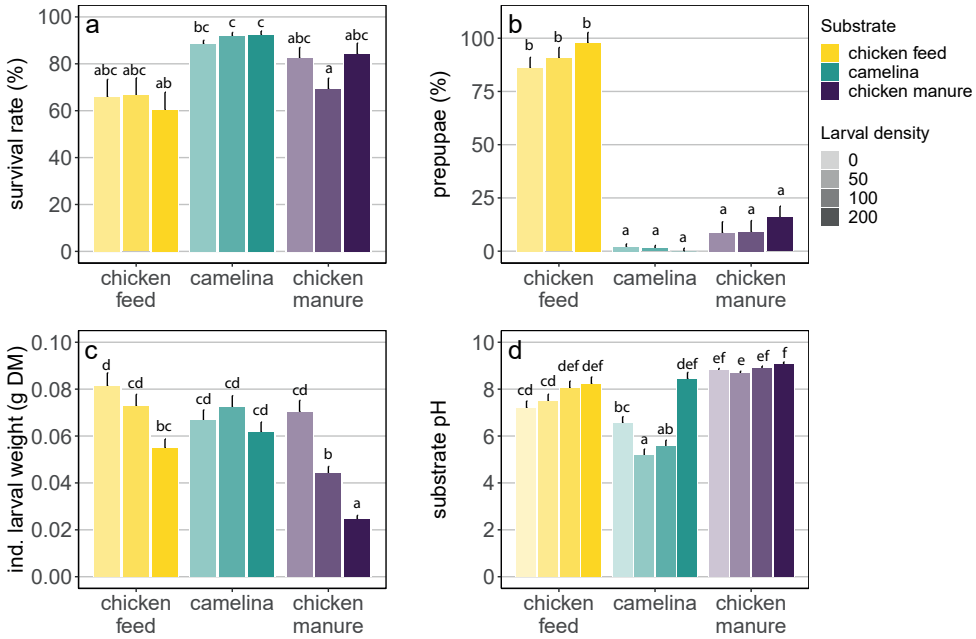

Figure 2. Larval performance parameters and substrate pH, on day 15 (estimated marginal means + SE, n = 4): (a) survival rate (%), (b) percentage of prepupae (%), (c) individual larval weight (g dry matter), and (d) substrate pH. Means with no shared letters are significantly different (α = 0.05, Tukey post-hoc comparisons on EMM from GLS (survival, prepupae), LMM (pH) or Gamma GLM (individual larval weight) regression).

Substrate pH

pH of freshly prepared substrates differed (LMM, $p = 0.002$; camelina pH 5.4, chicken feed 6.3, and chicken manure 7.7). Substrate pH on day 15 of the experiment showed differences among substrates too, and an effect of larval density in camelina substrate and chicken manure (LMM, main effect substrate: $p < 0.001$; main effect density: $p < 0.001$; interaction substrate x density: $p < 0.001$; Figure 2d). The pH of chicken manure was 8.7 – 9.1, and substrate pH at larval density of 50 larvae per container was lower (8.7) than at 200 larvae per container (9.1). Chicken feed pH ranged between 7.2 – 8.5, and camelina substrate between 5.2 – 8.4. In camelina, pH was lowest at larval densities of 50 and 100 larvae per container (5.2 and 5.5, respectively), intermediate in substrates without larvae (6.6) and highest in substrates with 200 larvae per container (8.4).

16S rRNA gene amplicon sequencing quality control

Bacterial community composition was assessed through PCR amplification and sequencing of the V5-V6 variable region of the 16S rRNA gene. This resulted in 68 million reads (after removal of mitochondrial and chloroplast DNA, which also resulted in removal of the day-0 substrate samples of camelina). No-template PCR controls contained 3,433 – 11,485 reads per sample, belonging to 28 genera (Table S1). 26 amplicon sequence variants (ASVs) were identified as contaminants and removed from the dataset (Table S2), mainly concerning known lab contaminants (Salter *et al.* 2014). Further analyses were performed on relative abundance data at genus level. Positive controls, *i.e.* synthetic mock communities of known composition (two different controls: mock 3 and 4) (Ramiro-Garcia *et al.* 2016), showed high correlation with the theoretical mock community composition (mock 3: Spearman correlation, $r = 0.78 – 0.87$; mock 4: $r = 0.68 – 0.77$) and between replicates of different sequencing libraries (mock 3: $r = 0.92 – 0.99$; mock 4: $r = 0.95 – 0.99$). Technical replicates of DNA isolation of substrate microbiota were highly correlated (Spearman $r = 0.73 – 0.94$), with a few exceptions ($r = 0.38$, 0.47, and 0.60, for chicken feed with 200 larvae per container on day 5, chicken feed without larvae on day 15, and camelina substrate without larvae on day 5, respectively), as were PCR replicates across sequencing libraries (Spearman $r = 0.88 – 0.98$).

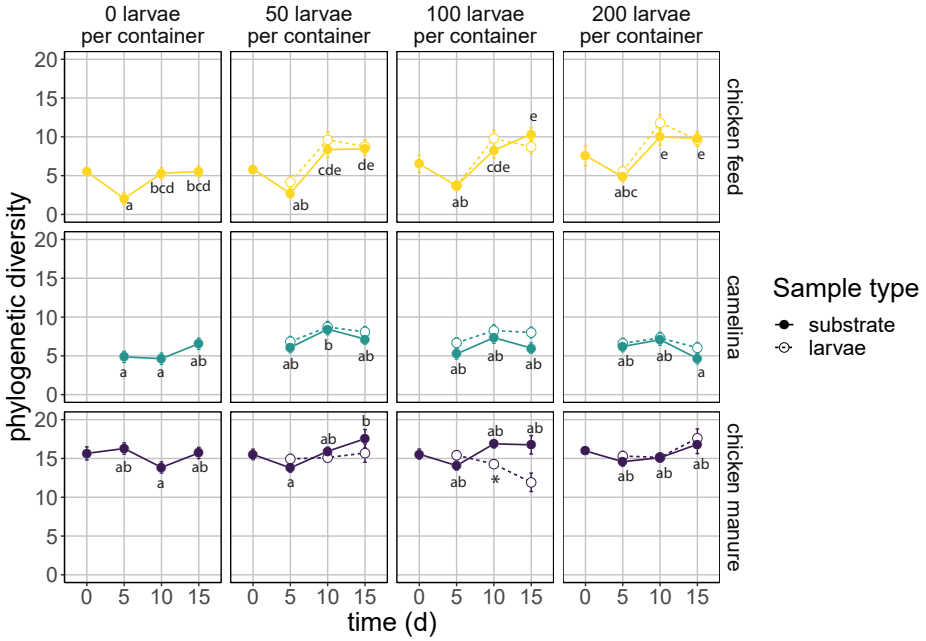

Figure 3. Faith's phylogenetic diversity (estimated marginal mean ± SE, n = 4) of larval and substrate microbiota over time, in chicken feed (top row), camelina (middle), and chicken manure (bottom), separated by larval density. Within a diet, means of substrate microbiota diversity with no shared letters are significantly different (α = 0.05, Tukey post-hoc comparisons on EMM from LMM regression on substrate samples, excluding day 0); means of larval microbiota diversity with an asterisk are significantly different from the corresponding substrate microbiota (α = 0.05, Tukey post-hoc comparisons on EMM from LMM regression on substrate and larval samples, excluding samples of day 0 or 0 larvae per container).

Phylogenetic diversity of substrate and larval microbiota

Alpha diversity was measured using Faith's phylogenetic diversity. The substrate microbiota (excluding day 0) of chicken manure was more diverse than that of the other substrates (LMM, main effect substrate: $p < 0.001$; Figure 3, Table S3). Over time, diversity did not change in camelina or chicken manure substrates. However, in chicken feed substrates, diversity increased from day 5 to 15 (main effect time: $p < 0.001$; interaction substrate x time: $p < 0.001$). Moreover, in chicken feed substrates with 100 or 200 larvae per container, diversity was higher than in substrate without larvae (main effect density: $p < 0.001$; interaction substrate x density: $p = 0.004$; interaction density x time: $p = 0.001$).

Larval and substrate bacterial diversity differed only in chicken manure with 100 larvae per container on day 10 (Figure 3, Table S4).

Effects of substrate and larval density on substrate microbiota

Bacterial community composition of substrates differed among substrates and over time (weighted UniFrac NMDS, Figure 4a), with Firmicutes, Proteobacteria, Actinobacteria and Bacteroidetes as the most predominant phyla (Figure S1). The substrate x density x time model explained 75% of the total microbiota variation (Table S5). Substrate and time explained 56% of microbiota variation. Microbiota of all three substrates differed from each other. Chicken manure substrate microbiota was most distinct from the other two substrates (dbRDA pairwise contrasts, $p = 0.001$; Figure 4a). Substrates of chicken manure contained twelve abundant genera that were absent in the other substrates, including *Petrimonas*, *Gallicola*, *Koukoulia*, *Aerosphaera*, and an unassigned genus of Clostridiales family XI (Figure 5). Chicken manure also lacked eight genera that were abundant in the other substrates, including *Klebsiella*, *Weissella*, *Serratia*, *Pediococcus*, and *Lachnoclostridium_5* (Figure 5).

Time explained most variation in substrate microbiota in each substrate, followed by larval density (Table S6). Within each substrate, there were differences among larval densities in substrate bacterial community composition (Figure 4b-d). In chicken feed, substrate microbiota with 200 larvae per container differed significantly from those without or with 50 larvae per container on day 15 (Figures 4b and S2a). Relative to the control without larvae on day 15, camelina substrate microbiota with 50 or 100 larvae per container differed from substrate microbiota with 200 larvae per container (Figures 4c and S2b). In chicken manure, substrate microbiota with 100 or 200 larvae per container started to differ on day 15 or 10, respectively, from substrate microbiota without or with 50 larvae (Figures 4d and S2c). This indicated that the change in chicken manure substrate microbiota happened at a faster rate with higher larval densities.

In all substrates, substrate microbiota at the lower larval densities were more associated with lactic acid producing bacteria (*Lactobacillus*, *Pediococcus*) (Figure S2). Apart from these shared patterns, each substrate had different genera associated with substrates of increasing larval densities (Figure S2): *Providencia* and *Proteus* in camelina substrate, *Petrimonas* and *Corynebacterium_1* in chicken manure, and an unassigned genus of Planococcaceae in chicken feed.

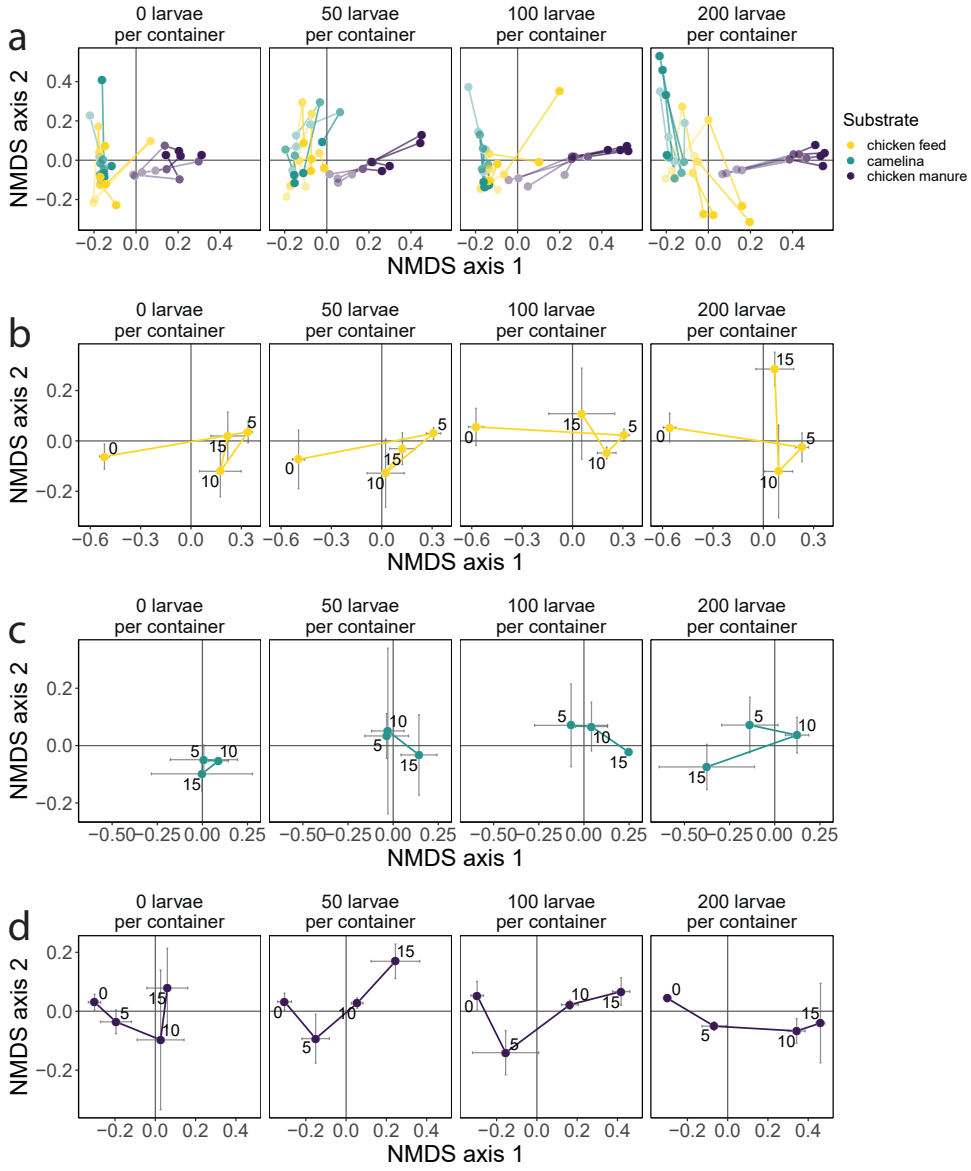

Figure 4. Microbiota composition of substrates (NMDS of weighted UniFrac distances): (a) all three feed substrates combined, (b) chicken feed, (c) camelina, (d) chicken manure. Plots show microbiota variation along the 1st and 2nd NMDS axes. Samples of day 0 are excluded from (a), because data were lacking for camelina diet. In (a), individual replicates (containers) are plotted, with timepoints (days) displayed as a transparency gradient (day 5 most transparent, day 15 non-transparent), and the timepoints of each container are connected by lines from day 5 to 15. In (b-d), timepoints are labels in the plot. (a-d) are separate NMDS ordinations, *i.e.* in (b-d) the NMDS is done only on samples of the respective feed substrate. Each row is one ordination split into four panels for visibility, corresponding with the four larval densities (0, 50, 100, or 200 larvae per container). Error bars in (b-d) are mean ± SD of axis scores (n = 4). Stress of NMDS solutions: a = 0.116, b = 0.070, c = 0.100, d = 0.073.

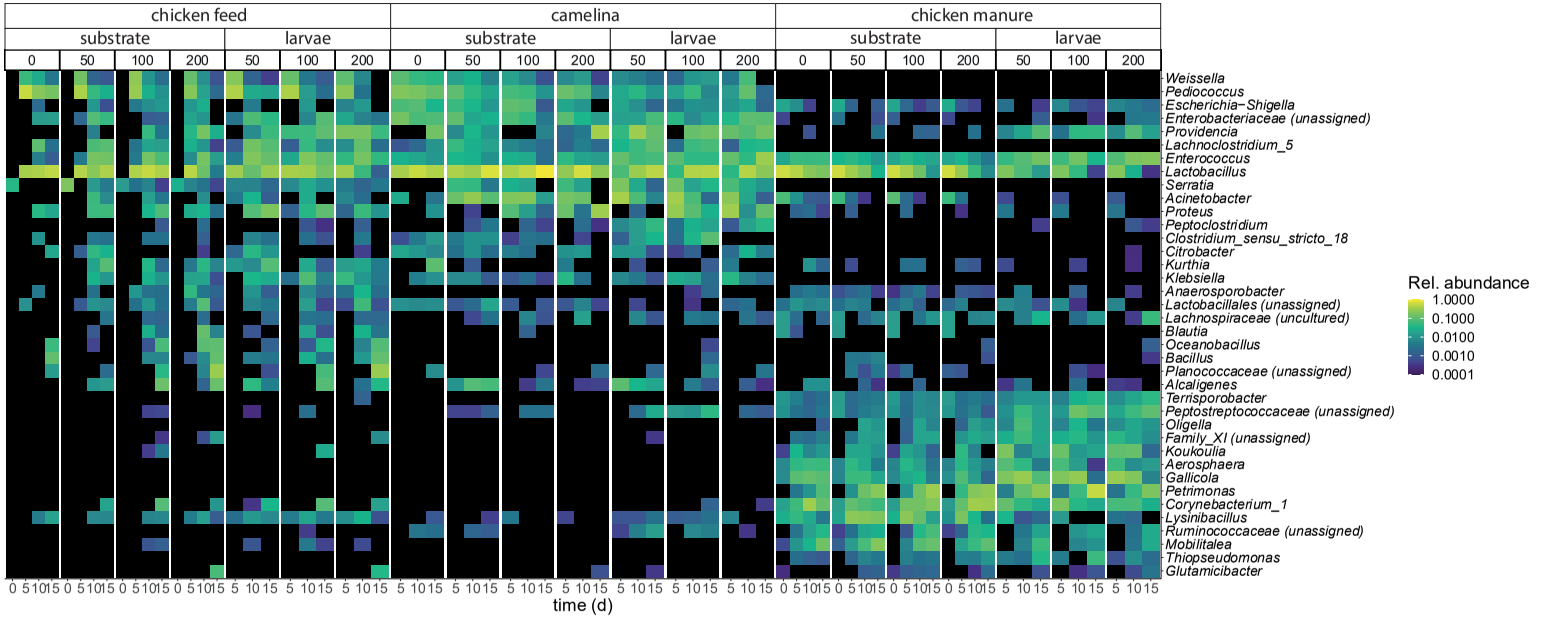

Figure 5. Heatmap of the most abundant bacterial genera over time (genera with a maximum relative abundance > 10% and present in > 10% of samples). Colour scale displays the median relative abundance (n = 4). The plot is divided into panels for feed substrate (chicken feed, camelina, chicken manure), sample type (substrate or larvae) and larval density (0, 50, 100 or 200 larvae per container), see top of plot.

Effects of substrate and larval density on similarity of larval and substrate microbiota

The similarity between larval and substrate microbiota depended on the substrate (Tables S7-S8, Figure 6). Larvae and substrate microbiota in chicken feed overlapped and changed in a similar way over time (Figure 6b), and although sample type (*i.e.* larvae vs substrate) had a significant effect, it only explained 3% of microbiota variation (Table S8). Across larval densities, larvae tended to be more associated with *Providencia*, whereas substrates tended to be more associated with *Lactobacillus* (Figures 5 and S3).

In camelina, microbiota of larvae and substrates developed differently over time (Figure 6c), and sample type and time together explained 31% of total microbiota variation (Table S8). Larval and substrate microbiota differed in composition at a larval density of 100 larvae per container on day 15, but they overlapped in the other densities (Figure 6c). At 100 larvae per container, larvae were associated with *Providencia* and *Proteus*, whereas substrates were more associated with *Lactobacillus* (Figures 5 and S3).

In chicken manure, larval and substrate microbiota were clearly separated at all densities (Figure 6d). In this substrate, sample type and time together significantly explained 58% of total variation (Table S8). Across larval densities, larvae were more associated with *Providencia*, an unassigned genus of Peptostreptococcaceae, *Gallicola*, *Enterococcus*, and *Koukoulia*, whereas substrates were more associated with *Lactobacillus*, *Mobilitalea*, *Lysinibacillus*, *Corynebacterium_1*, and *Petrimonas* (Figure S3).

Discussion

Bacterial community composition in substrates was mainly driven by the type of feed substrate and the density of BSF larvae. In addition, larvae and substrates differed in their microbiota composition, depending on the feed substrate and larval density.

Substrate-dependent microbiota

Chicken manure microbiota differed considerably from microbiota of chicken feed and camelina substrate (Figures 4a and 5, Table S6). These differences are likely related to nutrient composition (Cammack *et al.* 2018; Martin Jr *et al.* 1983; Schreven *et al.* 2021 (**Chapter 2**)) and substrate origin (De Smet *et al.* 2018). Chicken manure was

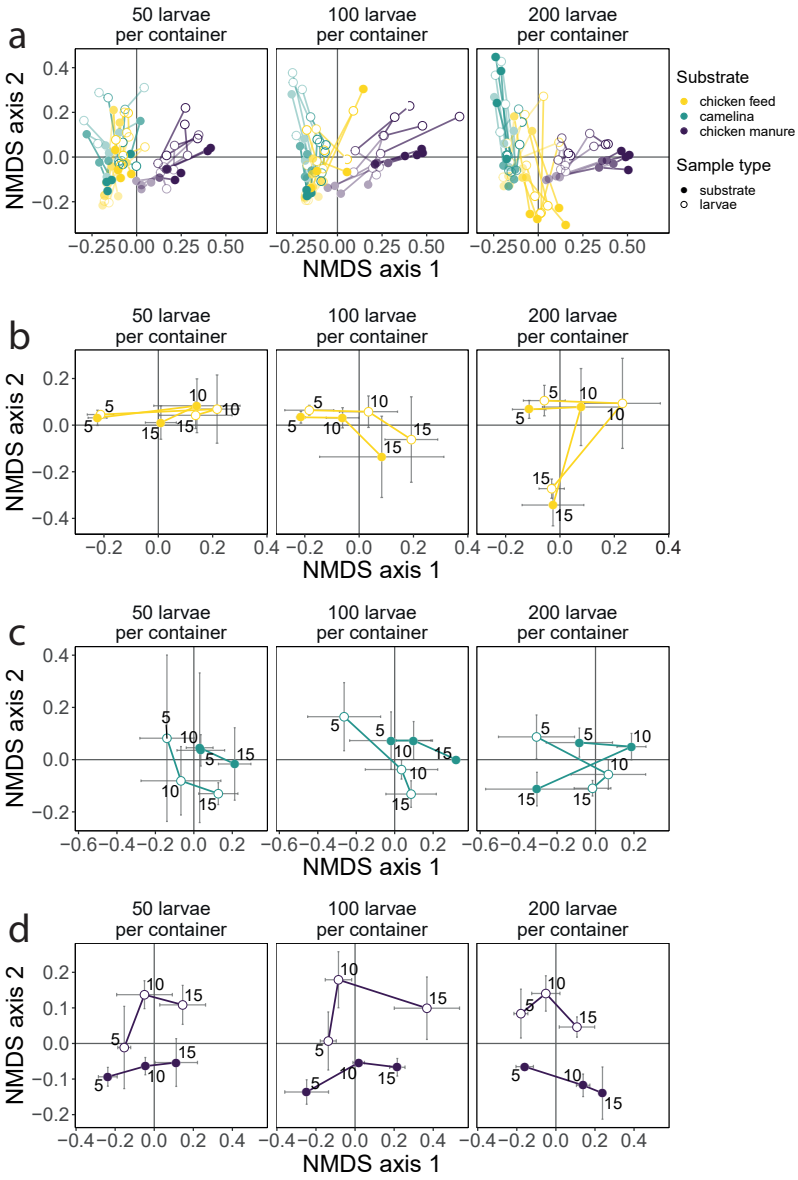

Figure 6. Microbiota composition of larvae and substrates (NMDS of weighted UniFrac distances): (a) all three feed substrates combined, (b) chicken feed, (c) camelina, (d) chicken manure. Plots show microbiota variation along the 1st and 2nd NMDS axes. Substrate samples of day 0 or 0 larvae per container were excluded. In (a), individual replicates (containers) are plotted, with timepoints (days) displayed as a transparency gradient (day 5 most transparent, day 15 non-transparent), and the timepoints of each container are connected by lines from day 5 to 15. In (b-d), timepoints are labels in the plot. (a-d) are separate NMDS ordinations, *i.e.* in (b-d) the NMDS is done only on samples of the respective substrate. Each row is one ordination split into three panels for visibility, corresponding with three larval densities (50, 100, or 200 larvae per container). Error bars in (b-d) are mean ± SD of axis scores (n = 4). Stress of NMDS solutions: a = 0.131, b = 0.125, c = 0.123, d = 0.142.

dominated by *Firmicutes* in accordance with previous studies, but the relative abundance of other phyla and their dynamics over time differed with other studies (Figure S1; Wadud *et al.* 2012; Wynants *et al.* 2019; Zhang *et al.* 2018). Apart from the effects of BSF larvae, the scale of the compost system and concomitant differences in *e.g.* moisture content (Wadud *et al.* 2012), temperature (Zhang *et al.* 2018) and the absence/presence of antibiotics, may underlie the variation in chicken manure microbiota among studies.

Fresh chicken feed (day 0) in our study was rich in *Curtobacterium* and *Pantoea*. Five days later, however, substrate bacterial communities had changed drastically, and were dominated by the lactic acid producing bacteria *Pediococcus*, *Lactobacillus* and *Weissella* (Figure 5). This latter group of bacteria may initially have been present below the detection threshold but outcompeted the former group as substrate characteristics (*e.g.* moisture content) changed.

Camelina substrate shared many bacterial genera with chicken feed, but these genera differed in relative abundance between the two substrates (Figure 5). The overlap in genera is most likely the result of camelina substrate containing 50 % DM chicken feed. The differences in relative abundances may be caused by nutrient composition and moisture content (Cammack *et al.* 2018), crop-associated bacteria (Vorholt 2012), and isothiocyanates from the camelina press cake (Amyot *et al.* 2019). Isothiocyanates are derivatives of secondary plant compounds (glucosinolates) of crucifer crops, that have antimicrobial effects (Dufour *et al.* 2015).

The impact of larvae on substrate microbiota

Larval density significantly altered bacterial community composition in all three feed substrates (Figures 4, 5, and S2). BSF larvae changed the relative abundance of some of the most abundant bacterial genera in all three substrates (Figures 5 and S2). With increasing larval density, *Lactobacillus* decreased across substrates, whereas different genera increased depending on the type of feed substrate. These changes can be caused by larval foraging in maggot masses. Maggot mass foraging generally impacts the substrate by increasing the local peak temperature, aeration, and pH, and decreasing moisture content (Beskin *et al.* 2018; Jiang *et al.* 2019; Ma *et al.* 2018; Meneguz *et al.* 2018; Putman 1978). In addition, it increases decomposition rate (Jiang *et al.* 2019) and alters microbial metabolism and resultant volatile emissions (Beskin *et al.* 2018).

We also found that larvae at a density of 100 – 200 larvae per container significantly increased bacterial phylogenetic diversity in chicken feed substrate on day 15, compared to substrates without larvae, and that larvae did not affect phylogenetic diversity in the other substrates (Figure 3, Table S3). This nuances the assertion in Gold *et al.* (Gold *et al.* 2018) that BSF larvae, like other fly species, would decrease bacterial diversity.

BSF larvae alter bacterial community composition of substrates by introducing gut-associated bacteria (Jiang *et al.* 2019), and/or changing population sizes of resident bacteria in the substrate (Erickson *et al.* 2004; Lalander *et al.* 2013; Liu *et al.* 2008). Gut-associated bacteria can make up 66% of substrate microbiota after two days of larval feeding (starting with 5-day-old larvae), before gradually decreasing to 13% on the tenth day (Jiang *et al.* 2019). Larvae of 10-15 days old decreased *Salmonella* and *E. coli* populations ($\log_{10}$ CFU g^{-1}) in contaminated manure after three or more days of feeding, compared to a control without larvae (Erickson *et al.* 2004; Lalander *et al.* 2013; Liu *et al.* 2008). In our study, larvae significantly altered the substrate microbiota on day 10-15, depending on the substrate. Administering the substrate at intervals, *i.e.* adding fresh substrate every few days, instead of as bulk from the start as applied here, may offset such larval impact on substrate microbiota (*e.g.* Bruno *et al.* 2019b).

Larval impact on bacterial community composition was different in each substrate, possibly related to larval performance, foraging behaviour and substrate nutrient composition and concentration. Larval performance (survival, development, and weight) differed significantly among substrates, and larval weight decreased at higher larval density in chicken feed and chicken manure (Figure 2a-c). Lower larval weight at higher larval density may indicate food shortage (Figure 2c), which can drastically impact BSF larval gut microbiota (Yang *et al.* 2018) and may have effects on larval foraging behaviour and substrate microbiota as well. Substrate nutritional quality, moisture content and initial pH influence larval performance (Cammack & Tomberlin 2017; Cheng *et al.* 2017; Ma *et al.* 2018; Meneguz *et al.* 2018). Additionally, the initial microbiota of the substrate may affect larval performance (De Smet *et al.* 2018), and the substrate may alter larval immune response and larval digestion (Pimentel *et al.* 2017; Vogel *et al.* 2018). This may cause the substrate-dependent impact of larvae on substrate microbiota composition.

Resident microbiota of the three substrates may have been differentially altered due to larval foraging behaviour. In chicken feed, the larvae were significantly advanced in development compared to the other substrates, with 86 – 98% having reached the prepupal stage on day 15 (Figure 2b). Once prepupae, larvae cease feeding and start wandering (Barros *et al.* 2019; Schremmer 1986), and likely impact the substrate and its microbiota in a different way than the penultimate larval instar.

The high pH of all substrates on day 15 (Figure 2d) likely resulted from proteolysis and accumulation of ammonia (Green & Popa 2012). The lower pH in substrates with 50 or 100 larvae per container in camelina substrate may relate to the increase in *Lactobacillus* in this substrate over time, as opposed to the other substrates (Figure 5). Furthermore, most larvae in camelina substrate initially foraged at the surface in biofilms of *Acinetobacter*, *Serratia* and *Comamonas* (Figure S4) and moved deeper into the substrate only after 5-7 days. This seemed to happen more extensively at higher larval density, and may have led to increased aeration, a shift to aerobic microbial metabolism (Putman 1978) and consequently reduced lactic acid fermentation, that only at the highest larval density impacted substrate pH. Indeed, the substrate microbiota at 200 larvae per container differed significantly from those at 50 or 100 larvae per container (Figure S2b).

Substrate-dependent and density-dependent differences between larval and substrate microbiota

Although larval and substrate microbiota mostly changed in a similar way over time, they differed significantly depending on the substrate and larval density (Figures 6 and S3). Firmicutes and Proteobacteria dominated the larval microbiota, along with Bacteroidetes and Actinobacteria in larvae fed chicken manure (Figure S1), similar to previous studies (Bruno *et al.* 2019b; Jeon *et al.* 2011; Zhan *et al.* 2020; Zheng *et al.* 2013a).

Despite the large impact of the substrate on larval gut microbiota (Bruno *et al.* 2019b; Jeon *et al.* 2011; Klammsteiner *et al.* 2020; Zhan *et al.* 2020), larval and substrate microbiota can differ (Shelomi *et al.* 2020; Wynants *et al.* 2019). In our study, larval and substrate microbiota composition significantly differed in chicken manure and camelina substrate (the latter at 100 larvae per container, on day 15) but overlapped in chicken feed (Figures 6b-d and S3, Table S8). This may be because the type of substrate influences larval

immune response and digestive function (Bonelli *et al.* 2020; Bonelli *et al.* 2019; Vogel *et al.* 2018; Zhan *et al.* 2020), resulting in a substrate-dependent selection pressure of larvae on ingested and resident gut bacteria (Bruno *et al.* 2019b). Chicken manure and camelina substrate may have triggered a more complex and stronger larval immune response compared to chicken feed (Vogel *et al.* 2018), since chicken manure has a high bacterial load and camelina substrate is rich in protein (32% DM) and camelina seed oil (8% DM; Schreven *et al.* 2021 (**Chapter 2**)). In all three substrates, *Providencia* was more associated with larvae (Figure S3). In addition, *Proteus* was associated with larvae fed camelina and *Gallicola* and *Enterococcus* with larvae fed chicken manure. Besides, *Providencia*, *Lactobacillus* and *Enterococcus* persisted in larvae across substrates and time (Figure 5). These genera may confer benefits to host functioning and survival. *Providencia* may be transmitted vertically from adult females to eggs (Zheng *et al.* 2013a), and a strain of this genus has been isolated from eggs of our BSF colony, along with a strain of *Lysinibacillus* (Schreven *et al.*, unpublished). Several egg-associated bacteria, *e.g. Enterococcus faecalis* and *Lysinibacillus boronitolerans*, increase BSF egg hatching rate, larval growth, and/or adult female fecundity (Mazza *et al.* 2020; Xiao *et al.* 2018a; Yang *et al.* 2018; Yang *et al.* 2017; Yu *et al.* 2011; Zheng *et al.* 2013b).

Some studies identify the shared bacterial taxa across substrates as a core microbiota of BSF larvae (Jeon *et al.* 2011; Klammsteiner *et al.* 2020; Wynants *et al.* 2019; Zhan *et al.* 2020), *i.e.* a taxonomic core microbiome (see **Glossary**). Although *Providencia* and *Enterococcus* have been identified as core taxa of BSF larvae in other studies, there is considerable variation in the identified core among studies (Jeon *et al.* 2011; Wynants *et al.* 2019). For instance, *Dysgonomonas*, *Parabacteroides*, *Pseudomonas* and *Morganella* have been reported, but the former three were rarely present whereas *Morganella* was absent from our study (Jeon *et al.* 2011; Jiang *et al.* 2019; Shelomi *et al.* 2020; Wynants *et al.* 2019). This variability may be due to rearing facility and host genotype but may also be because the identified core in some studies may include microbiota of a nursery substrate (Khamis *et al.* 2020; Wynants *et al.* 2019). In our study we found different taxa enriched in larvae depending on the substrate (Figure S3). The core microbiome of BSF larvae may be defined by critical functions (*i.e.* the functional core microbiome (see **Glossary**)) rather than specific taxa, and BSF larvae may be able to select for those (Vogel *et al.* 2018). Identifying these critical gut microbiome functions that complement host function, may result in an understanding of BSF microbial ecology that is more applicable to the edible insect industry.

Conclusion

BSF larvae altered bacterial community composition in all three substrates. However, this effect was different in each substrate and also dependent on larval densities. Causal factors involved are likely substrate nutritional value, larval foraging behaviour, and larval performance. Remarkably, larval and substrate microbiota were distinct in chicken manure and camelina diet, whereas they overlapped in chicken feed. These findings highlight the flexibility of association between bacteria and BSF larvae and support the concept of substrate-dependent selection of bacteria by BSF larvae. For the edible insect industry, our study indicates that substrate composition and larval density can alter microbial community composition and possibly improve insect microbial safety.

Methods

Insects and substrates

Insects originated from the Black Soldier Fly colony of the Laboratory of Entomology, Wageningen University, The Netherlands. The colony has been established with source material from the USA around 2008 and has since been reared at 27±2 °C, 70±10 % relative humidity and photoperiod L14:D10. The larvae were reared on chicken feed (Kuikenopfokmeel 1, Kasper Faunafoods BV, The Netherlands). Eggs < 24 h old were collected in cardboard strips deployed in the adult cage, incubated under the same abiotic conditions and neonate larvae were transferred to treatment feed substrates within 24 h after hatching.

Three experimental substrates were used. Chicken feed was the same as the colony substrate (Kuikenopfokmeel 1, Kasper Faunafoods BV); organic chicken manure free of antibiotics and pesticides was collected freshly from a belt system of layer hens at Carus experimental farm (Wageningen University), and used in the experiment on the same day. The Camelina press cake substrate was a 1:1 mixture on dry matter (DM) basis of chicken feed : *Camelina sativa* press cake. The press cake was produced from mechanical pressing of seeds (produced without application of insecticides) from the 2015 harvest of the University of Warmia and Mazury in Olsztyn, Poland. Substrates were prepared with 36.0 g DM of feed and 67.1% moisture (chicken feed) or 78.6% moisture (camelina substrate, controlled for higher water retention capacity than chicken feed), or 46.6 g DM feed and 75.4% moisture (fresh chicken manure, no water added).

Experimental design

To determine the influence of larval density (0, 50, 100 or 200 neonate larvae per container) on bacterial community dynamics in three different substrates (chicken feed, camelina press cake 50% and chicken manure) over the course of 15 days, we used four replicates per treatment, divided into two batches of two replicates each, started on consecutive days. The experiment was conducted in a climate chamber at 27 ± 2 °C, 70 ± 10% relative humidity and photoperiod L14:D10, from 15 June to 1 July 2017. Every day, the position of each container was randomly changed to control for any abiotic gradient in the climate chamber. Containers were Superfos™ UniPak™ 5012 polypropylene transparent containers of 520 ml volume, bottom diameter 75 mm, top diameter 95 mm (RPC Superfos, Taastrup, Denmark), with a mesh lid (mesh area 60 mm diameter, ~ 0.5 mm mesh size). Containers were disinfected with 70% ethanol prior to use.

Performance parameters and substrate pH

On day 15, the experiment was terminated and larvae were harvested. Larvae were counted to determine survival rate, rinsed with lukewarm tap water, gently dried using paper tissues, weighed as fresh biomass yield (Ohaus Adventurer Pro AV313, d = 0.001 g, Ohaus Corp. USA) and then frozen at -21 °C. The proportion of prepupae was determined using the degree of dark pigmentation of the cuticle. A subsample of ten larvae from each container was dried in a stove at 70 °C until stable weight (Mettler-Toledo ML54/01), to determine dry matter content and dry larval biomass. Substrates were stored at -21 °C, and subsamples of 2 – 7 g fresh matter (FM) were oven-dried at 70 °C until stable weight (Mettler-Toledo ML54/01). Larval survival rate was calculated as the number of living larvae at time of harvest divided by the number of larvae on day 0, minus 9 (*i.e.* the number of larvae collected for analysis, three larvae on three time points).

Substrate pH of samples on day 15 was measured in a suspension of approximately 1 g FM of harvested substrate (weighed at 0.0001 g precision, Mettler Toledo ML54/01) in 10 mL Milli-Q® water (Merck KGaA, Darmstadt, Germany). Within an hour, the pH was measured using a pH meter (ProLine B210, ProSense B.V., The Netherlands). After the experiment, the pH of newly prepared substrates of chicken feed and camelina

press cake, and of the fresh chicken manure batches (four batches, one per replicate) of which reference material had been stored at -21 °C, was measured in triplicate using the same method.

Molecular sample processing

DNA sample collection

For DNA isolation, substrate samples were collected on days 0, 5, 10 and 15; larval samples on day 5, 10 and 15. On day 0, substrate samples were collected 1 h after distribution of substrate into the containers and addition of water to the substrate, and prior to the addition of larvae. Substrate samples were taken by removing the top layer (top 1 – 5 mm) of the substrate and then taking a sample of the full depth of the substrate using a sterile plastic straw (7 mm diameter). This sample was then placed in a 1.5-mL tube and mixed thoroughly for 30 s using a small spatula. For each larval sample, three larvae were surface-sterilised using the following rinsing protocol in Petri dishes: Milli-Q® water (30 s), 70 % ethanol (30 s), 1 % Halamid®-D (chloramine-T, 20 s), and 2x 10 s in Milli-Q® water. The three larvae were then placed in a 1.5-mL tube. Substrate and larval samples were snap-frozen in liquid nitrogen, then stored at -80 °C.

Sample homogenization and DNA isolation

The following methods for cell lysis, repeated bead-beating and subsequent DNA extraction were adapted from Salonen *et al.* (2010) and Van Lingen *et al.* (2017).

Larvae samples – Samples were homogenized in 300 µL buffer for Stool Transport and Recovery (STAR, Roche) in sterile 2.0 mL screw-cap tubes with 0.25 g of 0.1 mm zirconia beads and 3 glass beads (2.5 mm). Small larvae were homogenized per pool of three larvae using a bead beater (Precellys 24, Bertin Technologies, France) at room temperature at 5.5 m s^{-1} thrice for 1 min with 20 s intervals, incubated in a shaker at 95 °C and 300 rpm for 15 min, and centrifuged at 4 °C and 16,100 x *g* for 5 min. Big larvae harvested on day 15 were homogenized individually, and prior to homogenization these frozen larvae were cut with a disinfected spatula behind the mesothoracal segment and before the second-last abdominal segment, to facilitate tissue destruction. The supernatant was transferred to a new 2.0 mL tube, and steps were repeated with 200 µL

STAR buffer to yield a total of approximately 500 µL supernatant. From this, 250 µL was transferred to a cartridge of a customized DNA isolation kit (Maxwell 16 Tissue LEV Total RNA Purification Kit, cat. no. XAS1220, Promega Corporation, USA), and DNA was isolated and eluted in 30 µL nuclease-free water using the Maxwell MDx robot (Promega Corporation, USA). The three supernatants of larvae from a single sample (container) were pooled in the cartridge using 83 µL of each supernatant (total 250 µL).

Substrate samples – Samples were homogenized in 700 µL buffer for Stool Transport and Recovery (STAR, Roche) in sterile 2.0 mL screw-cap tubes 0.5 g of 0.1 mm zirconia beads and five glass beads (2.5 mm). The samples (0.25 g) were then homogenized in a bead beater at room temperature at 5.5 m s^{-1} thrice for 1 min with 20 s intervals, then incubated in a shaker at 95 °C and 300 rpm, centrifuged at 4 °C and 16,100 x *g* for 5 min. The supernatant was transferred to a new 2.0 mL tube, and steps were repeated with 300 µL STAR buffer to yield a total of approximately 1 mL supernatant. DNA isolation was the same as described for larval samples (250 µL supernatant per cartridge).

Microbiota profiling

DNA concentrations were measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc, Wilmington, DE, USA) and samples were diluted to 20 ng DNA μL^{-1} prior to PCR. The V5-V6 region of the 16S rRNA gene was amplified using barcoded primers F784-1064R (Ramiro-Garcia *et al.* 2016) according to the following PCR program: 98 °C for 30 s, 25 cycles of 98 °C 10 s, 42 °C 10 s, 72 °C 10 s, and 72 °C for 7 min. Per reaction, the following 50 µL mix was prepared: 36.5 µL nuclease-free water, 10 µL 5x HF buffer (Thermo Fisher Scientific, USA), 1 µL dNTPs (10 mM), 0.5 µL Phusion™ Hot Start II DNA polymerase (2 U μL^{-1}) (Thermo Fisher Scientific, USA), 1 µL barcoded primers (10 µM) and 1 µL DNA template. Samples were amplified in duplicate. As positive controls we used synthetic Mock communities of known composition and comprising full length 16S rRNA gene amplicons of bacterial phylotypes associated with the human gut (Ramiro-Garcia *et al.* 2016). As negative control, no-template blanks (1 µL nuclease-free water as template) were included in the PCR. Products were checked for yield and correct size by agarose gel electrophoresis, and PCR amplification was repeated for samples with no or low yield with 5 µL DNA template. PCR products were purified using the CleanPCR magnetic bead suspension

(CleanNA, The Netherlands), 1.8x the volume of the PCR mix (duplicates combined), two washes with 200 µL 70 % ethanol, and eluted in 30 µL nuclease-free water. Purified DNA concentrations were measured using the Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific, USA) and pooled in equimolar concentrations per library of 70 samples (randomly assigned to each library), concentrated using magnetic beads and re-eluted in 20 µL nuclease-free water. Final DNA concentration per library was measured in Qubit, after which the libraries were sent to GATC Biotech AG (Konstanz, Germany; now part of Eurofins Genomics Germany GmbH) for 2 x 150 bp sequencing on an Illumina HiSeq4000 instrument.

Statistical analyses

All analyses were performed in R statistical software version 3.5.0 (R Core Team 2018).

Larval performance

Survival rate and percentage of prepupae were analysed using generalized least squares (GLS), and individual larval weight was analysed using generalized linear model regression (GLM), using the gls (nlme; Pinheiro *et al.* 2018) and glm function, respectively. Prior linear mixed model regression (LMM) showed that a random term for batch (*i.e.* batch 1 or 2) was not needed in any performance parameter, based on AIC (Sakamoto *et al.* 1986; Zuur *et al.* 2009). In GLS, we selected a variance structure based on AIC; a GLM with Gamma distribution was used if residuals violated GLS assumptions. The full model (substrate x density) was used as fixed term. Post-hoc comparisons were based on estimated marginal means (EMM) with Tukey-adjusted p-values (emmeans package; Lenth 2020).

Substrate pH

Initial substrate pH was compared between substrates using a LMM with a random intercept for batch and an AIC-selected variance structure. Substrate pH on day 15 was analysed with LMM because a mixed model with random term for batch fitted best based on AIC. Post-hoc comparisons for both initial and final substrate pH were based on EMM with Tukey-adjusted p-values.

Microbiota analysis

Raw amplicon sequence data were analysed using the NG-Tax pipeline with default settings (Ramiro-Garcia *et al.* 2016). In short, paired-end libraries were demultiplexed using read pairs with perfectly matching barcodes. Amplicon sequence variants (ASV) were picked as following: sequences were ordered by abundance per sample and reads were considered valid when their cumulative abundance was ≥ 0.1%. Taxonomy was assigned using the SILVA 128 database version 128 (Quast *et al.* 2013). ASVs are defined as individual sequence variants rather than a cluster of sequence variants with a shared similarity above a specified threshold such as Operational Taxonomic Units. Data were analysed using the phyloseq v1.24.2 (McMurdie & Holmes 2013) and microbiome v1.2.1 packages (Lahti & Shetty 2017). Chloroplast and mitochondrial 16S rRNA sequences were removed prior to analysis.

Contaminant ASVs were identified based on visual inspection of correlation plots between DNA concentration and the relative abundance of each ASV and these ASVs were removed from the dataset prior to further analysis. Data quality was assessed by comparing the composition of the sequenced positive controls to the known composition (Ramiro-Garcia *et al.* 2016) using Spearman's rank correlation. Reproducibility was assessed by Spearman's rank correlation of technical replicates (duplicate substrate samples within each substrate of one container with 0 or 200 larvae per container on day 5 and 15, and PCR duplicates (one substrate and two larval samples) across sequence libraries).

In the alpha (within sample) diversity and beta (between sample) diversity analysis, we tested models separately on data of substrates including density 0 larvae per container, and on data of larvae and substrates excluding this density and day 0 samples. This was done because parameter estimation and multivariate permutation tests required balanced datasets. Data were not normalized to equal sequencing depth because for data processed in NG-Tax diversity does not depend on sequencing depth (Muller *et al.* 2020).

Alpha diversity of microbiota at genus level was calculated as Faith's phylogenetic diversity, using the picante package (Faith 1992; Kembel *et al.* 2010), and tested for significance of treatment effects using LMM with variance structure after AIC-based model selection.

Beta diversity at genus level was visualised using non-metric multidimensional scaling (NMDS, minimum 100 iterations; Kruskal 1964), based on weighted UniFrac distances (Luzopone & Knight 2005). The relative importance of substrate, larval density, time and sample type in explaining microbiota composition, was determined by distance-based redundancy analysis (dbRDA) directly decomposing the weighted UniFrac distances (dbrda function of vegan package v2.5-6; McArdle & Anderson 2001; Oksanen *et al.* 2019; Shankar *et al.* 2017). We compared the full model (substrate x density x timepoint x sample type) with a null model and the significance of main effects and interaction terms was tested, using a permutational multivariate analysis of variance (999 permutations) stratified for container ID to account for repeated measures (anova.cca function of vegan package; Legendre *et al.* 2011).

To assess the effect of larval density on substrate microbiota within each substrate over time, we performed weighted UniFrac distance-based Principal Response Curves (dbPRC), with the control without larvae as a baseline (function prc in the vegan package) (Shankar *et al.* 2017; Van den Brink & Ter Braak 1999). Within each timepoint, we tested the effect of larval density on substrate microbiota composition, and pairwise compared the axis scores of the first principal coordinate between larval densities (analysis of variance with Tukey contrasts). The same analysis was done within each substrate x density combination, to assess the difference between larval and substrate microbiota over time, with substrate as a baseline.

Availability of data and material

The sequence datasets generated and analysed during the current study have been deposited in the European Nucleotide Archive (ENA) repository under the study accession number PRJEB40667 (https://www.ebi.ac.uk/ena/browser/view/PRJEB40667). The larval performance data, pH data, metadata of 16S rRNA samples, and R script used for analysing the datasets are available at the 4TU.ResearchData repository under DOI 10.4121/13118291.

List of abbreviations

AIC: Akaike's information criterion; ASV: amplicon sequence variant; BSF: black soldier fly; dbPRC: distance-based principal response curves; dbRDA: distance-based redundancy analysis; EMM: estimated marginal means; GLM: generalized linear model regression; GLS: generalized least-squares regression; LMM: linear mixed model regression; NMDS: non-metric multidimensional scaling; NTC: no-template control; OTU: operational taxonomic unit; PERMANOVA: permutational multivariate analysis of variance; rRNA: ribosomal ribonucleic acid.

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Author contributions

JvL acquired funding for the study. SS, HdV, GZ, HS, and JvL were involved in the experimental design. SS and GZ conducted the insect experiment, sample collection, and data collection. SS, GZ, and HdV processed samples for DNA isolation and PCR. SS analysed the data statistically with extensive help of HdV and GH, and all authors were involved in the data interpretation. SS wrote the first version of the manuscript and processed the revisions from all authors. All authors read and approved the final manuscript.

Supplementary information

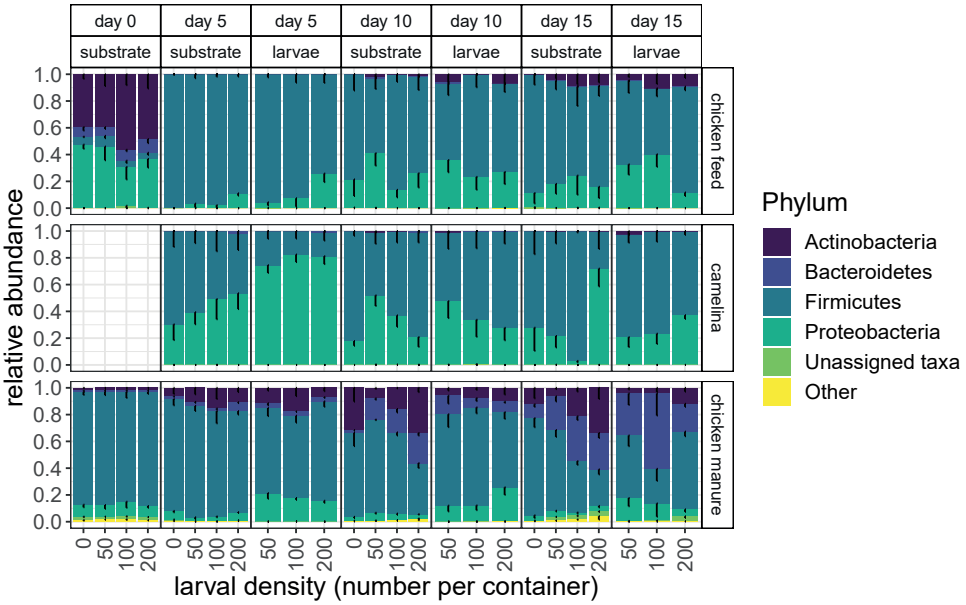

Figure S1. Relative abundance of the five most abundant bacterial phyla in substrate and larval microbiota (mean - SE, n = 4).

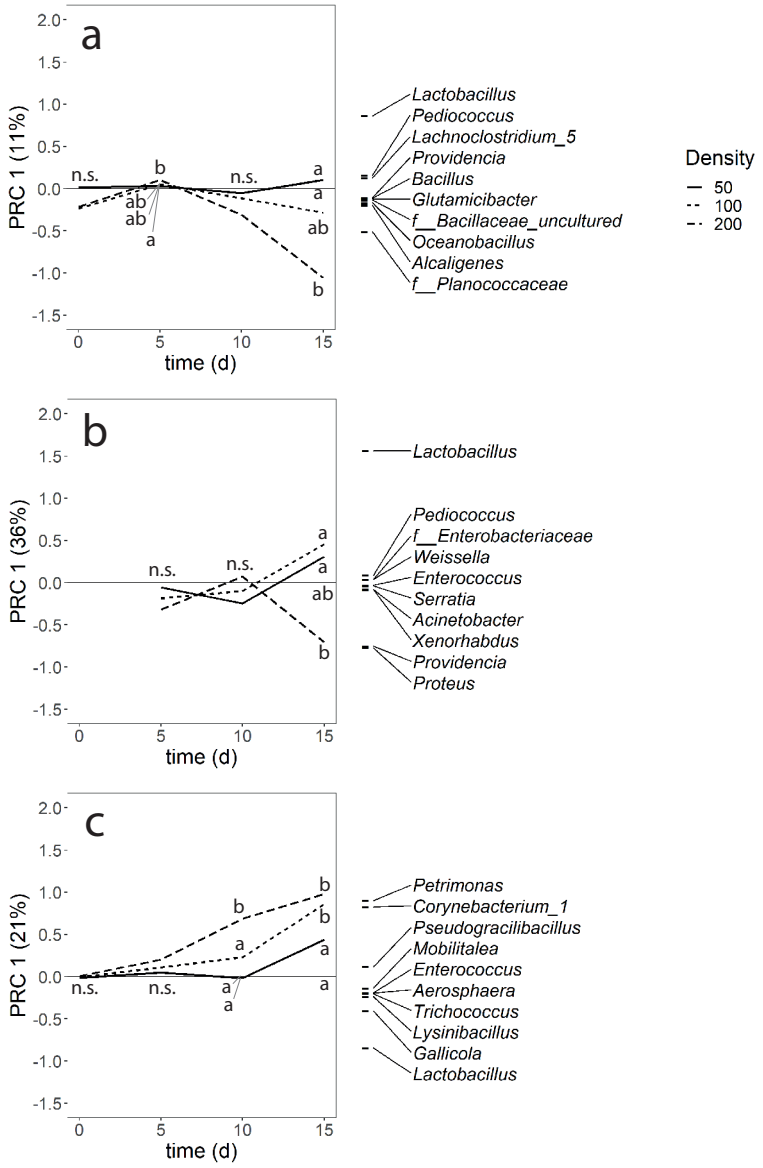

Figure S2. Weighted UniFrac distance-based Principal Response Curves (dbPRC) for substrate microbiota of different larval densities per feed substrate: (a) chicken feed, (b) camelina, (c) chicken manure. Baseline is the control treatment without larvae. The y-axis represents the microbiota variation along the first PRC axis, in parentheses the percentage the axis (and larval density) explained from the total microbiota variation. Points that share no letters are significantly different on that day ($\alpha = 0.05$, Tukey contrasts between densities, on 1st axis scores per timepoint). At the right margin of each plot, the genera with the top 10 absolute scores along the 1st PRC axis are displayed.

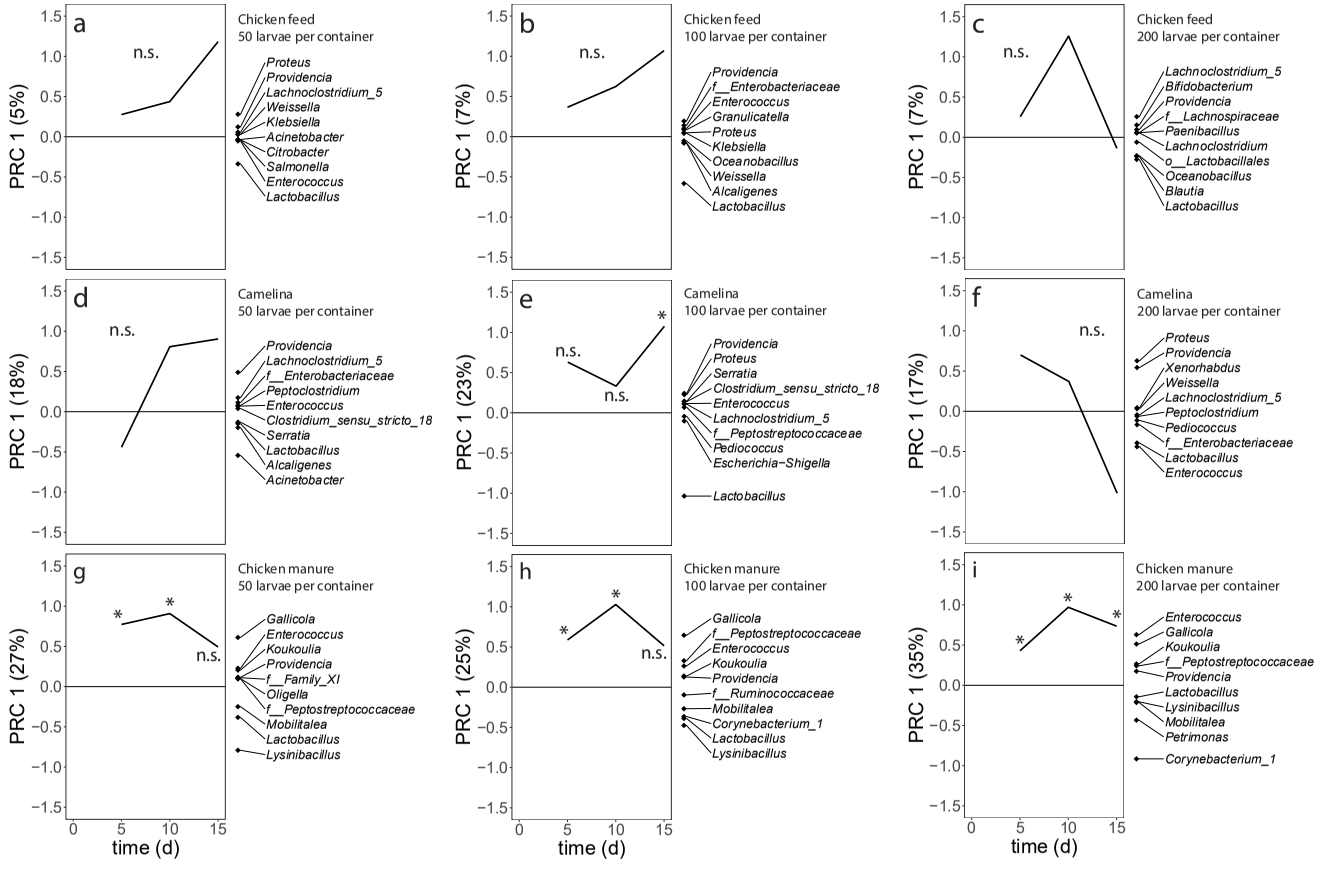

Figure S3. Weighted UniFrac distance-based Principal Response Curves (dbPRC) for larval and substrate microbiota per feed substrate and per larval density: (a-c) chicken feed, (d-f) camelina, and (g-i) chicken manure. Larval densities: (a, d, g): 50 larvae per container; (b, e, h): 100 larvae per container; (c, f, i): 200 larvae per container. Baseline is the substrate microbiota. The y-axis represents the microbiota variation along the first PRC axis, in parentheses the percentage the axis (and sample type) explained from the total microbiota variation. Points with an asterisk indicate significant difference between larval and substrate microbiota on that day ($\alpha = 0.05$, PERMANOVA test of dbPRC for sample type). At the right margin of each plot, the genera with the top 10 absolute scores along the 1st PRC axis are displayed.

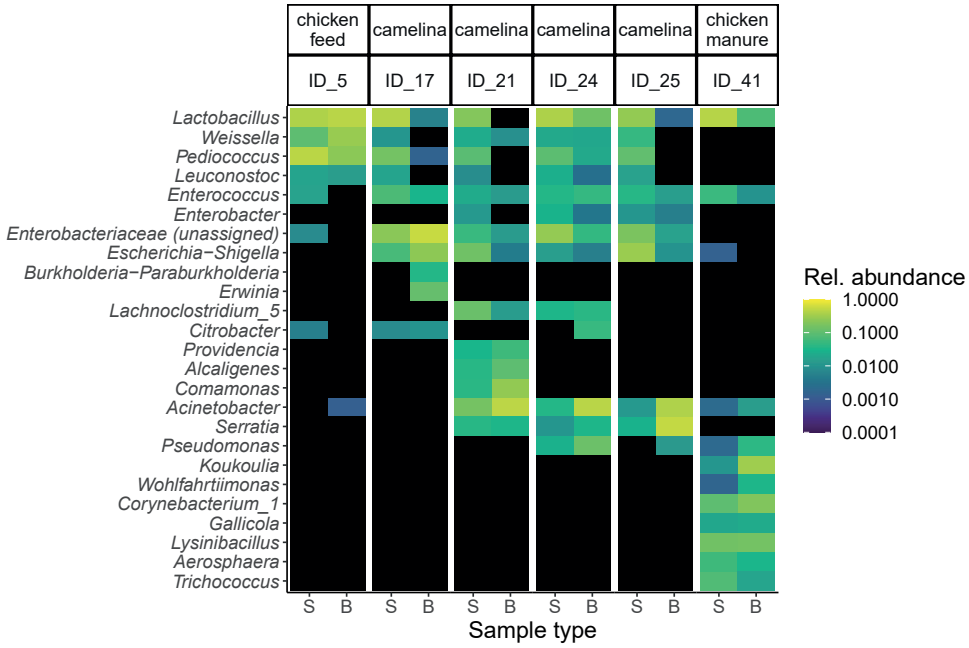

Figure S4. Heatmap of relative abundance of the 25 most abundant bacterial genera in substrates (sample type "S") and biofilms (sample type "B") on day 5. ID = container ID.

Table S1. Relative abundance of bacterial genera detected in NTCs of PCR, ranked by relative abundance. Each NTC is from a different sequencing library.

Genus	NTC 1	NTC 2	NTC 3	NTC 4	NTC 5
Nesterenkonia	0.276	0.326	0.430	0.420	0.357
Caldalkalibacillus	0.126	0.113	0.348	0.310	0.329
Ralstonia	0.191	0.130	0.055	0.081	0.069
Halomonas	0.132	0.133	0.039	0.056	0.079
Bacillus	0.075	0.061	0.052	0.045	0.060
Bacillaceae (unassigned)	0.073	0.058	0.037	0.031	0.032
Shewanella	0.035	0.067	0.021	0.031	0.033
Halomonadaceae (unassigned)	0.025	0.040	0.012	0.020	0.021
Xanthomonadaceae (unassigned)	0.028	0.029	0	0	0.004
Cupriavidus	0.012	0.013	0.004	0.002	0.009
Achromobacter	0.005	0.004	0	0.002	0.002
Dietzia	0.005	0.005	0	0	0
Geobacillus	0.002	0.007	0	0	0
Georgenia	0.003	0.003	0	0	0
Unassigned taxa	0	0	0.002	0	0.003
Delftia	0	0.002	0	0.001	0.001
Rhodococcus	0.002	0.002	0	0	0
Sphingomonas	0.002	0.002	0	0	0
Aerococcus	0.003	0	0	0	0
Mycobacterium	0.003	0	0	0	0
Lactobacillus	0	0	0	0	0.002
Dermacoccus	0	0.001	0	0	0
Staphylococcus	0	0.001	0	0	0
Rubrobacter	0.001	0	0	0	0
Tetragenococcus	0	0.001	0	0	0
Comamonadaceae (unassigned)	0.001	0	0	0	0
Glutamicibacter	0.001	0	0	0	0
Brevundimonas	0	0.001	0	0	0

Table S2. Total relative abundance (of all reads in dataset) of ASVs identified as contaminants. Contaminant identification based on assessment of correlation plots between ASV relative abundance and DNA concentration of samples.

ASV code	Genus	number of reads	% of total reads
301590670	Bacillaceae (unassigned)	34651	0.0511%
301590674	*Bacillus*	17336	0.0256%
301590679	*Bacillus*	10775	0.0159%
3015901111	*Bacillus*	1730	0.0026%
301590601	*Caldalkalibacillus*	88233	0.1302%
301590672	*Caldalkalibacillus*	34727	0.0512%
3015901123	*Caldalkalibacillus*	1624	0.0024%
3015901572	*Caldalkalibacillus*	7893	0.0116%
3015901605	*Caldalkalibacillus*	638	0.0009%
3015901109	*Cupriavidus*	4138	0.0061%
3015901814	*Delftia*	146	0.0002%
3015901112	*Dietzia*	1530	0.0023%
3015901113	*Georgenia*	751	0.0011%
301590676	Halomonadaceae (unassigned)	17890	0.0264%
301590660	*Halomonas*	43649	0.0644%
301590669	*Halomonas*	29361	0.0433%
3015901115	*Halomonas*	1106	0.0016%
301590591	*Nesterenkonia*	240340	0.3546%
3015901108	*Nesterenkonia*	16887	0.0249%
3015901126	*Nesterenkonia*	795	0.0012%
3015901798	*Nesterenkonia*	474	0.0007%
301590589	*Ralstonia*	123086	0.1816%
301590677	*Shewanella*	17149	0.0253%
301590741	*Shewanella*	7292	0.0108%
3015901110	*Shewanella*	2096	0.0031%
301590675	Xanthomonadaceae (unassigned)	11494	0.0170%
Total		**715791**	**1.0561%**

Table S3. ANOVA output of LMM of Faith's phylogenetic diversity of substrate microbiota (excluding samples of day 0).

Model term	df_{num}	df_{den}	F-value	p-value
(Intercept)	1	72	4853.11	< 0.001
Substrate	2	36	510.57	< 0.001
Density	3	36	8.10	< 0.001
Timepoint	2	72	53.26	< 0.001
Substrate × Density	6	36	3.96	0.004
Substrate × Timepoint	4	72	17.12	< 0.001
Density × Timepoint	6	72	4.10	0.001
Substrate × Density × Timepoint	12	72	1.83	0.059

Table S4. ANOVA output of LMM of Faith's phylogenetic diversity of larval and substrate microbiota (excluding samples of day 0 or 0 larvae per container).

Model term	df_{num}	df_{den}	F-value	p-value
(Intercept)	1	135	5367.63	< 0.001
Substrate	2	27	488.22	< 0.001
Density	2	27	0.21	0.812
Timepoint	2	135	76.28	< 0.001
Sample type	1	135	4.25	0.041
Substrate × Density	4	27	3.10	0.032
Substrate × Timepoint	4	135	37.63	< 0.001
Density × Timepoint	4	135	1.74	0.144
Substrate × Sample type	2	135	5.39	0.006
Density × Sample type	2	135	1.29	0.280
Timepoint × Sample type	2	135	4.76	0.010
Substrate × Density × Timepoint	8	135	1.76	0.091
Substrate × Density × Sample type	4	135	2.06	0.089
Substrate × Timepoint × Sample type	4	135	4.17	0.003
Density × Timepoint × Sample type	4	135	1.06	0.381
Substrate × Density × Timepoint × Sample type	8	135	0.82	0.590

Table S5. Substrate microbiota variation of the three feed substates combined, partitioned by model terms (PERMANOVA of the weighted UniFrac distances). This analysis excludes substrate samples of day 0. R^2 = 75% (*i.e.* % of total microbiota variation explained by model Substrate × Density × Timepoint). Permutation test against null model (999 permutations): χ^2 = 16.64, F = 9.23, p = 0.001. The percentage of microbiota variation explained by each model term, is calculated as the relative sum of squares (sum of squares divided by total sum of squares).

Model term	df	SSq	F	p	%
Substrate	2	8.49	82.35	0.001	38.2%
Density	3	0.99	6.37	0.003	4.4%
Timepoint	2	1.44	13.97	0.001	6.5%
Substrate × Density	6	1.16	3.75	0.016	5.2%
Substrate × Timepoint	4	2.45	11.87	0.001	11.0%
Density × Timepoint	6	0.64	2.07	0.001	2.9%
Substrate × Density × Timepoint	12	1.48	2.40	0.001	6.7%
Residual	108	5.56	NA	NA	

Table S6. Substrate microbiota variation within each feed substrate, partitioned by model terms (PERMANOVA of the weighted UniFrac distances). This analysis includes substrate samples of day 0 for chicken feed and chicken manure. R^2 is the percentage of total microbiota variation explained by the model Density × Timepoint. The percentage of microbiota variation explained by each model term, is calculated as the relative sum of squares (sum of squares divided by total sum of squares).

	Chicken feed					Camelina					Chicken manure				
R^2	79%					48%					84%				
Test against null model	χ^2	10.01				χ^2	2.20				χ^2	5.73			
	F	11.75				F	3.07				F	16.46			
	p	0.001				p	0.001				p	0.001			
Model term	**df**	**SSq**	**F**	**p**	**%**	**df**	**SSq**	**F**	**p**	**%**	**df**	**SSq**	**F**	**p**	**%**
Density	3	0.57	3.33	0.001	4.5%	3	0.67	3.45	0.002	14.9%	3	0.70	10.16	0.001	10.3%
Timepoint	3	8.57	50.31	0.001	67.3%	2	0.55	4.19	0.001	12.0%	3	4.22	61.69	0.001	62.8%
Density × Timepoint	9	0.87	1.70	0.147	6.8%	6	0.98	2.50	0.003	21.5%	9	0.71	3.48	0.001	10.6%
Residual	48	2.73	NA	NA		36	2.35	NA	NA		48	1.10	NA	NA	

Table S7. Larval and substrate microbiota variation of the three substrates partitioned by model terms (PERMANOVA of the weighted UniFrac distances). This analysis excludes substrate samples of day 0 or 0 larvae per container. R^2 = 75% (*i.e.* % of total microbiota variation explained by the model Substrate × Density × Timepoint × Sample type). Permutation test against null model (999 permutations): χ^2 = 27.83, F = 8.98, p = 0.001. The percentage of microbiota variation explained by each model term, is calculated as the relative sum of squares (sum of squares divided by total sum of squares).

Model term	df	SSq	F	p	%
Substrate	2	12.84	109.74	0.001	34.4%
Density	2	0.48	4.06	0.187	1.3%
Timepoint	2	2.19	18.74	0.001	5.9%
Sample type	1	1.12	19.10	0.001	3.0%
Substrate × Density	4	0.88	3.76	0.220	2.4%
Substrate × Timepoint	4	4.86	20.79	0.001	13.0%
Density × Timepoint	4	0.64	2.72	0.001	1.7%
Substrate × Sample type	2	0.75	6.42	0.001	2.0%
Density × Sample type	2	0.20	1.69	0.004	0.5%
Timepoint × Sample type	2	0.47	3.98	0.001	1.2%
Substrate × Density × Timepoint	8	1.67	3.57	0.001	4.5%
Substrate × Density × Sample type	4	0.24	1.03	0.048	0.6%
Substrate × Timepoint × Sample type	4	0.82	3.50	0.001	2.2%
Density × Timepoint × Sample type	4	0.28	1.19	0.007	0.7%
Substrate × Density × Timepoint × Sample type	8	0.40	0.86	0.058	1.1%
Residual	162	9.48	NA	NA	

Table S8. Larval and substrate microbiota variation within each substrate, partitioned by model terms (PERMANOVA of the weighted UniFrac distances). This analysis excludes substrate samples of day 0 or 0 larvae per container. R^2 is the percentage of total microbiota variation explained by the model Density × Timepoint × Sample type. The percentage of microbiota variation explained by each model term, is calculated as the relative sum of squares (sum of squares divided by total sum of squares).

	Chicken feed					Camelina					Chicken manure				
R^2	58%					52%					73%				
Test against null model	χ^2	4.61				χ^2	4.29				χ^2	6.10			
	F	4.45				F	3.44				F	8.70			
	p	0.001				p	0.001				p	0.001			
Model term	**df**	**SSq**	**F**	**p**	**%**	**df**	**SSq**	**F**	**p**	**%**	**df**	**SSq**	**F**	**p**	**%**
Density	2	0.53	4.32	0.001	6.7%	2	0.54	3.70	0.006	6.6%	2	0.29	3.46	0.001	3.4%
Timepoint	2	2.50	20.56	0.001	31.7%	2	1.51	10.30	0.001	18.3%	2	3.04	36.92	0.001	36.6%
Sample type	1	0.21	3.48	0.003	2.7%	1	0.48	6.52	0.001	5.8%	1	1.18	28.60	0.001	14.2%
Density × Timepoint	4	1.11	4.57	0.001	14.1%	4	0.74	2.51	0.001	8.9%	4	0.46	2.77	0.010	5.5%
Density × Sample type	2	0.03	0.27	0.965	0.4%	2	0.12	0.83	0.472	1.5%	2	0.28	3.43	0.003	3.4%
Timepoint × Sample type	2	0.12	1.02	0.305	1.6%	2	0.54	3.69	0.003	6.6%	2	0.62	7.48	0.001	7.4%
Density × Timepoint × Sample type	4	0.10	0.40	0.968	1.2%	4	0.35	1.20	0.192	4.3%	4	0.23	1.42	0.177	2.8%
Residual	54	3.29	NA	NA		54	3.97	NA	NA		54	2.22	NA	NA	

Chapter 4

Relative contributions of egg-associated and substrate-associated microorganisms to black soldier fly larval performance and microbiota

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Abstract

Larvae of the black soldier fly (BSF) can be used to convert organic waste into insect biomass for animal feed. In this process, they interact with microorganisms originating from the substrate, the insect, and the environment. The substrate is the main determinant of the larval gut microbiota composition, but inoculation of the substrate with egg-associated bacteria can improve larval performance. We aimed to quantify the relative importance of substrate-associated and egg-associated microorganisms on BSF larval performance, bacterial abundance, and microbiota composition, when larvae were fed with chicken feed or chicken manure. For this we inactivated substrate-associated microorganisms by autoclaving, or disinfected BSF eggs. Larval survival rate, weight, and proportion of prepupae were determined on day 15. We collected substrate and larval samples on days 0 and 15 and performed 16S rRNA gene targeted qPCR and amplicon sequencing. In both chicken feed and chicken manure, egg disinfection did not cause any difference in larval performance or overall microbiota composition. In contrast, in chicken manure, substrate-associated microorganisms increased larval biomass and autoclaving caused major shifts in microbiota. Thus, substrate-associated microorganisms not only impact the larval microbiota but also larval performance, whereas egg-associated microorganisms have a minor role in the densities present.

Keywords

Hermetia illucens, 16S rRNA gene, amplicon sequencing, qPCR, sterile, chicken manure

Introduction

The saprophagous larvae of the black soldier fly (BSF, *Hermetia illucens* (Linnaeus, 1758); Diptera: Stratiomyidae) can be used to convert organic waste streams into insect biomass for livestock feed (Barragán-Fonseca *et al.* 2017; Cickova *et al.* 2015; Pastor *et al.* 2015; Wang & Shelomi 2017). These fly larvae interact with a community of microorganisms such as bacteria and fungi during the consumption of decaying organic matter (De Smet *et al.* 2018; Gold *et al.* 2018). The microbial decomposers can originate from the organic waste substrate, the insect, or the environment (Benbow *et al.* 2019). Especially in nutrient-rich substrates, competition can be fierce and favours those who can monopolize the resource or exploit it fastest (Hanski 1987). The strong competition may also favour partnerships between insect hosts and their associated microbiome (Benbow *et al.* 2019). The insect may introduce microorganisms into the decomposing resource, *e.g.* during oviposition, that confer competitive benefits to their offspring (Lam *et al.* 2009a; 2009b).

The BSF larval gut microbiota consists of a combination of ingested substrate bacteria (Bonelli *et al.* 2019; Bruno *et al.* 2019b; Jiang *et al.* 2019), and bacteria that are found mainly in the larvae (Jiang *et al.* 2019; Wynants *et al.* 2019; **Chapter 3**) and may originate from the eggs (Zheng *et al.* 2013a). In general, the feed substrate is the main determinant of larval gut microbiota (Bruno *et al.* 2019b; Jeon *et al.* 2011; Jiang *et al.* 2019; Zhan *et al.* 2020; **Chapter 3**). Larval and substrate microbiota composition can differ depending on the feed substrate (**Chapter 3**), due to the flexible digestive system of the BSF larvae (Bonelli *et al.* 2020; Zhan *et al.* 2020). Over time, the larvae alter substrate microbiota composition (Jiang *et al.* 2019; Wynants *et al.* 2019; **Chapter 3**), by inhibiting certain bacteria (Lalander *et al.* 2013; Vogel *et al.* 2018), while dispersing gut bacteria into the substrate (Gold *et al.* 2018; Jiang *et al.* 2019; Wynants *et al.* 2019).

In other saprophagous fly species, bacteria serve directly as a larval food source (Thompson *et al.* 2013), and BSF are likely no exception, based on the low middle midgut pH and wide repertoire of lysozyme-encoding genes in the BSF genome (Bonelli *et al.* 2019; Zhan *et al.* 2020). Additionally, microorganisms can complement the digestive capabilities of an insect host (Engel & Moran 2013). BSF egg-associated and larval gut-associated bacteria possess specific enzymes to break down macronutrients and recalcitrant macromolecules such as cellulose (Kim *et al.* 2014; Lee *et al.* 2014; Yu *et al.* 2010).

Microorganisms impact all life stages of BSF: from eggs (Yang *et al.* 2018), to larvae (Yu *et al.* 2011) and adults (Yang *et al.* 2017; Zheng *et al.* 2013b). Despite the large effect of the substrate on larval gut microbiota (Bruno *et al.* 2019b; Jeon *et al.* 2011; Jiang *et al.* 2019; Zhan *et al.* 2020; **Chapter 3**), studies on the effects of microorganisms on larval performance have focused on egg-associated or larva-associated bacteria and commercially available probiotics (Mazza *et al.* 2020; Skaro 2018; Somroo *et al.* 2019; Xiao *et al.* 2018a; Yu *et al.* 2011; Zheng *et al.* 2012). The effects of substrate-associated microorganisms on larval performance are potentially much larger. Moreover, the effects of microorganisms on BSF performance have only been investigated by inoculation of single strains or mixtures of a few bacterial species, whereas both the substrate and larval gut harbour dozens to hundreds of bacterial species (Jeon *et al.* 2011; Jiang *et al.* 2019; Klammsteiner *et al.* 2020; Wynants *et al.* 2019; **Chapter 3**).

BSF larval performance can increase when the feed substrate is inoculated with single strains or mixtures of bacteria. When fed chicken manure inoculated with larval gut-associated strains of *Bacillus subtilis*, BSF larvae grew larger and developed faster, and conversion efficiency and adult size increased (Mazza *et al.* 2020; Xiao *et al.* 2018a; Yu *et al.* 2011). In a similar setup, egg-associated *Lysinibacillus boronitolerans*, *Kocuria marina*, or *Proteus mirabilis* inoculated into chicken manure, produced larger larvae and reduced the manure residue (Mazza *et al.* 2020). Mixtures of these three bacteria and *B. subtilis* also increased larval weight, fat content, and protein content, depending on the ratio of strains in the mixture (Mazza *et al.* 2020). However, some bacterial strains and mixture ratios had no effect or even an adverse effect (Mazza *et al.* 2020). Commercially available bacterial mixtures, probiotics (*e.g. Lactobacillus buchneri*), and egg-associated bacteria (*e.g. Klebsiella oxytoca*) from other fly species, can also improve BSF larval performance and alter BSF nutrient composition (Skaro 2018; Somroo *et al.* 2019; Zheng *et al.* 2012).

In this study, we aimed to quantify the relative importance of microorganisms originating from substrate or eggs in BSF larval performance and in shaping the larval and substrate microbiota. We investigated this in chicken feed and chicken manure. We experimentally heat-inactivated substrate-associated microorganisms and eliminated egg-associated microorganisms, then tested for differences in larval performance parameters (survival, weight, proportion of prepupae), bacterial abundance and community composition. Because BSF larvae are used in industrial-scale bioconversion of organic waste into animal feed products (Barragán-Fonseca *et al.* 2017), understanding these host-microbe

interactions may help improve conversion efficiency and microbiological safety of the insects as livestock feed (Bosch *et al.* 2019; EFSA 2015).

Methods

Insects

Eggs were collected in corrugated cardboard strips on a moist substrate of sawdust and larval frass, from the BSF colony of the Laboratory of Entomology, Wageningen University & Research. The colony has been established with source material from the United States in 2008 and is maintained in a controlled climate chamber at 27±1 °C, 70±10% relative humidity and photoperiod of 16 h light and 8 h dark. Larvae are reared on chicken feed ("Kuikenopfokmeel 1", Kasper Faunafood, Woerden, The Netherlands). Neonate larvae (< 24 h after hatching) were used in the experiments.

Egg disinfection

The egg disinfection protocol was inspired by previously developed methods to sterilize eggs of BSF or blowflies (Barnes & Gennard 2013; Brundage *et al.* 2016; Cai *et al.* 2018b; Limsopatham *et al.* 2017). Upon collection, eggs were divided per 3-4 clutches in 1.5-mL tubes using a sterile cotton swab soaked in sterile phosphate-buffered saline (PBS). Next, the egg clutches were agitated in PBS-Tween (PBS with 0.05% Tween-20) by vortexing for 10 s, then separating eggs of the remaining clutches by gently pressing and rolling a cotton swab in the tube, and vortexing again for 10 s. PBS-Tween was removed by pipetting. For disinfection we added 1 mL 70% ethanol to each 1.5-mL tube with eggs, vortexed 2x for 10 s, removed the liquid, added 1 mL 0.05% NaOCl, vortexed 2x for 10 s, and again removed the liquid. Eggs were then rinsed three times with each 1 mL sterile PBS (1x vortexing for 10 s each). After the third rinse, 800 µL of the liquid was removed, the remaining 200 µL liquid and eggs were plated on sterile lysogeny broth agar (tryptone 10 g L^{-1}, yeast extract g L^{-1}, sodium chloride 5 g L^{-1}, agar 15 g L^{-1}) and incubated in a controlled climate chamber at 27±1 °C and 70±10 % relative humidity. After 72 h incubation, sterility was assessed (colony forming units (CFU) per plate) and only the plates with no colonies were used in the experiment. For neonate collection, 2 mL sterile PBS was pipetted onto the lysogeny broth agar plate or its lid with neonate larvae. The suspension of PBS and neonate larvae was poured into

an empty sterile Petri dish, photographed, counted, and poured onto the substrates in containers of the experiment. From the agitation step onward, all steps were performed in a class II biological safety cabinet.

Preparation of feed substrates

Chicken feed and fresh chicken manure were used as feed substrates. Chicken feed was the same as used for maintaining the BSF colony. The chicken feed was sieved (mesh size 1.5-2 mm), after which 2 mL of autoclaved demineralised water (Milli-Q®, Merck KGaA, Darmstadt, Germany) was added per g dry matter (DM) of chicken feed. Part was stored in the fridge at 4 °C (non-sterile substrate) for two days; part was autoclaved at 140 °C for 3 h (sterile substrate) at Unifarm, Wageningen University & Research, stored at 4 °C before and after, and picked up two days later. Fresh chicken manure was collected from a local organic poultry farm on the morning of substrate preparation. Without the addition of water, non-sterile and sterile manure were stored and prepared as described above for chicken feed. We determined DM content of each batch (both sterile and non-sterile) of both chicken feed and manure in triplicate by oven-drying subsamples at 70 °C for one day. Per container (Microbox Container O95/114+OD95/114, 520 mL, with green filter, SacO2, Belgium; autoclaved before use) in the experiment, 20 g DM feed substrate was used. Substrate treatments consisted of untreated substrate (20 g DM), sterilized (autoclaved) substrate (20 g DM), or a sterilized bulk (18 g DM) amended with 10% w/w untreated inoculum (2 g DM untreated substrate). Based on the DM content, sterile Milli-Q® water was added to the substrate in the container, in order to obtain a DM content of 33% in all substrates. To control for loss of vitamins during autoclaving, we added 1 mL of 0.35 g mL^{-1} stock solution of Vanderzant vitamin mixture for insects (Sigma-Aldrich Inc., USA) to the substrate in each container. After these additions, the substrate in each container was mixed thoroughly using a sterile spatula. Inoculation, addition of water and vitamins and mixing was done in a class II biological safety cabinet. Containers with substrate were then incubated at 27±1 °C and 70±10% relative humidity until the next day (start of experiment, day 0). Although we aimed to standardize the amount of feed substrate and moisture content, treatments differed in these parameters in both chicken feed and chicken manure (Table S1).

Experimental setup

To test the contribution of substrate-associated and egg-associated microorganisms, we tested four treatments within both chicken feed and chicken manure (Figure 1): 1) control treatment, comprising untreated substrate and larvae from untreated eggs (S/E), 2) sterilized (autoclaved) substrate with 10% untreated substrate as inoculum (i) and larvae from untreated eggs (Si/E), 3) sterilized substrate with 10% untreated substrate as inoculum and larvae from sterilized (s; surface-disinfected) eggs (Si/Es), and 4) sterilized diet (s, without inoculum) and larvae from untreated eggs (Ss/E). Each treatment was replicated four times, divided over two batches. To each container, 100 neonate larvae (< 24 h since hatching) were added. For chicken manure, a third batch was included, because the second batch had larvae of almost 24 h since hatching; making a total of six replicates per treatment for chicken manure. The experiment was conducted in a controlled climate chamber of 27±1 °C, 70±10 % relative humidity and

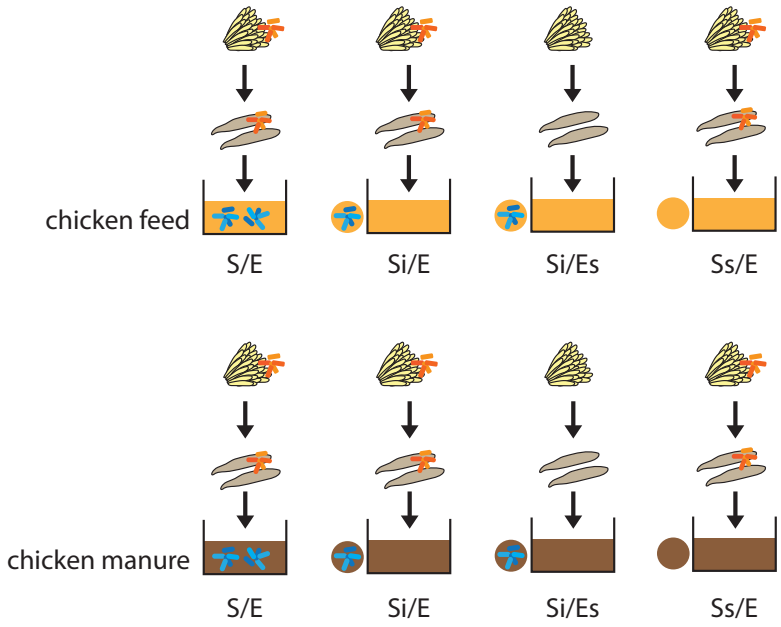

Figure 1. Experimental design of the study. We tested four treatments in chicken feed and chicken manure: S/E = untreated substrate with larvae from untreated eggs, Si/E = sterilized substrate with inoculum (10% w/w of untreated substrate) and larvae from untreated eggs, Si/Es = sterilized substrate with inoculum and larvae from disinfected eggs, and Ss/E = sterilized substrate without inoculum and with larvae from untreated eggs.

photoperiod of 16 h light and 8 h dark. Within each batch, containers were repositioned randomly each day to account for any temperature or humidity gradients in the climate chamber. Fifteen days after the addition of the larvae to the substrate, the batch was harvested. Only treatment of autoclaved chicken feed (Ss/E) was continued until day 22 to increase the chance of successful DNA isolation, because larvae were very small on day 15.

Sampling for molecular analyses of microbial composition

Samples for DNA extraction were collected from eggs, larvae, and substrates to assess sterility of disinfected eggs and autoclaved substrates, and to compare bacterial communities of substrates and eggs/larvae at the start and end of the experiment. Untreated eggs were transferred directly per 3-4 clutches from the cardboard strip into a 2-mL tube, using a sterile cotton swab soaked in sterile PBS. Disinfected eggs and the 200 µL remaining liquid of the third rinse PBS were transferred by pipet to a 2-mL tube. The egg samples were collected on the day of egg collection and disinfection, *i.e.* three days before adding the neonate larvae to the substrates. Substrate samples were collected on day 0 (onset of experiment, *i.e.* day that neonate larvae were added to the diet) and day 15, prior to larval sampling, using a sterilised plastic straw to take a vertical core from the substrate. In cases where this was unsuccessful, a sterile spatula was used. Larval samples of day 15 were collected by picking three average-sized larvae of a container using sterile tweezers (or six larvae in chicken feed Ss/E, since larvae were 3-5 mm length instead of 15-25 mm). Larvae were then surface-disinfected using the same rinsing protocol as in **Chapter 3**: 30 s sterile Milli-Q® water, 30 s 70% ethanol, 30 s 1% Halamid®-D (chloramine-T), and 2x 10 s in sterile Milli-Q® water. Each rinsing step was done in a separate 65-mm Petri dish. Sampling was done in a class II biological safety cabinet, and all samples for molecular analyses were snap-frozen in liquid nitrogen.

pH measurements

On day 0 and 15, additional substrate samples of 1-2 g were collected from each container for pH measurement. These samples were stored at -20 °C. pH was measured after thawing and suspending 1 g of each sample in 10 mL Milli-Q® water, using a pH meter (ProLine B210, ProSense B.V.).

Larval performance

After sampling for molecular analyses, the content of the container was harvested outside the biological safety cabinet. Larvae were separated from residue, washed in a sieve under lukewarm tap water, dried with tissue, and counted. Larval biomass samples were then stored at -20 °C. DM content of the residue was determined by weighing a fresh residue sample and drying it in an oven at 70 °C until stable weight. Additionally, fresh samples of each residue were stored at -20 °C. Subsamples of 10 average-sized larvae of each frozen sample were also weighed and oven-dried at 70 °C until stable weight, to determine DM content and individual larval weight (g DM). Total larval biomass (g DM) was calculated as the individual larval weight (g DM) multiplied by the total number of surviving larvae on the day of harvest.

Processing of samples for molecular analyses

Samples were ground in liquid nitrogen using disinfected mortar and pestle. Approximately 50 mg of sample was then weighed (to 0.001 g precision) and transferred to a 1.5 mL Eppendorf tube. Samples were randomly processed in batches of 16 samples, using the method of cell lysis, repeated bead-beating and DNA extraction adapted from Salonen *et al.* (2010) and Van Lingen *et al.* (2017). Per 70 samples, two no-template controls (NTC) were included to control for DNA isolation kit contaminants (isolation blank). 300 µL buffer for Stool Transport and Recovery (STAR, Roche) was added to the tube and vortexed until all frozen sample was suspended (10-20 s). The suspension was transferred to a sterile 2.0 mL screw-cap tube containing 0.1 g zirconia beads and 3 glass beads of 2.5 mm diameter. The samples were then homogenized in a bead beater (Precellys 24, Bertin Technologies, France) for 3 x 1 min at 5.5 m s^{-1} with a waiting step of 20 s in between, followed by incubation for 15 min at 95 °C and 300 rpm, and centrifugation for 5 min at 16,100 x *g* and 4 °C. Supernatant was transferred to a new tube. The homogenization, incubation and centrifugation were repeated with fresh 200 µL STAR buffer, and the supernatant was combined with the first supernatant. DNA was then isolated from 250 µL pooled supernatant by adding it to a cartridge of the Maxwell 16 Tissue LEV Total RNA Purification Kit (cat. no. XAS1220, Promega Corporation, USA) and eluted in 30 µL nuclease-free water using the Maxwell MDx robot (Promega Corporation, USA). DNA concentration was measured using a Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific, USA), after which samples with a DNA concentration above 50 ng μL^{-1} were diluted to 20 ng μL^{-1} for barcoded PCR.

qPCR

Absolute quantification of bacteria was carried out using qPCR targeting the 16S rRNA gene. Extracted and purified DNA template was diluted 1:5 – 1:125 depending on pilot runs of qPCR with dilution series. We used the universal primers BACT1369F (5'-CGGTGAATACGTTCYCGG-3') and PROK1492R (5'-GGWTACCTTGTTACGACTT-3') (Van Lingen *et al.* 2017). Per reaction, a mix of 10 µL BioLine SensiFAST SYBR, 1 µL 10 µM forward primer, 1 µL 10 µM reverse primer, 3 µL nuclease-free water and 5 µL (diluted) DNA template were added. qPCR was performed in a BioRad CFX96 C1000 real-time PCR machine, as follows: initial denaturation at 95 °C for 3 min, then 40 cycles of denaturation at 95 °C (10 s), annealing at 60 °C (10 s), and elongation at 72 °C (30 s), followed by a melt curve analysis from 65 °C to 95 °C in 0.5 °C increments for 5 s each. All reactions were run in duplicate. Each 96-well plate contained a dilution series of a standard (and inter-run calibrator) of 2.38 x 10^3 to 1.49 x 10^6 16S rRNA gene amplicons of *Bacillus circulans*, in five steps of 1:5 dilutions. Besides, each plate contained five NTCs, two of which used 5 µL of nuclease-free water from the dilutions, three used 5 µL nuclease-free water from the master mix.

Amplification curves and melting curves were checked in the BioRad CFX Manager. Sample quality assessment, run efficiency, inter-run calibration, and calculation of copy numbers were done using qbase+ (Hellemans *et al.* 2007). PCR efficiency ranged between 74.1 – 90.9% for the five 96-well plates. The standard curves were used for inter-run calibration, and only the standard curve of the first run (highest efficiency, 90.9%) was used to calculate copy numbers of all samples. NTCs showed C_q values between 36.1 – 39.5. Samples within 5 cycles differences of the lowest NTC with lowest C_q value in that plate, were scored as negative and excluded from analysis (22 samples and eight DNA isolation blanks; Hellemans *et al.* 2007). Additionally, eleven out of twenty egg samples were excluded from analysis because they scored negative and/or melting curves indicated low sample quality. Calibrated quantities of duplicates were averaged, and these averages were used to calculate the number of 16S rRNA gene copies per g fresh matter of starting material.

Barcoded PCR

Bacterial community composition of samples was determined using Illumina HiSeq sequencing of amplicons of the V5-V6 region of the 16S rRNA gene. We performed

barcoded PCR on samples in duplicate, with barcoded primers F784-1064R (Ramiro-Garcia *et al.* 2016). Per PCR run, we included one NTC (1 µL nuclease-free water as template) as a negative control. As positive controls we used synthetic mock communities of known composition (Ramiro-Garcia *et al.* 2016). The below procedure is largely the same as in **Chapter 3**. For each reaction, the following 50 µL mix was prepared in duplicate: 36.5 µL nuclease-free water, 10 µL 5x HF buffer (Thermo Fisher Scientific, USA), 1 µL dNTPs (10 mM), 0.5 µL Phusion Hot Start II DNA polymerase (2U μL^{-1}) (Thermo Fisher Scientific, USA), 1 µL barcoded primers (10 µM) and 1 µL DNA template. The following PCR program was used: 98°C for 30 s, 25 cycles of 98°C 10 s, 42°C 10 s, 72°C 10 s, and 72°C for 7 min. PCR products were checked for yield and correct size by agarose gel electrophoresis. Duplicate reaction products were pooled and amplified DNA was purified using the CleanPCR magnetic bead suspension (CleanNA, The Netherlands), 1.8x the volume of the PCR mix, two washes with 200 µL 70% ethanol, and eluted in 30 µL nuclease-free water. Purified DNA concentrations were measured using the Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific, USA) and pooled in equimolar concentrations per library of 70 samples (randomly assigned to each library), concentrated using magnetic beads and re-eluted in 20 µL nuclease-free water. Final DNA concentration per sequencing library was measured in Qubit, after which the libraries were shipped to Eurofins Genomics Germany GmbH (Konstanz, Germany) for 2 x 150 bp sequencing on an Illumina NovaSeq 6000 instrument.

Amplicon sequence data were processed using NG-Tax 2.0 (Poncheewin *et al.* 2019) and annotated using the SILVA 132 reference database (Quast *et al.* 2013).

Statistical analyses

All analyses were conducted in R version 3.5.0 (R Core Team 2018).

Larval performance

The effect of treatment on larval survival rate, percentage of prepupae, individual weight and total larval biomass was investigated per feed substrate separately. Linear mixed model (LMM) selection of a random intercept for batch effect and a variance structure for treatment was performed based on Akaike's information criterion (AIC, Sakamoto *et al.* 1986; nlme package, Pinheiro *et al.* 2018). If the random term did not improve the model, linear model or generalized least squares regression was used (LM

or GLS, respectively). Non-parametric testing for differences between treatments was done with Kruskal-Wallis tests. Post-hoc pairwise comparisons for linear models were performed using estimated marginal means (EMM) with Tukey-corrected P-values (emmeans package; Lenth 2020). Non-parametric post-hoc comparisons were made using the Wilcoxon rank sum test with P-values corrected for false discovery rate (FDR; Benjamini & Hochberg 1995).

Substrate moisture content and pH

Substrate moisture content was tested for treatment effects following the same procedure as for the larval performance traits. Substrate pH was tested using a generalized linear mixed model regression (GLMM) with Gamma distribution and inverse link function since LMM residuals were not normally distributed, with a random intercept for container ID (lme4 package; Bates *et al.* 2015). Post-hoc comparisons were made using EMM with Tukey-corrected P-values.

Bacterial abundance

Bacterial 16S rRNA gene abundances resulting from qPCR were tested separately per day. For substrate samples on day 0, we investigated the effect of treatment using linear model regression with AIC-based selection of a variance structure. This was done only for chicken manure, because chicken feed had insufficient replicates in other treatments than control (S/E). For larval and substrate samples of day 15, we tested the effects of treatment and sample type using an LMM regression with a random intercept for container ID, and AIC-based model selection of a variance structure. If model residuals were not normal, we performed GLMM with Gamma distribution and inverse link function. Post-hoc comparisons were made in EMM with Tukey-corrected P-values.

Microbiota composition

Sequence data were explored and analysed using the phyloseq (McMurdie & Holmes 2013) and microbiome packages (Lahti & Shetty 2017). Chloroplast and mitochondrial reads were excluded from analysis, as well as reads of contaminant Amplicon Sequence Variants (ASVs). Contaminant ASVs were identified by visual inspection of correlation plots of relative ASV abundance against DNA concentration (ng μL^{-1}) in the PCR

product. Samples similar to blanks in qPCR were also excluded from analysis of sequencing data, as well as samples with fewer than 5000 reads (excl. mitochondrial, chloroplast or contaminant ASVs) since microbiota composition of these samples was considered unreliable (36 out of 140 samples). One substrate sample of autoclaved chicken manure with inoculum (Si/E) of day 0 and one substrate sample of untreated chicken manure (S/E) of day 15 were excluded from analysis because they were suspected to be erroneously mixed up in the lab workflow. Additionally, samples of two containers in the autoclaved chicken feed with untreated eggs (Ss/E) were excluded because these were heavily contaminated with a green fungus, unlikely to originate from the eggs. All subsequent analyses were performed with relative abundance data at genus level.

Alpha diversity was measured as Faith's phylogenetic diversity, which is phylogenetically weighted richness, *i.e.* the sum of all phylogenetic tree branch lengths in a sample (Faith 1992). For substrates samples of day 0, we performed a linear model regression to test for a treatment effect. This was done only for chicken manure, since chicken feed had insufficient replicates in other treatments than control (S/E). For larval and substrate samples of day 15, we tested for the effects of treatment and sample type using an LMM regression with a random intercept for container ID and AIC-based selection of a variance structure. Post-hoc comparisons were made using EMM with Tukey-corrected P-values.

Total microbiota variation was analysed per feed substrate and day separately using non-metric multidimensional scaling based on weighted UniFrac distances (Kruskal 1964; Luzopone & Knight 2005). The effects of treatment and sample type were quantified using distance-based redundancy analysis (dbRDA; McArdle & Anderson 2001) and statistically tested using a permutational multivariate analysis of variance (anova.cca function, vegan package, Oksanen *et al.* 2019).

Weighted UniFrac distance between larval and substrate microbiota composition was assessed for differences between treatments using an analysis of variance and post-hoc comparisons with Tukey-correction.

Per feed substrate, the most abundant and prevalent genera (present in at least 10% of samples and comprising at least 1% of reads in a sample (or 10% in chicken manure)),

were displayed in heatmaps of mean relative abundance. Differences in relative abundance of these genera between treatments were tested using Kruskal-Wallis tests and post-hoc Wilcoxon rank sum tests. P-values were FDR-corrected.

All sequence data have been deposited in the European Nucleotide Archive under the study accession number PRJEB40821 (https://www.ebi.ac.uk/ena/browser/view/PRJEB40821). Larval performance data, substrate pH data, metadata of 16S rRNA samples, and all software code have been deposited in the 4TU.ResearchData repository under DOI 10.4121/13118294.

Results

Larval performance

Larval performance was not affected by microorganisms associated with eggs in either of the feed substrates, and only by substrate-associated microorganisms in chicken manure. In chicken feed, more larvae survived in the autoclaved substrates with inoculum (Si/E, 84%; Si/Es, 86%) versus the control treatment (S/E, 51%; GLS, $P < 0.001$), but larvae tended to be heavier in the control treatment (0.063 g DM) than in the autoclaved substrates with inoculum (0.018 – 0.022 g DM; Kruskal-Wallis, $P = 0.037$ but no significant pairwise differences; Figure 2A-B). No differences were observed in total larval biomass and the percentage of prepupae (Kruskal-Wallis, $P = 0.232$ and $P = 0.070$, respectively; Figures 2C and S1).

In chicken manure, larvae from autoclaved manure without inoculum were lighter (Ss/E, 0.006 g DM) compared to the other treatments (0.010 – 0.012 g DM; Kruskal-Wallis, $P = 0.005$; Figure 2B). This also resulted in lower total larval biomass from this treatment (Ss/E, 0.531 g DM) compared to the other manure treatments (0.739 – 0.887 g DM; Kruskal-Wallis, $P = 0.003$; Figure 2C). Survival rate and percentage of prepupae did not differ among treatments in chicken manure (ANOVA, $P = 0.110$; Kruskal-Wallis, $P = 0.235$, respectively; Figures 2A and S1).

Substrate pH

In chicken feed, substrate pH increased from day 0 (5.6 – 5.7) to day 15 (7.5 – 8.2) in all treatments except autoclaved chicken feed without inoculum (5.5 to 5.6; Figure S2,

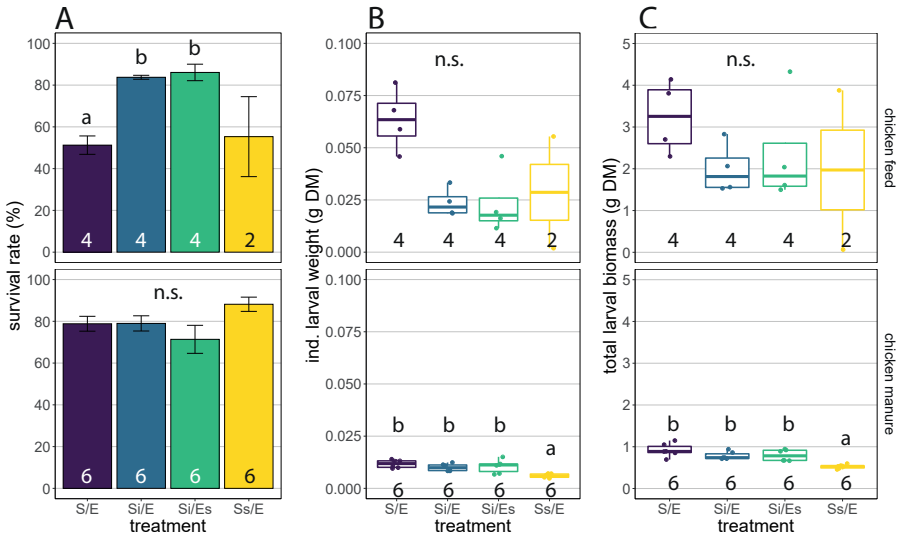

Figure 2. Larval performance: A) survival rate (%, mean ± SE), B) individual larval weight (g dry matter, boxplots), C) total larval biomass (g dry matter, boxplots). Top panels are for chicken feed, bottom panels for chicken manure. Treatment codes: S/E = control treatment (untreated substrate and untreated eggs), Si/E = sterilized substrate with inoculum and untreated eggs, Si/Es = sterilized substrate with inoculum and disinfected eggs, Ss/E = sterilized substrate and untreated eggs. All treatments were harvested on day 15, except chicken feed Ss/E which was harvested on day 22 and excluded from statistics. Numbers in bars indicate sample sizes (number of containers). Means or medians with different letters are significantly different (α = 0.05, different test used per substrate and parameter). n.s. = not significant.

Table S2). Substrate pH of inoculated chicken feed with disinfected eggs on day 15 (Si/Es, 8.2) was higher than in inoculated chicken feed with untreated eggs (Si/E, 7.5). In chicken manure, substrate pH also increased over time (from 7.5 – 8.0 to 9.0 – 9.3) in all treatments except untreated manure (8.7 to 9.1; Figure S2, Table S3). Additionally, on day 0, autoclaved manure without inoculum had a significantly lower pH (7.5) than untreated manure (8.7).

Substrate moisture content

Substrate moisture content on day 15 differed among treatments in both chicken feed and chicken manure (Kruskal-Wallis, chicken feed: P = 0.021; chicken manure: P = 0.004; Figure S3). Untreated chicken feed was wetter (83%) than the other treatments (79 – 80%); and autoclaved chicken manure without inoculum was drier (64%) than the other manure treatments (67 – 68%).

Total bacterial abundance

Treatments in both chicken feed and chicken manure differed in bacterial 16S rRNA gene abundance on day 0 (chicken feed, LM, $P < 0.001$; chicken manure, GLS, $P < 0.001$; Figure 3). In chicken feed, larval samples in all treatments except Ss/E contained fewer 16S rRNA gene copies than substrates (10^9-10^{10} vs 10^{11} copies g^{-1} sample; GLMM, $P < 0.001$; Figure 3). 16S rRNA gene abundance did not differ between chicken feed S/E, Si/E, and Si/Es treatments ($P = 0.154$). All substrate samples and three larval samples of Ss/E scored as negative (C_q values within 5 cycles of the negative control with the lowest C_q value).

Autoclaved manure without inoculum (Ss/E) still contained considerable (10^9) numbers of 16S rRNA gene copies per g sample on day 0. After 15 days, no differences in bacterial 16S rRNA gene abundance were found between treatments of chicken manure (LMM, $P = 0.020$ but no significant post-hoc comparisons), but in treatments S/E and Si/E, larval samples contained fewer 16S rRNA gene copies than substrates (10^{11} vs 10^{12}; $P < 0.001$).

Out of ten egg samples per egg treatment, four disinfected and five untreated egg samples scored positive in qPCR, *i.e.* with a C_q value more than five cycles lower than the lowest C_q value of an NTC. From these positive samples, disinfected eggs had significantly fewer bacterial 16S rRNA gene copies per g sample than untreated eggs, although the difference was small ($10^{6.6}$ vs $10^{7.4}$; GLMM, $P = 0.002$; Figure S4).

Bacterial community composition – quality controls

Amplicon sequencing resulted in 31 million reads assigned to 4231 ASVs (excluding chloroplast or mitochondrial reads (2.2% of all reads) and contaminant ASVs (2.4%)). We identified 188 contaminant ASVs, which were mostly assigned to known lab contaminant genera, *e.g. Ralstonia* and *Cupriavidus*, and were filtered from our dataset (Salter *et al.* 2014; Table S4). In the positive controls (synthetic mock communities), Spearman rank correlations at genus level between replicates were high: 0.89 – 0.99 (mean 0.95) for mock community 3 and 0.91 – 0.99 (mean 0.95) for mock community 4. Spearman rank correlations at genus level between positive controls and corresponding theoretical mock composition were 0.79 ± 0.04 for mock 3 and 0.73 ± 0.02 for mock

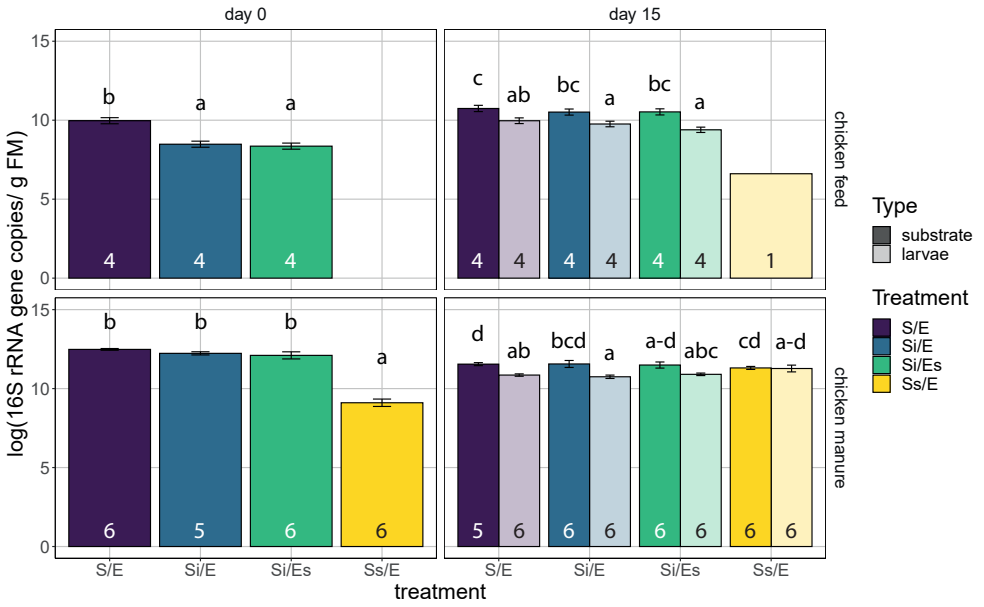

Figure 3. Total bacterial 16S rRNA gene abundance (estimated marginal mean ± SE, $\log_{10}$ 16S rRNA gene copies per g fresh matter), on day 0 and 15 in substrate and larval samples in the different treatments of chicken feed (top panels) and chicken manure (bottom). Treatment codes: S/E = control treatment (untreated substrate and untreated eggs), Si/E = sterilized substrate with inoculum and untreated eggs, Si/Es = sterilized substrate with inoculum and disinfected eggs, Ss/E = sterilized substrate and untreated eggs. Numbers in bars indicate sample sizes (number of containers). Larvae were only sampled on day 15. Substrate samples of chicken feed Ss/E were similar to blanks. Means without shared letters are significantly different (tested per feed substrate and day; α = 0.05; post-hoc comparisons with Tukey-corrected P-values).

4, which is in accordance with routinely observed values, indicating accurate and reproducible sequencing of bacterial communities across sequencing runs.

DNA isolation and PCR replicates were highly correlated in both untreated chicken feed and chicken manure for substrates of day 0 and for larvae (Table S5). PCR replicates of egg samples did not result in reproducible bacterial communities (untreated eggs: r = 0.22 – 0.39; disinfected eggs r = 0.12), and so we decided not to analyse the egg samples further.

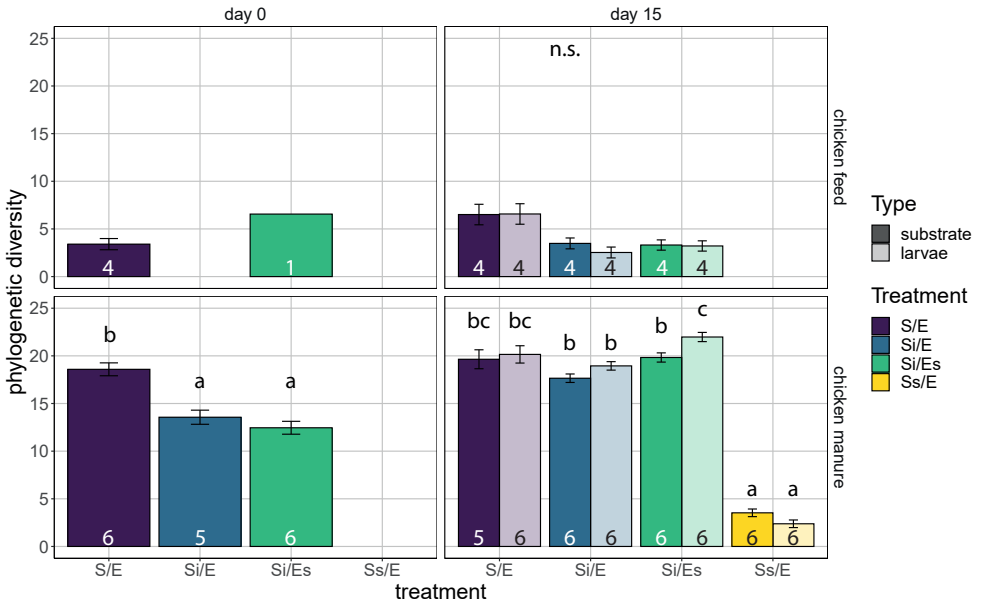

Figure 4. Faith's phylogenetic diversity (mean ± SE) of substrate and larvae samples of the different treatments in both feed substrates. Top panels are for chicken feed, bottom panels for chicken manure. Treatment codes: S/E = control treatment (untreated substrate and untreated eggs), Si/E = sterilized substrate with inoculum and untreated eggs, Si/Es = sterilized substrate with inoculum and disinfected eggs, Ss/E = sterilized substrate and untreated eggs. Numbers in bars indicate sample sizes (number of containers). Larvae were only sampled on day 15. Means that share no letters are significantly different (tested per substrate and day; α = 0.05; post-hoc comparisons with Tukey-corrected P-values).

4

Bacterial community composition – α diversity

In chicken feed on day 15, there was a significant treatment effect on Faith's phylogenetic diversity, but pairwise comparisons showed no differences (LMM, P = 0.015; Figure 4). In chicken manure on day 0, untreated manure had a higher phylogenetic diversity (S/E, 18.6) than inoculated manure (Si/E, 13.6; Si/Es, 12.5) (ANOVA, P < 0.001; Figure 4). Fifteen days later, substrate microbiota of untreated and inoculated manure groups did not differ (17.7 – 19.8), but the microbiota of autoclaved manure was less diverse than the rest (Ss/E, 3.5) (LMM, P < 0.001; Figure 4). In addition, larval microbiota was more diverse than substrate microbiota in the inoculated manure with disinfected eggs (Si/Es, 22.0 vs 19.8) (P = 0.001; Figure 4).

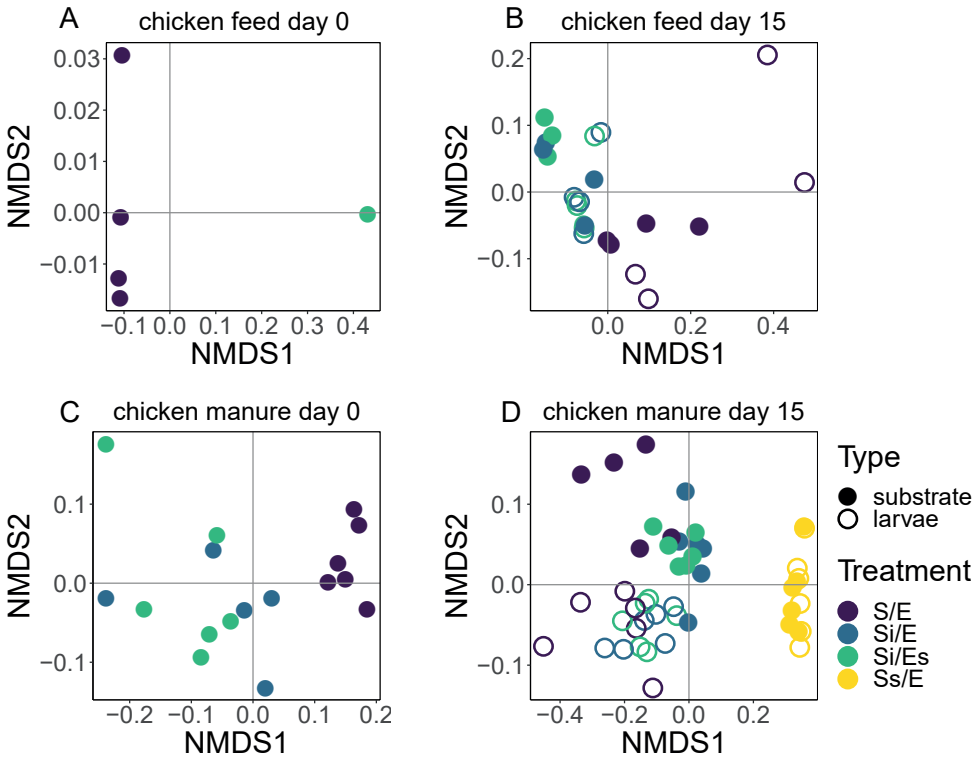

Figure 5. Bacterial community composition (NMDS) based on weighted UniFrac distances at genus level: A) chicken feed substrates on day 0; B) chicken feed larvae and substrates on day 15; C) chicken manure substrates on day 0; D) chicken manure larvae and substrates on day 15. Treatment codes: S/E = control treatment (untreated substrate and untreated eggs), Si/E = sterilized substrate with inoculum and untreated eggs, Si/Es = sterilized substrate with inoculum and disinfected eggs, Ss/E = sterilized substrate and untreated eggs. Stress of NMDS solutions: A = 0, B = 0.063, C = 0.066, D = 0.060.

Bacterial community composition – β diversity

Treatments affected microbiota composition in both chicken feed and chicken manure (weighted UniFrac NMDS and dbRDA; Figure 5, Tables S6-S7). The most abundant phyla were Firmicutes, Proteobacteria, Actinobacteria, and Bacteroidetes (Figure S5).

In chicken feed on day 15, samples of inoculated groups (Si/E, Si/Es) overlapped, suggesting no effect of egg-associated microorganisms on microbiota composition (NMDS, Figure 5B). However, microbiota of untreated chicken feed differed from the

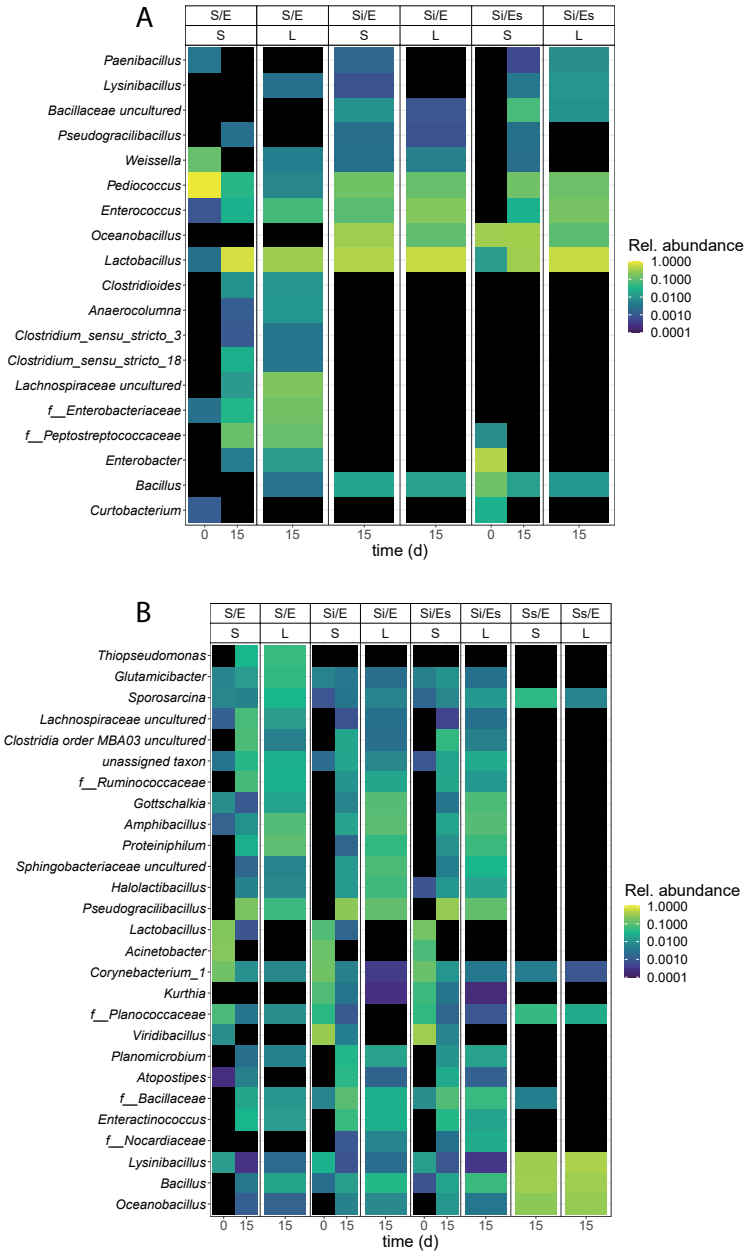

Figure 6. Heatmaps of the most abundant genera per substrate, mean relative abundance: A) chicken feed; B) chicken manure. Treatment codes: S/E = control treatment (untreated substrate and untreated eggs), Si/E = sterilized substrate with inoculum and untreated eggs, Si/Es = sterilized substrate with inoculum and disinfected eggs, Ss/E = sterilized substrate and untreated eggs. S = substrate microbiota, L = larval microbiota. For chicken feed, genera are displayed if relative abundance > 1% in a sample and occurring in > 10% of all samples; for chicken manure, genera are displayed if relative abundance > 10% in a sample and occurring in > 10% of all samples.

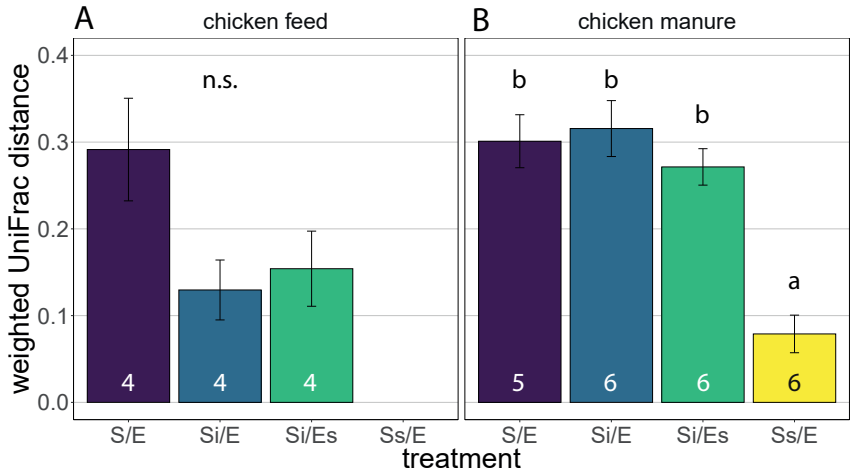

Figure 7. Weighted UniFrac distance between larval and substrate microbiota (mean ± SE): A) chicken feed, B) chicken manure. Treatment codes: S/E = control treatment (untreated substrate and untreated eggs), Si/E = sterilized substrate with inoculum and untreated eggs, Si/Es = sterilized substrate with inoculum and disinfected eggs, Ss/E = sterilized substrate and untreated eggs. Numbers below bars indicate sample sizes (number of containers). Means with different letters are significantly different (α = 0.05; ANOVA with Tukey contrasts). n.s. = not significant.

inoculated groups (Si/E, Si/Es) (dbRDA, treatment effect: P = 0.002; Table S6). Eight of the most abundant genera on day 15 were only present in larvae and substrates from untreated chicken feed (S/E; Figure 6A). Weighted UniFrac distances between larval and substrate microbiota did not differ among treatments (ANOVA, P = 0.076; Figure 7A).

In chicken manure on day 0, substrate microbiota composition of untreated manure (S/E) differed from the inoculated manure (Si/E, Si/Es) (dbRDA, R^2 = 51%, P = 0.001; Figure 5C). There was also a treatment effect on day 15, explaining 63% of total larval and substrate microbiota variation in chicken manure (dbRDA, P = 0.001; Figure 5D, Table S7). Samples of autoclaved manure (Ss/E) differed from the remaining treatment groups, and untreated manure (S/E) differed from the inoculated manure microbiota (Si/E and Si/Es; Figure 5D). Twelve of the 22 most abundant genera differed in abundance among larval microbiota of different treatments (Kruskal-Wallis test, Table S8; Figure 6B). Larval and substrate microbiota differed in composition in untreated and inoculated manure, but not in autoclaved manure (Figure 5D). The weighted UniFrac distance between larval and substrate microbiota in autoclaved manure was lower than in the other treatments (ANOVA, P < 0.001; Figure 7B).

Discussion

This study shows that substrate-associated microorganisms affected larval performance and caused major changes in larval and substrate microbiota, whereas egg-associated microorganisms did not affect performance and only had a minor effect on larval and substrate microbiota.

Effects of substrate-associated microorganisms on larval performance

BSF larvae performed better on substrates with associated bacteria than on autoclaved substrate without the inoculum (*i.e.* 10% w/w untreated substrate) (Figure 2). Larval biomass was lower in the latter treatment in chicken manure compared to all other manure treatments. Moreover, larval development and growth tended to be much retarded in autoclaved chicken feed without inoculum. These effects may have been caused by the differences in bacterial abundance, since initial bacterial abundance in the autoclaved treatments without inoculum was much lower than in the inoculated or untreated manure and chicken feed (Figure 3). Bacteria serve directly as food for fly larvae and help decompose macronutrients (Gold *et al.* 2018). Improved nutrition is also likely the reason why bacterial inoculation of substrates can lead to increased larval weight (Mazza *et al.* 2020; Somroo *et al.* 2019).

Differences in substrate pH of the manure treatments on day 0 were likely caused by autoclaving, due to the evaporation of ammonia and elimination of bacteria responsible for its production (Erickson *et al.* 2004). The elimination of nitrogen-mineralizing bacteria may also explain why the pH of autoclaved chicken feed on day 15 was so much lower than the other chicken feed treatments (Figure S2), since bacterial abundance in this substrate remained similar to NTCs in qPCR (Figure 3).

Larvae fed autoclaved chicken feed with inoculum (Si/E, Si/Es) tended to weigh less than larvae fed untreated chicken feed (Figure 2B). This difference may be due to the effect autoclaving has on nutritional properties of the feed substrate: nutrient digestibility and quality may have changed after autoclaving due to complex reactions between sugars and amino acids, known as the Maillard reaction or non-enzymatic browning (O'Brien *et al.* 1989). This can have positive and negative effects on the nutritional value of the substrate (O'Brien *et al.* 1989). Alternatively, the lower larval

weight in the inoculated chicken feed groups may be the result of fiercer competition for food due to the higher larval survival in these groups compared to the untreated chicken feed (Figure 2A). Larval survival in the untreated chicken feed may have been lower because of excessive moisture. BSF larvae can differ in survival in substrates of different moisture content, but this relationship depends on the type of substrate (Cammack & Tomberlin 2017; Cheng *et al.* 2017; Fatchurochim *et al.* 1989). In our study, moisture content among chicken feed treatments varied much less than the values compared in the three aforementioned studies (*i.e.* 68 – 71% fresh matter on day 0, 79 – 84% on day 15; Table S1, Figure S3).

The Maillard reaction may also explain differences in microbiota composition between larvae of untreated substrates and of autoclaved substrates with inoculum. Besides the inactivation of microorganisms during autoclaving, Maillard reaction products can inhibit growth of *e.g. Salmonella*, *Escherichia*, *Bacillus* and *Lactobacillus* species (O'Brien *et al.* 1989).

Effects of substrate-associated microorganisms on larval microbiota

The present study as well as previous studies suggest that especially substrate-associated bacteria influenced the gut microbiota of larvae, and less so the other way around (Bruno *et al.* 2019b; Jiang *et al.* 2019; Zhan *et al.* 2020; **Chapter 3**). The BSF larval gut and substrate exchange large proportions of microbiota over time, with 86% of bacteria in the larval gut samples originating from the substrate and 13% of bacteria in the substrate originating from the larval gut, after larvae were reared on food waste for ten days (Jiang *et al.* 2019). The difference between larval and substrate microbiota is caused by differences in prevailing environmental conditions, including the selection pressure of the larval digestive and immune systems (Bonelli *et al.* 2019; Bruno *et al.* 2019b; Vogel *et al.* 2018; **Chapter 3**).

Larval and substrate microbiota differed significantly in both untreated and inoculated manure, like previously found (**Chapter 3**), but did not differ in autoclaved manure (Figures 5-7). This may have several explanations related to the immune response of BSF larvae. A high bacterial load of a mixture of bacteria, present in the untreated and inoculated manure, can trigger a strong and complex larval immune response (Vogel *et al.* 2018), but the initial bacterial abundance in the autoclaved manure was a thousand

times lower, compared to the other treatments (Figure 3). Secondly, the bacterial species that survived in the autoclaved manure could have triggered a different immune response than the ones present in the other treatments (Zdybicka-Barabas *et al.* 2017). Finally, Erickson *et al.* (2004) suggest that active larval growth is linked to a decrease in *Salmonella* populations in manure, and absence of growth may imply a loss of this effect. Hence, the reduced larval growth we observed in autoclaved manure (Figure 2B) may have led to reduced suppression of certain bacteria as well, and consequently the higher similarity between larval and substrate microbiota in this treatment (Figure 7B).

In contrast to what we observed in chicken feed, larvae from autoclaved manure without inoculum and with untreated eggs (Ss/E) harboured a bacterial community as abundant as that of the other treatments after 15 days, and these bacteria may have originated from the eggs. *Bacillus*, *Lysinibacillus*, and *Oceanobacillus* dominated larval and substrate microbiota in this treatment (Figure 6). *Lysinibacillus fusiformis* was previously isolated from the eggs of our BSF colony (Schreven *et al.* unpublished data) and can increase larval weight and survival (Portela Cardenas 2020). This could indicate that in the absence of competition from substrate-associated microorganisms, egg-associated microbiota can colonise the substrate. On the other hand, *Lysinibacillus* as well as *Bacillus* and *Oceanobacillus* can form endospores that are heat-resistant and may survive autoclaving (Flores & Popham 2020). Since bacterial abundance in the autoclaved manure without inoculum was considerable on day 0 (10^9 16S rRNA gene copies g^{-1}), it is more likely that these genera originated from the manure as spores, survived autoclaving, multiplied throughout the substrate and colonized the larval gut. The copy numbers resulting from qPCR may, however, have included DNA from dead bacteria (Carini *et al.* 2016; Emerson *et al.* 2017), and therefore the viable bacterial population may be overestimated.

The role of egg-associated microorganisms on larval performance and microbiota

Our study suggests that the egg-associated microorganisms were so few compared to the substrate-associated microorganisms, that they had no effect on overall microbiota composition or larval performance in both chicken feed and chicken manure (Figures 3-7). They did, however, cause differences in the relative abundance of individual genera and phylogenetic diversity in larvae fed chicken manure (Si/E vs Si/Es; Figure 4, Table S8), and in substrate pH in chicken feed (Figure S2). Among the most abundant

genera, an unassigned genus of Bacillaceae was less abundant in larvae of Si/E than in those of Si/Es manure (Figure 6, Table S8). In chicken feed, substrate pH was higher in inoculated diet with disinfected eggs than in inoculated diet with untreated eggs. The higher pH is likely due to increased ammonia production from proteolysis (Erickson *et al.* 2004; Green & Popa 2012). Since there are no differences in larval weight, bacterial abundance, or community composition, this suggests that egg-associated microorganisms would suppress the rate of nitrogen mineralization in the chicken feed substrate, through yet unknown mechanisms.

Our findings on the limited role of egg-associated microorganisms on larval performance contrast to previously reported effects of egg-associated or larva-associated bacteria on larval growth (Mazza *et al.* 2020; Xiao *et al.* 2018a; Yu *et al.* 2011). A fundamental difference between these studies and ours, is that we tested the effect of the total community of microorganisms residing on the untreated eggs, whereas other studies tested single species or mixtures of up to four species of bacteria (Mazza *et al.* 2020; Xiao *et al.* 2018a; Yu *et al.* 2011). Moreover, the number of bacteria on the eggs may have been much smaller than applied in the inoculation studies, *i.e.* 10^8 – 10^9 CFU mL^{-1} inoculum resulting in 10^6 CFU g^{-1} substrate (Mazza *et al.* 2020; Xiao *et al.* 2018a; Yu *et al.* 2011). In our qPCR results, the number of bacterial 16S rRNA gene copies in egg samples was at most 10^7 copies g^{-1} eggs (Figure S4), which would be more diluted still in the substrate. The bacteria that are present on the eggs could still be beneficial to BSF, *e.g.* during larval hatching from the eggs (Yang *et al.* 2018) – a developmental stage we did not include in our performance study since we used neonate larvae that successfully hatched. Alternatively, Gold *et al.* (2020a) suggested a role of larva-associated microorganisms in providing essential nutrients such as vitamins, because they found that sterile BSF larvae failed to grow on autoclaved substrates whereas non-sterile larvae were able to grow. We may have missed this effect, because we supplemented vitamins to all substrates and did not test the combination of disinfected eggs on autoclaved substrate.

We could not consistently detect and describe the bacterial community present on untreated eggs. Egg samples, untreated or disinfected, showed C_q values close to or within the range of NTCs. Bacterial densities on BSF eggs may simply be very low and, when extracted from limited starting material (on average 40 mg eggs per sample in our study), too low to be detected by qPCR. In that case, DNA of laboratory and kit contaminants may be present in similar, or higher quantities than egg bacterial DNA.

Additionally, eukaryotic DNA of the insect may interfere with or be co-amplified by the 16S rRNA gene primers, besides other inhibitors and contaminants extracted with the DNA (Huys *et al.* 2008; Prosdocimi *et al.* 2015). The barcoded PCR of egg samples yielded little product after 30 cycles (< 5 ng DNA μl^{-1}) and the composition of PCR replicates showed low reproducibility. Zheng *et al.* (2013a) successfully sequenced the BSF egg microbiota but used 250 mg eggs. This suggests that with a higher amount of starting material, sequencing of egg-associated bacterial DNA can be successful.

Characterizing the egg-associated microbiota and quantifying its consistency within and among BSF populations over time, may provide insights into the flexibility of host-microbe associations in BSF and help explain the variability in members of a core community of BSF larvae across studies (Khamis *et al.* 2020; Wynants *et al.* 2019; **Chapter 3**). In the present study, *Providencia* was virtually absent, whereas it was strongly associated with larvae regardless of feed substrate in a previous study using eggs of the same BSF colony (**Chapter 3**). This suggests that besides variability due to host strain, there may be inter-batch variation in egg-associated microbiota. It would be very useful if future research would quantify this variation and investigate its causes.

Conclusion

Our study shows that substrate-associated microorganisms have a larger effect on BSF larval performance and microbiota than egg-associated microorganisms. Substrate-associated microorganisms increased larval biomass in chicken manure, and larval survival and biomass tended to be lower in autoclaved as compared to inoculated chicken feed. Besides, substrate-associated microorganisms increased substrate pH in chicken feed, likely related to increased ammonia production. In chicken manure, substrate-associated microorganisms accounted for major shifts in larval and substrate microbiota: autoclaving resulted in a high similarity between larval and substrate microbiota, different from the microbiota in the other manure treatments. This may indicate that the larval digestive or immune systems were triggered differently in this treatment compared to the other manure treatments.

Although previous studies showed that egg-associated bacteria can increase larval performance if applied to the substrate in higher concentrations, we found no such effect of the egg-associated microorganisms as present in resident concentrations on

the eggs. We also did not record an effect of egg-associated microorganisms on overall microbiota composition. However, their presence resulted in decreased pH in chicken feed and increased phylogenetic diversity of larval microbiota from chicken manure. In conclusion, we found large effects of substrate-associated microorganisms and only minor effects of egg-associated microorganisms, indicating that BSF producers would better focus on manipulation of the former to improve BSF performance and microbiological safety.

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Author contributions

JvL acquired funding for the study. SS planned and designed the study together with all authors. SS conducted the insect experiment, collected the samples and data, and processed samples for molecular analysis. SS analysed the data statistically with advice

from GH, HdV, and HS. SS interpreted data in collaboration with all authors. SS wrote the first version of the manuscript and processed comments from all authors. All authors read and approved the final manuscript.

Supplementary information

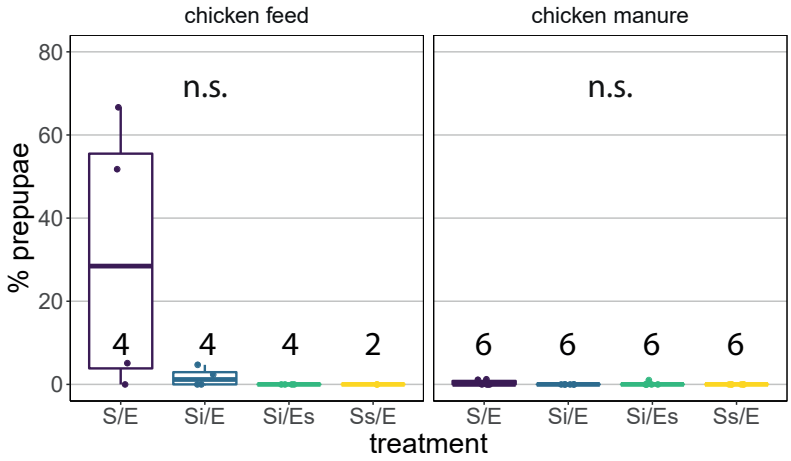

Figure S1. Percentage of prepupae on day 15, in chicken feed (left panel) and chicken manure (right). Treatment codes: S/E = control treatment (untreated substrate and untreated eggs), Si/E = sterilized substrate with inoculum and untreated eggs, Si/Es = sterilized substrate with inoculum and disinfected eggs, Ss/E = sterilized substrate and untreated eggs. Numbers in bars indicate sample sizes (number of containers). Data for chicken feed Ss/E are from day 22. N.s. = not significant (Kruskal-Wallis test, $\alpha = 0.05$).

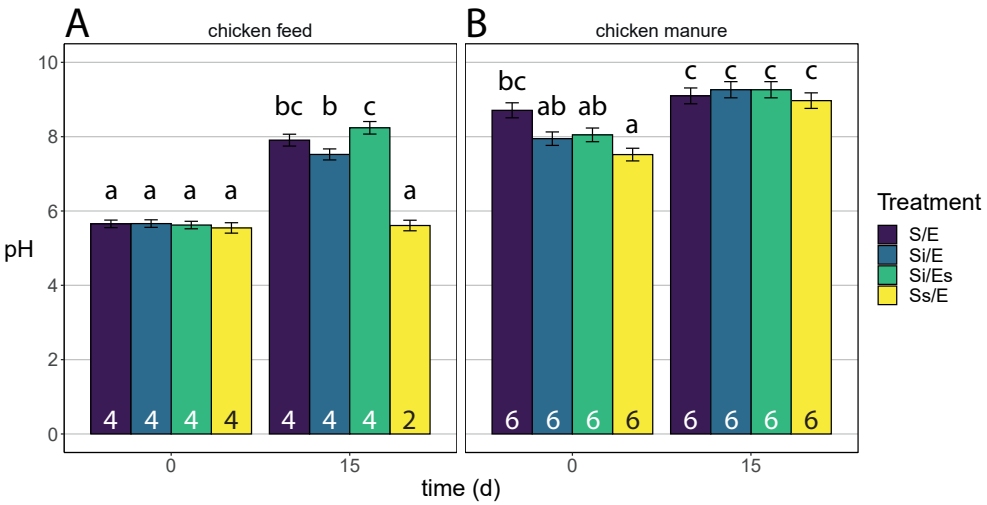

Figure S2. Substrate pH (mean ± SE) of A) chicken feed and B) chicken manure over time. Treatment codes: S/E = control treatment (untreated substrate and untreated eggs), Si/E = sterilized substrate with inoculum and untreated eggs, Si/Es = sterilized substrate with inoculum and disinfected eggs, Ss/E = sterilized substrate and untreated eggs. Numbers in bars indicate sample sizes (number of containers). Means without shared letters are significantly different ($\alpha = 0.05$; GLMM per feed substrate; posthoc comparisons with Tukey-corrected P-values).

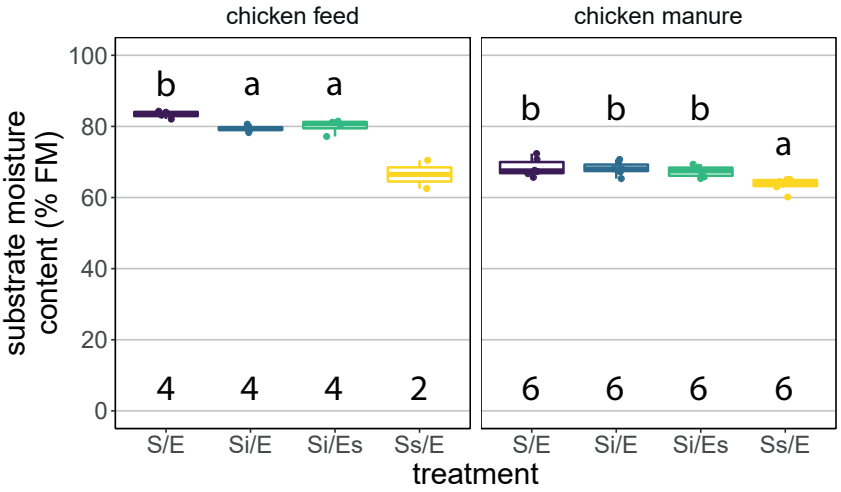

Figure S3. Boxplots of substrate moisture content at time of harvest (% fresh matter). Treatment codes: S/E = control treatment (untreated substrate and untreated eggs), Si/E = sterilized substrate with inoculum and untreated eggs, Si/Es = sterilized substrate with inoculum and disinfected eggs, Ss/E = sterilized substrate and untreated eggs. All treatments were harvested on day 15, except chicken feed Ss/E which was harvested on day 22 and therefore excluded from statistics. Numbers below bars indicate sample sizes. Medians with no shared letters are significantly different ($P < 0.05$, Kruskal-Wallis test with Wilcoxon posthoc comparisons and FDR-corrected P-values).

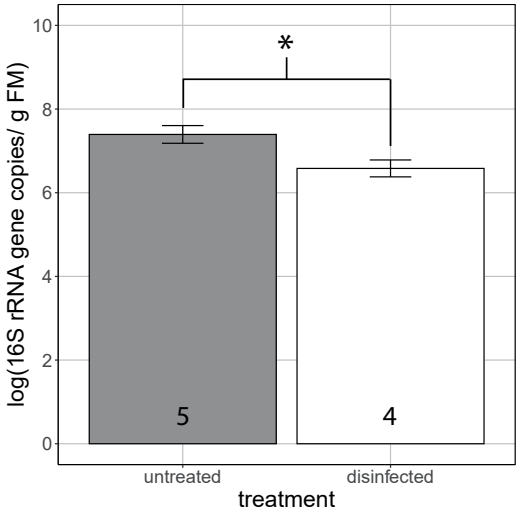

Figure S4. Log_{10} transformed 16S rRNA gene copies per g fresh matter sample material, in egg samples. Disinfected eggs have a lower 16S rRNA gene abundance than untreated eggs (GLMM, P = 0.002). Numbers in bars indicate sample sizes (number of batches).

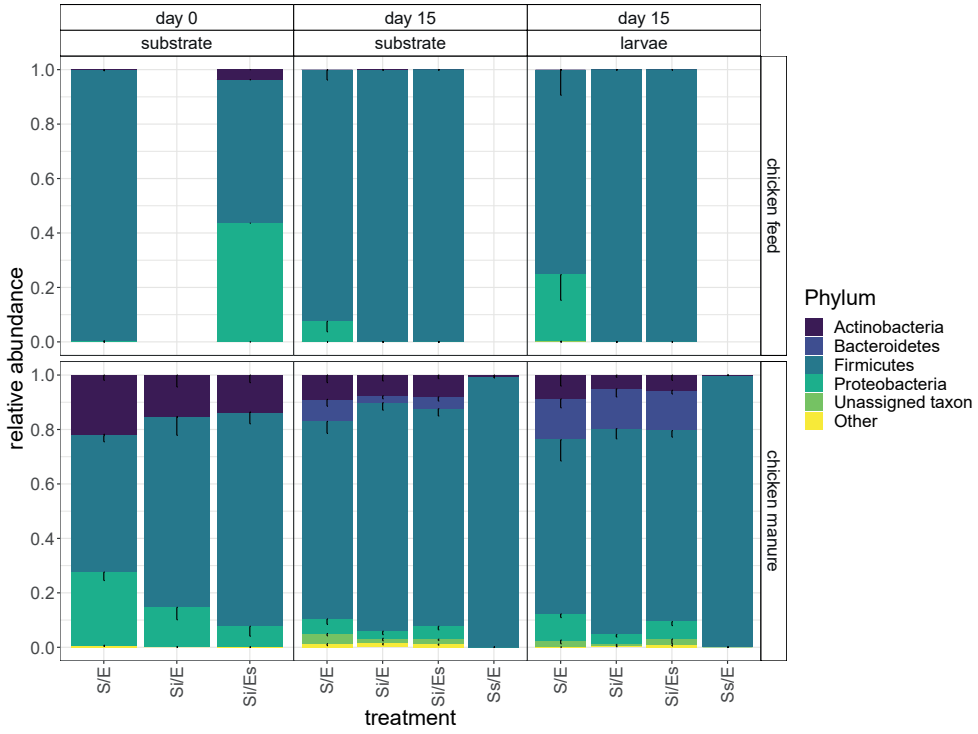

Figure S5. Relative abundance (mean – SE) of the five most abundant bacterial phyla in substrate and larvae samples from different treatments in both feed substrates on day 0 and 15. Treatment codes: S/E = control treatment (untreated substrate and untreated eggs), Si/E = sterilized substrate with inoculum and untreated eggs, Si/Es = sterilized substrate with inoculum and disinfected eggs, Ss/E = sterilized substrate and untreated eggs.

Table S1. Amount of feed (g dry matter) and moisture content (% of fresh matter) per feed substrate and treatment on day 0, mean ± SE. Treatment codes: S/E = control treatment (untreated substrate and untreated eggs), Si/E = sterilized substrate with inoculum and untreated eggs, Si/Es = sterilized substrate with inoculum and disinfected eggs, Ss/E = sterilized substrate and untreated eggs. Means that share no letters are significantly different (per parameter and per feed substrate, LMM with Tukey contrasts, $\alpha = 0.05$).

Feed substrate	Treatment	Amount of feed (g dry matter)	Moisture content (% fresh matter)
chicken feed	S/E	18.1 ± 0.3a	71.3 ± 0.4d
chicken feed	Si/E	19.9 ± 0.3b	68.4 ± 0.4b
chicken feed	Si/Es	20.0 ± 0.3bc	69.2 ± 0.4c
chicken feed	Ss/E	20.1 ± 0.3c	68.1 ± 0.4a
chicken manure	S/E	20.6 ± 0.4a	67.1 ± 0.5b
chicken manure	Si/E	22.1 ± 0.4b	65.9 ± 0.5a
chicken manure	Si/Es	21.9 ± 0.4b	65.8 ± 0.5a
chicken manure	Ss/E	22.0 ± 0.4b	65.9 ± 0.5a

Table S2. Analysis of deviance table for substrate pH of chicken feed, GLMM regression model.

Model term	df	χ^2	p
Treatment	3	60.20	< 0.001
Timepoint	1	609.56	< 0.001
Treatment × Timepoint	3	75.47	< 0.001

Table S3. Analysis of deviance table for substrate pH of chicken manure, GLMM regression model.

Model term	df	χ^2	p
Treatment	3	9.65	0.022
Timepoint	1	73.32	< 0.001
Treatment × Timepoint	3	13.32	0.004

Table S4. Total relative abundance (of all reads in dataset) of ASVs identified as contaminants, grouped per genus and ordered by relative abundance. The thirty most abundant genera are displayed, the rest is summed under "Other". Contaminant identification based on assessment of correlation plots between ASV relative abundance and DNA concentration of samples.

Genus	number of ASVs	number of reads	% of total reads
Ralstonia	62	494163	1.5469%
Unassigned taxon	26	80241	0.2512%
Clostridium_sensu_stricto_1	7	39161	0.1226%
Peptostreptococcaceae (unassigned genus)	7	30281	0.0948%
Fusobacterium	3	30187	0.0945%
Cupriavidus	5	23194	0.0726%
Turicibacter	1	11178	0.0350%
Syntrophococcus	5	9545	0.0299%
Candidatus_Nucleicultrix	1	9320	0.0292%
Lactobacillus	6	6234	0.0195%
Subdoligranulum	6	4987	0.0156%
Burkholderiaceae (unassigned genus)	1	4737	0.0148%
Achromobacter	1	3259	0.0102%
Stenotrophomonas	1	3083	0.0097%
Sphingomonas	4	2893	0.0091%
Shewanella	2	2740	0.0086%
Desulfovibrio	1	2594	0.0081%
Shuttleworthia	1	2321	0.0073%
Aquabacterium	1	1954	0.0061%
Staphylococcus	3	1713	0.0054%
Holdemanella	1	1522	0.0048%
Collinsella	1	1478	0.0046%
Blautia	3	1442	0.0045%
Catenisphaera	1	1411	0.0044%
Solobacterium	2	1390	0.0044%
Catenibacterium	1	1372	0.0043%
Delftia	1	1115	0.0035%
Faecalibacterium	1	950	0.0030%
Methylobacterium	2	947	0.0030%
Lactobacillaceae (unassigned genus)	2	896	0.0028%
Other	29	8440	0.0264%
Total	**188**	**784748**	**2.4565%**

Table S5. Spearman rank correlations (mean ± SD) between technical replicates of samples. Treatment codes: S/E = control treatment (untreated substrate and untreated eggs), Si/Es = sterilized substrate with inoculum and disinfected eggs.

Replication	Sample ID	Sample type	Feed substrate	Treatment	Timepoint	N replicates	Spearman r
DNA extraction	15.K	substrate	chicken feed	S/E	0	4	1.000 ± 0
DNA extraction	15.N	larvae	chicken feed	S/E	15	4	0.968 ± 0.035
DNA extraction	18.K	substrate	chicken manure	Si/Es	0	4	0.828 ± 0.104
DNA extraction	32.K	substrate	chicken manure	S/E	0	4	0.940 ± 0.007
DNA extraction	30.N	larvae	chicken manure	S/E	15	4	0.958 ± 0.008
PCR	16.K	substrate	chicken feed	S/E	0	2	1.000
PCR	15.N	larvae	chicken feed	S/E	15	2	1.000
PCR	18.N	larvae	chicken manure	Si/Es	15	2	0.972
PCR	33.M	substrate	chicken manure	Si/Es	15	2	0.954

Table S6. Output of permutational multivariate ANOVA of weighted UniFrac dbRDA of chicken feed on day 15. 999 permutations, stratified for container ID. R^2 = 54%.

Model term	df	SSq	F	P	% explained
Treatment	2	0.509	8.103	0.002	41.0%
Sample type	1	0.048	1.532	0.017	3.9%
Treatment × Sample type	2	0.119	1.886	0.002	9.5%
Residual	18	0.566			

Table S7. Output of permutational multivariate ANOVA of weighted UniFrac dbRDA of chicken manure on day 15. 999 permutations, stratified for container ID. R^2 = 75%.

Model term	df	SSq	F	P	% explained
Treatment	3	2.665	32.350	0.001	63.3%
Sample type	1	0.325	11.837	0.001	7.7%
Treatment × Sample type	3	0.179	2.167	0.001	4.2%
Residual	38	1.043			

Table S8. Genera with differential relative abundance among larval microbiota of different treatments of chicken manure on day 15. Treatment codes: S/E = control treatment (untreated substrate and untreated eggs), Si/E = sterilized substrate with inoculum and untreated eggs, Si/Es = sterilized substrate with inoculum and disinfected eggs, Ss/E = sterilized substrate and untreated eggs. Kruskal-Wallis test and posthoc Wilcoxon tests, with FDR-corrected P-values. Treatment groups without shared letters have significantly different medians (α = 0.05), letter a is lowest median. * = only present in S/E.

Genus	P	Multiple comparisons			
		S/E	Si/E	Si/Es	Ss/E
Amphibacillus	0.010	b	b	b	a
Bacillaceae (unassigned)	0.004	b	b	c	a
Clostridia order MBA03 (uncultured)	0.021	b	ab	b	a
Enteractinococcus	0.007	b	b	b	a
Glutamicibacter	0.021	b	ab	ab	a
Gottschalkia	0.004	b	c	c	a
Planomicrobium	0.029	ab	b	b	a
Proteiniphilum	0.007	b	b	b	a
Pseudogracilibacillus	0.006	b	b	b	a
Ruminococcaceae (unassigned)	0.007	b	b	b	a
Sphingobacteriaceae (uncultured)	0.007	a	b	b	a
Thiopseudomonas	0.004	*			

Chapter 5

General discussion

Introduction

The black soldier fly *Hermetia illucens* (L.), BSF, is a tropical fly species that receives increasing attention in science and industry for its suitability as a livestock feed ingredient (Van Huis 2020). BSF larvae can improve the sustainability of food production, by partially replacing soymeal and fishmeal in livestock feed (Smetana *et al.* 2019). Soybean cultivation is linked to deforestation in the tropics, concurrent carbon emissions and biodiversity loss, as well as environmental costs of transoceanic freight (Gasparri *et al.* 2013; Gasparri *et al.* 2016; He *et al.* 2019; Taherzadeh & Caro 2019). Fishmeal is mainly produced from wild-caught fish, associated with overfishing and marine biodiversity loss (Alder *et al.* 2008; FAO 2016; Smith *et al.* 2011). BSF larvae, however, can be grown on a wide range of organic by-products and side streams from the food production system (Barragán-Fonseca *et al.* 2017; Cickova *et al.* 2015; Pastor *et al.* 2015). The annual wastage of over 1 billion tons of food is a major problem in global food security (Alexander *et al.* 2017b; FAO 2011), which is all the more pressing with a world population that is projected to increase to over 10 billion people in 2050 (United Nations 2019) and a concomitant growth in demand for animal protein (Alexandratos & Bruinsma 2012). By using organic waste as feed, BSF production requires much less land than used for crops and can be competitive in sustainability compared to fishmeal and soymeal (Alexander *et al.* 2017a; Smetana *et al.* 2016; Smetana *et al.* 2019; Van Huis & Oonincx 2017).

Partial replacement of soymeal and fishmeal in livestock and aquaculture feeds yields comparable animal growth, health, and product quality (Chia *et al.* 2019b; Dörper *et al.* in press; Gasco *et al.* 2019; Henry *et al.* 2015; Moula & Detilleux 2019). Although n-3 polyunsaturated fatty acids generally decrease in fish/livestock products when fed insect-based diets (Gasco *et al.* 2019), these fatty acids can be enriched in BSF larvae depending on their diet (Barroso *et al.* 2019; Oonincx *et al.* 2020; St-Hilaire *et al.* 2007). Additionally, BSF larval meal in animal feed can improve immunological parameters in pigs, poultry, and fish, possibly through lauric acid, chitin, and antimicrobials (Dörper *et al.* in press; Spranghers *et al.* 2018; Xiao *et al.* 2018b).

The efficiency with which BSF larvae convert organic waste into insect biomass, however, varies considerably among waste streams and studies (Bosch *et al.* 2019; Lalander *et al.* 2019). Besides the influence of *e.g.* macronutrients (Barragán-Fonseca *et al.* 2019; Barragán-Fonseca *et al.* 2018b; Gold *et al.* 2020b), this variability may be due to variation

in composition and functioning of the microbial community associated with the waste substrate and larval gut (De Smet *et al.* 2018; Gold *et al.* 2018). As saprophagous animals, BSF larvae share their food source with a microbial community of bacteria (Jiang *et al.* 2019), fungi (Boccazzi *et al.* 2017; Zhang *et al.* 2021), viruses (Chen *et al.* 2019), archaea (Klammsteiner *et al.* 2020) and possibly protists (Benbow *et al.* 2019; Gurung *et al.* 2019).

Additionally, several studies have detected food pathogens in edible insects, emphasizing the need for measures to control microbiological safety of insect products, *e.g.* by standardizing company hygiene or heat treatment of insect products (Campbell *et al.* 2020; EFSA 2015; Klunder *et al.* 2012; Stoops *et al.* 2016; Vandeweyer *et al.* 2017; Wynants *et al.* 2019). Although intuitively this risk may seem even higher when BSF larvae are fed organic waste, this does not need to be true. In livestock manure and aquaculture waste spiked with *Escherichia coli* or *Salmonella spp.*, BSF larvae decreased populations of said pathogens (Erickson *et al.* 2004; Lalander *et al.* 2013; Liu *et al.* 2008; Lopes *et al.* 2020). One reason for this suppression may be that BSF possesses the largest known repertoire of genes encoding antimicrobial peptides in insects so far (Zhan *et al.* 2020) and produces different antimicrobial peptides and lysozymes depending on the feed substrate (Vogel *et al.* 2018).

When reared on biowaste, BSF larvae may be challenged not only by the substrate-associated microbiota, but also by chemical contaminants. These chemicals, such as toxins, pharmaceuticals, pesticides, and heavy metals, are potential hazards for human and livestock health, if not for BSF health (EFSA 2015; Van der Spiegel *et al.* 2013). Some heavy metals such as cadmium and zinc can accumulate in BSF larvae (Bulak *et al.* 2018; Wang *et al.* 2019; Wu *et al.* 2020), and can alter BSF microbiota composition (Wu *et al.* 2020). On the other hand, mycotoxins are not accumulated but degraded by BSF larvae (Bosch *et al.* 2017; Camenzuli *et al.* 2018; Leni *et al.* 2019; Meijer *et al.* 2019), and so are certain pharmaceuticals and pesticides (Lalander *et al.* 2016). However, very little is known so far on BSF tolerance of plant toxins and the fate of these toxins in BSF larvae.

In this thesis, I aimed to study the performance and microbial ecology of BSF larvae on different feed substrates. Understanding BSF microbial ecology may contribute to controlling and improving bioconversion efficiency, BSF production, and product quality and safety. In **Chapter 2**, I assessed larval performance on feed substrates with

varying proportions of oilseed by-products containing plant toxins and economically interesting fatty acids. **Chapters 3** and **4** aimed to explore the contributions of feed substrate, larval density, and egg-associated microorganisms on the variation in microbiota in larvae and substrate. **Chapter 3** assessed to what extent BSF larvae can control their own microbiota and manipulate substrate microbiota. To that purpose, I quantified the relative importance of larval density and feed substrate on bacterial community dynamics over time. **Chapter 4** assessed the contributions of egg-associated and substrate-associated microorganisms to bacterial community assembly and larval performance. This chapter gave an indication of the relative importance of potentially vertically (egg-associated) versus horizontally (substrate-associated) transmitted microorganisms. In this synthesis I discuss the implications of my findings for our understanding of BSF-microbe interactions embedded in ecological theory (Christian *et al.* 2015; Costello *et al.* 2012), address potential future scientific endeavours, and envisage the possible applications of this knowledge.

Host control of the gut microbiome

An animal microbiome can be viewed as an ecological community or ecosystem that is under some control by the host (Christian *et al.* 2015; Costello *et al.* 2012; Coyte *et al.* 2015; Douglas & Werren 2016; Savage 1977); its functions can be viewed as ecosystem services provided to the host (McKenney *et al.* 2018). A more specific framework is the holobiont / hologenome theory of evolution, where the holobiont (host and associated microbiome) is regarded as a primary unit of natural selection (Rosenberg & Zilber-Rosenberg 2013; Zilber-Rosenberg & Rosenberg 2008). There is, however, a heated debate on the applicability of the assumptions underlying this framework, especially concerning host-microbiome coevolution (Bordenstein & Theis 2015; Douglas & Werren 2016; Hurst 2017; Moran & Sloan 2015; Theis *et al.* 2016). In this synthesis, I argue that vertical transmission of microorganisms in BSF thus far appears of minor importance to host performance, and that the associations between BSF host and microorganisms are dynamic within and among (captive) host populations. Therefore, I decided to discuss the BSF microbiome in the wider concept of community ecology without the restrictive assumptions of the holobiont framework.

That being said, despite large variation in BSF gut microbiota composition, the BSF host has a range of mechanisms at its disposal to influence its gut microbiota. The

BSF gut in itself poses strong constraints on ingested and resident microorganisms, via physicochemical properties of the gut, host digestive enzymes, and the host immune system (Bonelli *et al.* 2019; Bruno *et al.* 2019b; De Smet *et al.* 2018; Gold *et al.* 2018). Some of these selection pressures are relatively stable, such as the low and high luminal pH in middle and posterior midgut, respectively, the oxygenation of gut regions, the peritrophic membrane forming a barrier between midgut lumen and epithelia, and a cuticle layer protecting the epithelia in other gut compartments (Bonelli *et al.* 2020; Bonelli *et al.* 2019; Engel & Moran 2013). Others are dynamic in response to the gut microbiota and feed substrate, such as the composition and concentrations of digestive enzymes and immune compounds (Bonelli *et al.* 2020; Vogel *et al.* 2018; Zhan *et al.* 2020).

BSF has an extensive repertoire of gene families encoding receptor proteins and immune compounds (Zhan *et al.* 2020). The diet-dependent expression of these genes enables BSF larvae to target defence against specific microorganisms ingested with the substrate (Vogel *et al.* 2018). This suggests that BSF larvae are able to "garden" their intestinal microbiome similar to epithelial cells in the intestines of humans and mice (Jacobs & Braun 2014). In humans, gut microbiome stability is achieved by limiting positive feedbacks through immune suppression, compartmentalization, and epithelial feeding of microbial community members (Coyte *et al.* 2015). Predatory phages possibly provide an additional restriction on positive feedbacks by infecting specific bacteria, resulting in "kill-the-winner" dynamics, *i.e.* a type of Lotka-Volterra dynamics for phage and bacterial populations (Lim *et al.* 2015; Mirzaei & Maurice 2017; Sharon *et al.* 2013). In BSF, immune function and gut compartmentalization may play similar roles in promoting stability as suggested for humans; the importance of predatory phages and of BSF gut epithelial feeding of nutrients to microbes is unknown so far.

Beyond the gut: BSF larval impact on substrate microbiota

BSF larvae forage in an aggregation, or maggot mass. Larval aggregation may improve larval digestion of the substrate, accelerate development, and reduce susceptibility to toxins (Green *et al.* 2002; Green *et al.* 2003), but can also increase intraspecific competition for food (Barragán-Fonseca *et al.* 2018a; Diener *et al.* 2009; Dzepe *et al.* 2020; Jones *et al.* 2019; Parra Paz *et al.* 2015).

BSF larval foraging changes the relative abundance of dominant bacterial taxa in the substrate, and this impact is different depending on the type of feed substrate (**Chapter 3**). BSF larval aggregation can impact microbiota via heat generation, substrate aeration, fragmentation of food particles, increase in pH, and secretion of enzymes and antimicrobials. The generation of heat from a larval aggregation (Parra Paz *et al.* 2015; Slone & Gruner 2007; Turner & Howard 1992) may drastically change microbiota, because temperature can be a major determinant of BSF microbiota composition (Raimondi *et al.* 2020). Possibly related to the increased local temperature, larval foraging increases the decomposition rate of the substrate (Jiang *et al.* 2019). Besides, BSF larvae alter microbial metabolism (Beskin *et al.* 2018; Jiang *et al.* 2019; Zhang *et al.* 2021).

Interactions within the gut microbiome

Besides host-microbe interactions, there can be diverse interactions between gut microorganisms, including competition, cooperation, predation, commensalism, and amensalism. Fungi and bacteria can compete for nutrients. In order to kill competitors, fungi can produce antimicrobial compounds such as aflatoxins (Arai *et al.* 1967), and bacteria can produce bacteriocins (Granato *et al.* 2019). At the same time, bacterial species may cooperate by syntrophy (cross-feeding), *i.e.* exchanging metabolites as nutrients (Coyte *et al.* 2015). Microbial cells communicate via chemicals that can initiate collective behaviour, *e.g.* swarming or biofilm formation, by a process called quorum sensing; other microbial species or the host animal can interfere with this chemical communication via quorum quenching (Jordan *et al.* 2016). The role of quorum sensing and quorum quenching in the BSF gut is still unknown. In addition, gut microorganisms may exchange genetic material via horizontal gene transfer through bacterial conjugation or phage transformation (Cai *et al.* 2018b; Keen *et al.* 2017; Liu *et al.* 2020; Mukhopadhya *et al.* 2019).

Bacteriophages and mycoviruses act as predator analogues feeding on bacteria and fungi, respectively (Christian *et al.* 2015; Gurung *et al.* 2019; Hunter 2013; Mukhopadhya *et al.* 2019; Xie & Jiang 2014). The virome in the human gut outnumbers the bacterial cells by up to 10:1 (Mukhopadhya *et al.* 2019). The BSF virome is largely unknown but a recent study described a novel bacteriophage of *E. coli* from the BSF gut (Chen *et al.* 2019). Moreover, their results suggested that prophage induction, *i.e.* the switch from the latent to the virulent form of the phage, depends on the type of substrate. Protists

may be another group of microbial predators in the gut. They play key roles in soil microbial food webs as consumers of bacteria, fungi, and other microeukaryotes (Gao *et al.* 2019b; Geisen *et al.* 2018), but also include cellulose-degrading symbionts in the hindgut of lower termites (Ohkuma 2008).

Black soldier fly digestion: the role of the gut microbiome in larval performance

The dietary flexibility of BSF larvae is directly related to the combined digestive capabilities of the larva and its gut microbiota (De Smet *et al.* 2018; Gold *et al.* 2018). The BSF gut microbiome can expand the nutritional capacities of its host, providing essential nutrients or breaking down recalcitrant compounds (De Smet *et al.* 2018; Douglas 2015; Engel & Moran 2013; Gold *et al.* 2018; Wang *et al.* 2020).

BSF larvae may depend on microorganisms for survival and growth, similar to the saprophagous larvae of the stable fly *Stomoxys calcitrans* and housefly *Musca domestica* (Lam *et al.* 2009b; Lysyk *et al.* 1999; Schmidtmann & Martin 1992). There was no 100% sterile treatment in **Chapter 4**, but the large reductions in bacterial load (measured by qPCR) after substrate autoclaving did result in retarded larval growth. In the same chapter, egg-associated microorganisms did not affect larval performance in the concentrations present on the eggs, although vitamin supplementation may have obscured a role of microorganisms in providing these essential nutrients to the host. Egg-associated bacteria can improve BSF hatching rate (Yang *et al.* 2018) and, depending on the number and mixture of bacterial strains in an inoculum, they can improve larval performance when applied in high concentrations to the feed substrate (Mazza *et al.* 2020; Yu *et al.* 2011). Preliminary studies also found that BSF larvae have reduced growth and survival when fed sterilised substrates (Gold *et al.* 2020a; Portela Cardenas 2020). In another preliminary study, I observed that sterile BSF larvae fed sterile chicken feed were delayed for at least two weeks in development and growth but recovered after that; for most of that delay, larvae seemed to be in first instar. Mouth morphology is conserved across BSF larval instars but increases in complexity with larval development (Bruno *et al.* 2020). My hypothesis is that the first instar depends more on nutrients derived from digestion of bacteria than later instars, because its mouthparts may only sieve fine food particles and may lack the strength to grind coarse food particles compared to later instars. Growth assays on sterile diet starting with sterile larvae of different instars could test this hypothesis.

The digestive activities of BSF and gut microbes are flexible in response to the diet (Bonelli *et al.* 2020; Gold *et al.* 2020c). The BSF genome encodes a diversity of enzymes, which can be differentially expressed depending on larval age and diet (Bonelli *et al.* 2020; Pimentel *et al.* 2017; Zhan *et al.* 2020; Zhu *et al.* 2019). Bacteria isolated from the BSF larval gut produce amylases, proteases, and lipases (Kim *et al.* 2014; Ng *et al.* 2019), in addition to endogenous host enzymes. Moreover, several gut bacterial isolates produce cellulases and enzymes involved in phosphate metabolism and nitrogen cycling, complementing BSF host metabolism (Callegari *et al.* 2020; Kim *et al.* 2014; Lee *et al.* 2014; Ng *et al.* 2019; Yu *et al.* 2010). These bacteria may also be dispersed into the feed substrate by BSF (Jiang *et al.* 2019) or *M. domestica* (Zhao *et al.* 2017), to function as an "external rumen" (Louzada & Nichols 2012). Inoculating feed substrates with these bacteria can significantly improve BSF growth (Callegari *et al.* 2020; Yu *et al.* 2011). In addition, it has been found that inoculation of substrates with bacteria or yeasts can alter protein content, fat content, and fatty acid composition of BSF larvae (Abduh *et al.* 2017; Mazza *et al.* 2020; Richard *et al.* 2019; Wong *et al.* 2020).

BSF larvae possess a wide range of detoxification enzymes (Zhan *et al.* 2020), and their gut microbiota may have additional roles in detoxification. BSF larvae can tolerate and degrade high concentrations of mycotoxins (Bosch *et al.* 2017; Camenzuli *et al.* 2018; Leni *et al.* 2019; Meijer *et al.* 2019), but the relative contributions of host and microbiota to this degradation remain unknown. Bacteria can degrade mycotoxins (Hathout & Aly 2014). BSF gut microbiota may rapidly degrade antibiotics through multiplication of bacteria containing antibiotic resistance genes, possibly enhanced by horizontal gene transfer as observed in *M. domestica* (Wang *et al.* 2017). Liu *et al.* (2020) showed that the antibiotic oxytetracycline altered BSF gut microbiota composition, with *Providencia*, *Enterococcus*, and *Proteus* linked to most antibiotic resistance genes. Non-sterile BSF larvae degraded tetracycline at a faster rate compared to sterile larvae in chicken manure, indicating an important role of gut microbiota (Cai *et al.* 2018b).

In **Chapter 2**, BSF larvae appeared tolerant to certain levels of glucosinolates and their highly toxic degradation products, isothiocyanates and nitriles, from camelina and crambe oilseed by-products. It is unknown to what extent these toxins were degraded by BSF or their microbiota. Isothiocyanates have antimicrobial activity and thus may select for resistant gut bacteria (Dufour *et al.* 2015). Herbivorous insects can contain microorganisms that degrade the plant toxins they are consistently exposed to (Hammer

& Bowers 2015; Mason *et al.* 2014; Van den Bosch & Welte 2017). Future studies should investigate to what extent the saprophagous BSF and its gut microbiota can metabolize plant toxins, in order to determine the fate of these toxins and implications for chemical safety. Additionally, transcriptomics may identify the detoxification genes involved (Bonelli *et al.* 2020). Depending on the occurrence of such plant toxins in organic waste streams, and the severity of antinutritional effects on livestock or humans, this research can have high priority in order to allow the use of particular crop-derived biowastes for BSF conversion.

Apart from bacteria, BSF larvae harbour a diet-dependent fungal community (Boccazzi *et al.* 2017; Zhang *et al.* 2021) but it is unclear how this influences the bacteria (Boucias *et al.* 2018) and BSF larvae. In **Chapter 3**, we observed considerable fungal overgrowth of chicken feed, which can reduce neonate survival (Samayoa *et al.* 2016). However, fungi may also be capable of degrading recalcitrant chemicals and toxins (Dowd 1992), and recent studies showed that supplementation of feed substrates with specific yeasts or fungi improved BSF performance (Isibika *et al.* 2019; Richard *et al.* 2019; Wong *et al.* 2020).

Microbial volatile organic compounds may play an important role as chemical cues indicating *e.g.* nutrient quality, microbial activity, and presence of competitors (Cammack *et al.* 2016; Davis *et al.* 2013). Adult flies, including BSF, are well-known to respond to microbial volatiles in their oviposition behaviour (Yang *et al.* 2017; Zheng *et al.* 2013b). Larvae of other saprophagous fly species can smell a wide range of volatile organic compounds (Cobb 1999), use such chemical cues *e.g.* to detect microorganisms able to produce essential amino acids and alter their foraging behaviour accordingly (Rhinesmith-Carranza *et al.* 2018). BSF larvae seem to have a similar ability to detect, and respond to, volatile or non-volatile microbial metabolites in a feed substrate. In one example, BSF larvae preferred to feed on pig manure rather than plant by-products regardless of age and previous diet, possibly due to microbial diversity and the associated chemosensory cues (Parodi *et al.* 2020b). Future studies using choice assays with feed substrates of different composition of nutrients and microorganisms (Parodi *et al.* 2020b; Rhinesmith-Carranza *et al.* 2018), may not only inform which nutrients are essential in larval foraging behaviour, but also which volatile compounds the larvae use in order to assess these differences – if combined with techniques to analyse volatile blends, such as gas chromatography.

Undoubtedly, more functions of BSF gut microbiota await discovery. Rearing BSF larvae on substrates with nutrient deficiencies, nutritional imbalance, xenobiotics, or recalcitrant compounds, may reveal specific roles of the gut microorganisms that were not revealed in the biowaste types studied so far. Moreover, experimentally eliminating microbial components of substrate and/or eggs (like in **Chapter 4**) will quantify their importance for BSF performance in such challenging substrates. Besides aiding in digestion and producing essential nutrients, the gut microbiota may also be involved in modulating host immune defence and host gut morphology (Bonelli *et al.* 2019; Broderick & Lemaitre 2012; Bruno *et al.* 2019b). The mechanisms behind these functions are still unknown.

Community assembly of the larval gut microbiome

Microorganisms originating from the environment (including the feed substrate) and the insect eggs can colonize the BSF larval gut (Crippen *et al.* 2016; Wynants *et al.* 2019; Zheng *et al.* 2013a). By inactivating or eliminating the microorganisms of these potential sources in **Chapter 4**, I found that the egg microbial source had only minor effects on BSF bacterial community assembly compared to the feed substrate source. This suggests that most bacteria colonize the BSF larva via horizontal transmission rather than vertical transmission.

In order for gut microbes to be vertically transmitted, they need to survive the drastic remodelling of the host gut during metamorphosis (Bruno *et al.* 2019a; Hammer & Moran 2019), be passed on from adult to egg, and establish in the larval gut. Zheng *et al.* (2013a) suggested that BSF may vertically transmit bacteria from the genus *Providencia*, because this genus was found in all life stages of the insect. But egg-associated bacteria may also come from the oviposition substrate. A generic technique providing more conclusive evidence would be *in vivo* stable isotope probing of bacteria in the BSF parent generation followed by Illumina sequencing of microbiota in the larval offspring (Alonso-Pernas *et al.* 2017). This would enable tracking of bacteria across host generations and besides may reveal their functions in host metabolism (Alonso-Pernas *et al.* 2017). An alternative but more selective method to test for vertical transmission of specific bacterial taxa, would be to feed the BSF mother with fluorescent protein-tagged bacteria and trace these in the larval offspring (Prosdocimi *et al.* 2015; Teh *et al.* 2016). The same labelling techniques can be used to track microbial inocula applied to the feed

substrate or insect eggs. It is important to determine which microbes are transmitted and how, *i.e.* vertically or horizontally. If there is a role of the larval feed substrate in the transmission of microbes from adult to offspring, BSF producers may want to tailor their substrates to safeguard transmission and persistence of specific microbes that benefit reproduction and/or microbial safety.

The strong influence of the feed substrate on larval microbiota calls into question the existence of a core microbiome of BSF larvae. There is large variation among studies in bacterial taxa that are more abundant in the larval gut than the substrate, or are shared across larvae reared on different substrates, which in these cases are regarded as core taxa (Bruno *et al.* 2019b; De Smet *et al.* 2018; Khamis *et al.* 2020; Klammsteiner *et al.* 2020; Wynants *et al.* 2019; **Chapters 3** and **4**). Nonetheless, my comparison focused on the dominant taxa and on those taxa identified as core by the authors of the respective studies. A meta-analysis comparing the complete datasets of 16S rRNA amplicon sequencing studies of BSF to date, including rare taxa, would provide more conclusive evidence in this debate. As discussed in **Chapter 3**, BSF larvae may rather be selecting for microbial metabolic functions instead of specific taxa (Vogel *et al.* 2018). If such selection pressures are consistent across larvae fed different feed substrates, a functional core may exist across these larvae, with different – but functionally redundant – taxa maintained upon ingestion from each substrate (Lemanceau *et al.* 2017; Risely 2020). For example, Ao *et al.* (in press) found that KEGG-predicted microbial metabolic functions were comparable among BSF fed chicken manure or swine manure despite differences in community composition. However, considering the diversity of biowaste substrates that BSF larvae can process and the fact that BSF physiology can be substrate-dependent (Bonelli *et al.* 2020; Vogel *et al.* 2018), it is even questionable whether a functional core microbiome exists.

The large contribution of the feed substrate to bacterial community assembly in BSF larvae (**Chapter 4**) adds a new dimension to the role of nursery diets in BSF experiments and commercial BSF production. Defining the nursery diet as the first feed substrate the neonate larvae are exposed to, it can be assumed that microorganisms from this diet are among the first to colonize the neonate larval gut, and therefore make a major contribution to the gut microbiota (Bruno *et al.* 2019b; Klammsteiner *et al.* 2020; **Chapters 3** and **4**). Any study that wishes to partition the BSF larval gut microbiota into its separate microbial sources, should include microbial analysis of the nursery diets

used. For example, Jiang *et al.* (2019) partitioned the gut bacterial community of BSF larvae fed food waste into a "larval gut" source and "raw food waste" source, but larvae were fed a nursery diet for five days prior to the experiment. Thus, their analysis may in fact reflect the contributions of bacterial taxa from the nursery diet and the subsequent experimental diet of food waste. From that perspective, the study by Jiang *et al.* (2019) would suggest that bacteria from the nursery diet dominate the BSF gut until six days after the diet shift, but thereafter bacteria originating from the food waste substrate dominate. Fungal community composition of the BSF larval gut also changes when larvae shift from one diet to another and depends on the exposure time of the larvae to each diet (Boccazzi *et al.* 2017).

Disturbances of the BSF gut microbiome

Intestinal microbial communities change in composition over the course of host development and this process of succession may eventually result in a stable, complex microbiota (Savage 1977). This concept of a climax community is, however, much disputed in traditional ecology as well as in the context of the gut microbiome (Fierer *et al.* 2012). Although BSF are short-lived and the larvae usually forage only for a few weeks before metamorphosis, their feed substrate decomposes quickly and microbiota composition changes concomitantly. BSF microbiota composition changes during the lifetime of the host (Ao *et al.* in press; Cifuentes *et al.* 2020; Jiang *et al.* 2019; Klammsteiner *et al.* 2020; Raimondi *et al.* 2020; Zheng *et al.* 2013a; **Chapter 3**). Microbial succession patterns in the larval gut depend on the type of feed substrate, larval density, and temperature (Jeon *et al.* 2011; Klammsteiner *et al.* 2020; Raimondi *et al.* 2020; Zhang *et al.* 2021; **Chapter 3**). Microbial succession in the BSF gut is regularly disrupted during host development due to removal of resident microbes or immigration of new ones, and therefore it is unlikely that the BSF gut microbiome reaches a stable composition (Figure 1).

Intrinsic disturbances

Some disturbances are inherent to host development and result in a removal of microorganisms from the BSF gut. During its development, the BSF host larva undergoes rapid growth while passing through six moults (Barros *et al.* 2019; Bruno *et al.* 2020; Kim *et al.* 2010), where head capsule and appendages, foregut, and hindgut

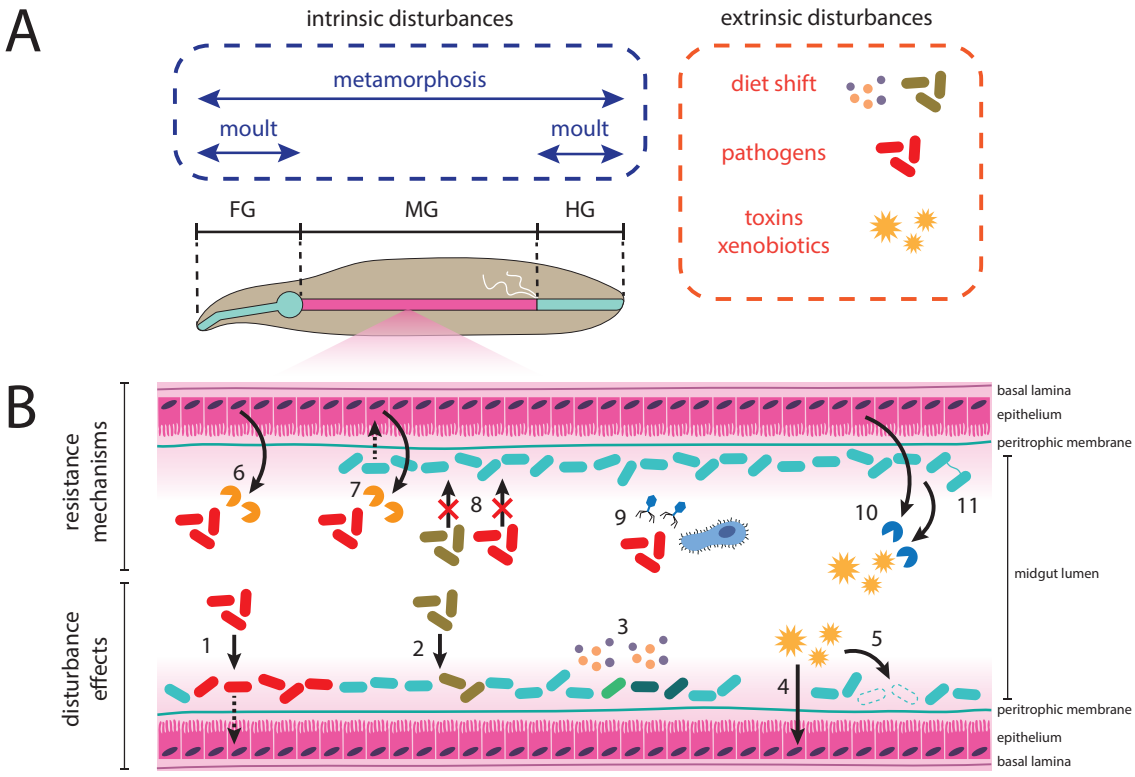

Figure 1. The potential disturbance and resistance mechanisms in the gut of the black soldier fly (BSF). A: The BSF host is exposed to intrinsic and extrinsic disturbances during its development. Inherent to host development, moults and metamorphosis cause intrinsic disturbances to the gut microbiota through expulsion of epithelia and lumen contents. The larva passes through six moults in which the epithelium of the foregut (FG) and hindgut (HG) are replaced, but midgut (MG) is retained. Extrinsic disturbances are mainly caused by diet shifts, including a change in ingested nutrients and microbiota, but the diet can also introduce pathogens, toxins, and xenobiotics into the gut. B: In the absence of resistance, the extrinsic disturbances can disrupt the gut microbiota in several ways. Pathogens may colonize the gut, drastically change gut microbiota composition and functioning, and result in reduced host health (1). Similarly, other ingested microorganisms may establish in the gut, but may not necessarily be detrimental to the host (2). A change in the composition of ingested nutrients can favour some resident microorganisms over others, with differential multiplication as a result (3). Xenobiotics and toxins may directly affect host tissues and physiology (4) or kill resident microbiota (5). However, the black soldier fly host and gut microbiota may resist these disturbances in a diversity of ways: host immune function is extensive and targets potential pathogens (6), possibly aided by the colonization resistance of the resident gut microbiota through immune priming (7) or competition for niches and nutrients (8). In addition, predatory phages and protists may keep microbial populations in check (9). Toxins and xenobiotics may be metabolized by endogenous host enzymes (10) or gut microorganisms, in which resistance genes can spread through horizontal gene transfer (11). Cyan, dark bluegreen, and green rod-shaped symbols are resident gut microbes. Illustration by author, partly based on figures in Engel & Moran (2013), Gold *et al.* (2018), and Bonelli *et al.* (2019).

are shed together with the larval skin (Engel & Moran 2013). Since the midgut remains intact, however, these moults may have limited impact on gut microbiota. Nonetheless, the peritrophic membrane inside the midgut is continuously renewed and shed and gut contents are transported to the posterior end of the gut (Engel & Moran 2013). The residence time of food in the BSF larval midgut is estimated to be 154 – 195 min, depending on the diet (Gold *et al.* 2020c).

The gut microbiota is much more drastically disturbed by the metamorphosis of the immature into the adult BSF. During this transformation, the entire gut is disintegrated and remodelled within the puparium (Barros-Cordeiro *et al.* 2014; Bruno *et al.* 2019a; Li *et al.* 2016). Much of the gut contents is expelled as meconium (Nayduch & Burrus 2017). Some microbes can survive this process into the adult fly via transstadial carriage, as shown in *M. domestica* and *S. calcitrans* (Nayduch & Burrus 2017; Rochon *et al.* 2005; Zurek & Nayduch 2016). These microbial species have the potential to be vertically transmitted from the adult female fly to her offspring during egg deposition. In one study, BSF adults and eggs shared several bacterial genera, among which *Providencia* (Zheng *et al.* 2013a). In **Chapter 3**, this genus was associated more with larvae regardless of the feed substrate, and it was also isolated from the eggs (unpublished data). However, in **Chapter 4**, the genus was hardly present. Thus, its potential vertical transmission may be haphazard throughout the colony and may not constitute a benefit for BSF.

Extrinsic disturbances

A dietary shift inadvertently causes a major disturbance in BSF larval gut microbiota (Boccazzi *et al.* 2017; Klammsteiner *et al.* 2020). It is common practice to feed neonates a nursery diet during 3 – 7 days before transferring them to a biowaste substrate to be converted (Jiang *et al.* 2019; Klammsteiner *et al.* 2020; Wynants *et al.* 2019; Zhan *et al.* 2020). Upon ingestion by the BSF larva, the feed substrate can directly or indirectly exert selection pressures on the resident gut microbiota. The direct impact of a dietary shift happens via a shift in the composition of ingested nutrients, the introduction of substrate-associated microorganisms and viruses, as well as possible introduction of toxins and xenobiotics (antibiotics, pesticides, pharmaceuticals). The nutrient composition of the ingested substrate can shift trophic interactions between resident gut microorganisms, as some will be better adapted to digest the new substrate than others, resulting in differential microbial amplification. Besides, the substrate introduces

an entirely new assemblage of microorganisms into the gut, which needs to compete with the established community (Boccazzi *et al.* 2017; Jiang *et al.* 2019; Klammsteiner *et al.* 2020). Xenobiotics, toxins, and heavy metals can exert strong selection pressures on gut microbes by interfering with their physiology, differentially affecting microbial survival and reproduction (Cai *et al.* 2018b; Liu *et al.* 2020; Wu *et al.* 2020). Moreover, antibiotics and quorum quenching compounds may render the gut microbial community more prone to pathogen invasion (Engel & Moran 2013; Jordan *et al.* 2016). Indirectly, the feed substrate may impact the gut microbiota by triggering digestive and immune responses of the BSF larval host (Bonelli *et al.* 2020; Vogel *et al.* 2018).

Feed substrates or the rearing environment may also introduce pathogens into the BSF larval gut. No major disease outbreaks have been reported from the BSF industry so far (Joosten *et al.* 2020), but a recent study showed that BSF adults are susceptible to *Beauveria bassiana* infection (Lecocq *et al.* 2021). If a pathogen invades and establishes in the host gut, it may drastically change the gut microbiome and disrupt healthy host gut function (Costello *et al.* 2012; Fassarella *et al.* in press; Maes *et al.* 2016). Similarly, introduction of predatory phages may result in cascading effects on the gut microbiome and consequently host functioning (Mukhopadhya *et al.* 2019; Santiago-Rodriguez & Hollister 2019). Protists are another group of microbial predators that may be introduced into the system via the feed substrate (Gao *et al.* 2019b; Geisen *et al.* 2018). Their role in BSF is unknown to date.

The gut microbiome response to disturbance

In the wake of a disturbance, the gut microbiome can respond in three ways, with increasing degree of change in community composition (Figure 2): resistance, resilience, or an alternative stable state (Christian *et al.* 2015; Fierer *et al.* 2012). Resistance implies little to no change in microbial community composition, whereas resilience implies a temporary change in community composition followed by recovery to the pre-disturbed composition. In the absence of recovery, the community may reach an alternative stable state. Although different in composition, this community may still fulfil the same functions as prior to disturbance if taxa are functionally redundant (Christian *et al.* 2015). In the worst case, however, the altered community results in a loss of functions and a decrease in host health (Fassarella *et al.* in press; Maes *et al.* 2016).

Resistance mechanisms

To establish in the BSF gut, ingested microbiota and potential pathogens need to survive the physicochemical and physiological constraints of the larval gut, including pH gradients and immune defence (Bonelli *et al.* 2019; Bruno *et al.* 2019b; Figure 1B). In addition, the resident gut microbial community may confer resistance to the host against pathogen invasion by colonization resistance, resulting from competition for nutrients and niches and immune priming (Engel & Moran 2013). Commensal and mutualist microbes can prime the host immune system and improve host defence against subsequent infections (Engel & Moran 2013). BSF larvae have an extensive immune system that can be modulated in diet-dependent, and microbe-dependent fashion (Vogel *et al.* 2018; Zhan *et al.* 2020), so immune priming likely plays a role in host resistance against pathogens. Following a shift in diet and accompanying ingested microbiota, the replacement of the resident gut microbiota may depend on the time the latter has had to establish in the BSF gut prior to the dietary shift (*i.e.* the exposure time of the host larva to the previous diet) and the competitive strength of ingested and resident microbiota (Boccazzi *et al.* 2017; Klammsteiner *et al.* 2020). The host may strengthen colonization resistance by limiting positive feedbacks among gut microorganisms and promoting resident microbial population growth through epithelial feeding (Coyte *et al.* 2015). Predatory phages and protists may further limit positive feedbacks (Mirzaei & Maurice 2017; Sharon *et al.* 2013).

If not metabolized by the host, xenobiotics and toxins in the ingested substrate introduce novel selection pressures that may cause rapid evolutionary responses in the resident larval gut microbiome. Antibiotic resistance genes can spread through a microbial community through horizontal gene transfer, a process that may occur in the BSF gut microbiome too (Cai *et al.* 2018b; Liu *et al.* 2020). Despite changes in BSF microbiota composition following antibiotic exposure (Cai *et al.* 2018b; Liu *et al.* 2020), horizontal gene transfer may allow the BSF larval host to retain specific members and their functions.

Resilience: the microbial mosaics in the maggot mass and substrate

The maggot mass and surrounding substrate can be viewed as a heterogenous metacommunity of microorganisms. Conspecifics in the maggot mass may enhance

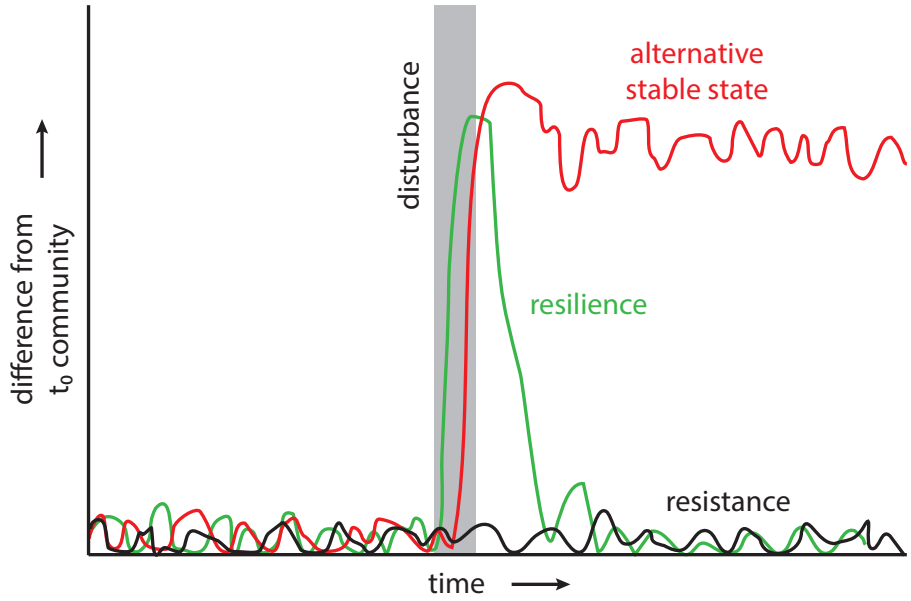

Figure 2. Possible responses of the gut microbiome to disturbance: following a disturbance (grey), the microbial community composition remains comparable to the initial state (resistance, black), the community changes in composition but recovers to the initial state (resilience, green), or the community composition changes and stabilizes in an alternative state (alternative stable state, red). The latter option includes, among others, a scenario of functional redundancy, where community composition has changed but reflects the same functions as the initial state. Based on Fierer *et al.* (2012), Christian *et al.* (2015), and Fassarella *et al.* (in press).

the resilience of each other's gut microbiome. Just as there can be intraspecific variation in BSF larval size within a maggot mass, there can be intraspecific variation in BSF gut microbiota. It is likely that the maggot mass encompasses a mosaic of healthy and disturbed larval microbiota that interact with each other via horizontal transmission through larval excretion and ingestion of mixed frass and feed substrate. Currently, the magnitude of intraspecific variation of gut microbiota in BSF larvae remains unquantified, as is the rate of exchange of microorganisms between individuals. The same labelling techniques as discussed for the study of vertical transmission, *i.e.* stable isotope probing with Illumina sequencing or tracking of fluorescent protein-tagged microorganisms (Alonso-Pernas *et al.* 2017; Prosdocimi *et al.* 2015; Teh *et al.* 2016), may be suitable to monitor the spread, persistence, and disappearance of microorganisms, *e.g.* inocula or pathogens, in a maggot mass over time and space.

Although larvae impact the microbiota composition of the substrate they forage in (**Chapter 3**), this impact may be attenuated in large-scale setups where larvae aggregate in local maggot masses moving through the substrate. Thus, the substrate is likely a mosaic of microbial communities more or less affected by larval foraging and in different stages of the decomposition process (Jiang *et al.* 2019; Wynants *et al.* 2019; Zhang *et al.* 2021; **Chapter 3**). Just like this spatial attenuation of larval impact, there can be a temporal attenuation of larval impact on substrate microbiota by feeding regime with a daily feeding rate instead of one-time bulk feeding at the start (Bruno *et al.* 2019b).

Future applications

Improving larval performance

The variable microbiota of biowaste and BSF larvae may be a considerable factor influencing variation in larval performance (De Smet *et al.* 2018; Gold *et al.* 2018). The findings presented in this thesis suggest that the microbiota of the feed substrate affect BSF larval performance. Substrate-associated microorganisms dominate the BSF larval microbiota and affect BSF larval biomass, compared to only minor effects of egg-associated microorganisms (**Chapter 4**). Understanding the microbial components of the system will allow to control their variation and manipulate them to benefit larval growth and survival. To reach that understanding we can treat the BSF larval microbiota as an ecological community as described above and may eventually apply methods analogous to those used for human intestinal microbiota and crop plant microbiota in order to improve BSF health and safety (Christian *et al.* 2015; Rosenberg & Zilber-Rosenberg 2013). These findings are relevant information for BSF producers that allow greater control over production predictability and stability.

To date, there are several ways to alter the microbiota of the substrate and impact the BSF larvae. The feed substrate can be supplemented with probiotics, prebiotics, or synbiotics, *i.e.* adding specific microbial strains, nutrients that stimulate growth of specific microbes, or a combination of both, respectively. Several studies showed positive effects on BSF performance after substrate inoculation with bacteria (Callegari *et al.* 2020; Kooienga *et al.* 2020; Mazza *et al.* 2020; Skaro 2018; Xiao *et al.* 2018a; Yu *et al.* 2011), yeasts (Richard *et al.* 2019; Wong *et al.* 2020), and fungi (Isibika *et al.* 2019), indicating potential application as probiotics in specific substrates. To that end, it may

also be worthwhile to survey the gut microbiota of wild BSF larvae, which may harbour a more diverse microbial community with digestive capabilities that may have been lost in laboratory populations (as suggested in *Drosophila*: Chandler *et al.* 2011). An alternative method is to pre-treat the substrate via fermentation, possibly combined with inoculation of specific microbial strains, in order to improve substrate digestibility (Gao *et al.* 2019a; Van Campenhout 2020). In addition, several studies have mixed different biowaste types to achieve a balanced macronutrient composition of the feed substrate for BSF larvae (Barragán-Fonseca *et al.* 2018b; Gold *et al.* 2020b; Ur Rehman *et al.* 2017). Such mixing of different substrates also combines their respective microbiota, which may influence the bioconversion efficiency of the larvae. Future studies can quantify the relative importance of nutritional and microbial components of mixed substrates in BSF larval performance.

The place of action of probiotic strains for BSF is often not known for the inoculation studies conducted so far. One question may be whether the inoculated strain needs to establish in the gut or feed substrate in order to be beneficial to the BSF larvae. If establishment of the inoculum in the larval gut is required, host immune function and colonization resistance of resident gut microbiota may be important constraints to successful inoculation. Selecting highly competitive microbial strains, increasing the applied inoculum concentration, or prolonging the exposure time of larvae to the inoculated substrate may facilitate inoculum establishment in the gut. Alternatively, the inoculum can be administered to the diet of neonate larvae. Resident microbial populations in the neonate gut may still be small (Costello *et al.* 2012) and microorganisms from the substrate may more readily establish in the microbial community. The effectiveness of this method depends on subsequent transfer of larvae to another substrate and the resistance/ resilience of the larval gut microbiota (including the inoculum). If the gut microbial community is largely replaced – as current literature seems to suggest (Boccazzi *et al.* 2017; Jiang *et al.* 2019; Klammsteiner *et al.* 2020) – inoculating after, rather than before, a dietary switch would be more effective (Kooienga *et al.* 2020).

At an industrial scale, processes may be different from the mostly small-scale experiments used to obtain scientific results. BSF performance and microbiota can differ between laboratory and industrial scales (Wynants *et al.* 2019; Yang & Tomberlin 2020), and so can the effects of bacterial supplementation on larval performance and microbiota (Kooienga *et al.* 2020). Hence, it is important to experimentally compare the different

scales of BSF-based bioconversion in order to translate knowledge on BSF microbial ecology into viable industrial applications. The application of metacommunity theory to the microbiota of larvae and surrounding substrate may facilitate this translation.

Improving microbiological and chemical safety

Our understanding of BSF microbial ecology may also enable producers to control BSF microbiological safety and chemical safety. Inoculation of feed substrates with specific bacteria can alter BSF gut microbial diversity (Kooienga *et al.* 2020). Several bacterial strains isolated from the BSF gut have antimicrobial activity against human pathogens (De Smet *et al.* 2020; Kim *et al.* 2014). If these strains express the same inhibition in the larval gut, and besides may improve larval performance such as other gut isolates (Mazza *et al.* 2020; Xiao *et al.* 2018a; Yu *et al.* 2011), they may be promising probiotics to improve both BSF biosafety and performance.

The immune system of BSF larvae may be tailored in several ways in order to improve microbial safety. Depending on the feed substrate nutrient composition, BSF larvae can suppress different microorganisms through the production of antimicrobial peptides and lysozymes (Vogel *et al.* 2018). These compounds may underlie the suppression of *E. coli* and *Salmonella spp.* in manure and aquaculture waste (Erickson *et al.* 2004; Lalander *et al.* 2013; Liu *et al.* 2008; Lopes *et al.* 2020). Besides, an increased larval density may enhance or alter such an immune response, because different larval densities can result in different substrate microbiota (**Chapter 3**). Further research could unravel whether these density-dependent effects are indeed related to immune or digestive responses of the larvae, for instance by transcriptomic analysis (Bonelli *et al.* 2020; Vogel *et al.* 2018). If so, BSF producers may be able to improve microbiological safety of the BSF production system to some extent by adjusting substrate nutrient composition and larval density. Moreover, the antimicrobial peptides produced by BSF larvae could function as in-feed antimicrobials for livestock animals (Xiao *et al.* 2018b).

Application of phages may be another way to biologically control potential human pathogens or treat pathogen infections in BSF larvae (Dalmasso *et al.* 2014; Potera 2013), although the latter have not been reported in BSF industry to date (Joosten *et al.* 2020). Phages and mycoviruses are specialist predators of bacteria and fungi, respectively (Potera 2013; Xie & Jiang 2014). In humans, phage therapy has been used to treat

bacterial infections in the early 20th century and is now regaining interest because of the surge in antibiotic resistance (Laxminarayan *et al.* 2013; Potera 2013). Phages are also successfully applied as food preservatives (Dalmasso *et al.* 2014). Although knowledge on the BSF virome, including phages, is still very limited (Chen *et al.* 2019), it may be a promising biocontrol method targeting specific microorganisms. Other microbial predators, such as protists, may also be used for biocontrol, as suggested from studies on plant rhizospheres (Gao *et al.* 2019b; Xiong *et al.* 2020).

In terms of chemical safety, microorganisms may play a significant role in bioremediation of substrates contaminated with xenobiotics or toxins (De Smet *et al.* 2018; Dowd 1992; Engel & Moran 2013). In my thesis, I showed that BSF larvae are able to tolerate certain levels of plant toxins (isothiocyanates), but it is unknown if they were able to metabolize these compounds (**Chapter 2**). Cabbage rootfly maggots (*Delia radicum*), a root herbivore specialized on the plant family producing the precursors of the isothiocyanates, can resist such toxins thanks to the catabolic activity of several species of gut bacteria (Welte *et al.* 2016b; Welte *et al.* 2016a). Although antibiotic resistance genes can spread and decline rapidly in BSF larvae and their feed substrate (Cai *et al.* 2018a; Cai *et al.* 2018b; Liu *et al.* 2020), it is unknown whether the BSF gut microbiome also contains resistance genes against phytotoxins or mycotoxins and whether they would spread at a similar rate. Welte *et al.* (2016b) present an example of exploring and characterising such detoxification potential in the larval gut microbiome. If present, further analysis should be done to elucidate what metabolites are formed in the process. Ideally, some gut isolates may be used as a probiotic and inoculated into the contaminated substrate to enhance detoxification and improve both chemical safety and larval performance.

Bioprospecting for biotechnology and pharmaceutics

The enzymes and antimicrobials produced by BSF and their gut microbiome have the potential to serve other applications than the food/feed industry. The detoxification enzymes from host or microbes may be characterized, isolated, and produced for bioremediation of contaminated waste (Almeida *et al.* 2017; Berasategui *et al.* 2016; Dowd 1992; Van den Bosch & Welte 2017). The antimicrobial peptides produced by BSF may provide an alternative to conventional antibiotics for human and livestock medicine, because the receptor-independent mode of action of such peptides likely overcomes most resistance mechanisms of pathogens (Li *et al.* 2012; Mor 2000; Reddy *et al.* 2004).

BSF have a wide repertoire of genes encoding immune compounds (Moretta *et al.* 2020; Zhan *et al.* 2020), and studies on immunization and nutritional immunology indicate that BSF larvae can be triggered to produce a selection of antimicrobial compounds targeting specific microbial species (Choi *et al.* 2018; Elhag *et al.* 2017; Lee *et al.* 2020; Vogel *et al.* 2018; Zdybicka-Barabas *et al.* 2017). Several studies provide methods to extract, identify, and test antimicrobial compounds from BSF (Choi *et al.* 2012; Choi *et al.* 2018; Elhag *et al.* 2017; Lee *et al.* 2020; Moretta *et al.* 2020; Park *et al.* 2014; Shin & Park 2019; Vogel *et al.* 2018; Zdybicka-Barabas *et al.* 2017).

Conclusion

With this thesis, I contribute to the understanding of BSF larval microbial ecology and performance on organic side streams. By placing the findings in an ecological community framework, this thesis paves the way for future studies to identify the key players and processes in the community dynamics and functions of the microbiota of the BSF gut and feed substrate during bioconversion. I showed that both the impact of larval density on substrate microbiota and the impact of egg-associated microorganisms on gut microbiota were subordinate to the contribution of the feed substrate microorganisms. In addition, BSF larvae were found to be tolerant to certain levels of phytotoxins in oilseed by-products, and such feed substrates resulted in larval fatty acid profiles with a desirable ratio of n-6 and n-3 polyunsaturated fatty acids.

Although current research is biased towards bacteria, other microbial groups such as fungi, viruses, and protists may play significant roles in the BSF larval gut microbiome. Fungi may be important in detoxification and digestion but can also include pathogens of BSF and competitors of beneficial bacteria. Viruses and protists may act as predators in the gut system, and viruses can additionally be involved in horizontal gene transfer between microbes.

This thesis provides insights into the relative importance of different microbial sources in community assembly of the BSF gut microbiota. It suggests that potentially vertically transmitted microorganisms play a minor role in BSF larval gut microbiota and larval performance, compared to environmentally acquired microorganisms. Moreover, I suggest that the common practice of diet switching likely inflicts drastic changes to BSF larval gut microbiota composition and functioning. Quantifying the resilience of

the gut microbiota to such disturbances and identifying key contributors and conserved functions has high research priority in order to predict and control host health, microbial safety, and the successful application of microbial inocula.

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Summary

The black soldier fly, *Hermetia illucens* (L.) (BSF; Diptera: Stratiomyidae), is one of the most promising insect species to provide an alternative protein source for animal feed. With a growing global human population and increasing income levels, the food production needs to increase by at least 70% to meet the projected global demand of 2050, and meat production needs to increase by an annual 200 million tons. The protein component of the feed needed to raise these animals is mainly provided by soymeal and fishmeal, and an increased demand for these two sources would aggravate environmental issues and increase feed prices. Insects reared on organic side streams can be a more sustainable alternative protein source than soymeal or fishmeal. Black soldier fly larvae can survive and grow on a wide range of organic side streams and by-products. However, the nutritional quality and performance of BSF larvae depends on the type of feed substrate. Besides substrate nutrient composition, substrate-associated microorganisms and chemical contaminants may affect larval performance and nutritional quality and introduce microbial and chemical safety risks to the use of BSF larvae as a feed ingredient. Microorganisms can colonize the larval gut from the environment (including feed substrate) and insect eggs. The larval gut exerts selection pressures on ingested and resident microbiota and excretes a microbiota of altered community composition into the substrate. Foraging in aggregations, the larvae may thus alter the substrate microbiota and physicochemical properties. However, the relative contributions of substrate, eggs, and larval aggregations on larval performance and microbiota have not been investigated to date. Therefore, this thesis focused on studying and understanding the performance of BSF larvae and their interactions with microbial communities in organic side streams.

In **Chapter 2**, BSF larval performance was investigated on diets of chicken feed with partial substitution by oilseed by-products. The oilseed crops crambe and camelina produce oils rich in erucic acid and n-3 polyunsaturated fatty acids (PUFA), respectively. After oil pressing, a protein-rich seed cake remains, which can be defatted to seed meal. These by-products have limited value as livestock feed because they contain glucosinolates which are enzymatically broken down into toxic isothiocyanates and nitriles. BSF larvae may be hindered by these toxins or an excess of protein. I tested the effects of 25%, 50% and 100% oilseed by-product inclusion in the diet on survival, development, biomass production and fatty acid composition of BSF larvae. Larvae fed up to 50% camelina by-product or 25% crambe by-product performed similarly to larvae fed control diet (chicken feed), and performance decreased with higher inclusion

percentages. Larvae fed camelina press cake had more α-linolenic acid, whereas larvae fed crambe contained most oleic acid. The n-6 : n-3 PUFA ratio decreased with increasing proportion of by-product, especially on camelina diets. Lauric acid content was highest in larvae fed 100% camelina meal or 50% crambe meal. These findings indicate that BSF larvae successfully grow on diets with camelina or crambe oilseed by-products, and that the resulting larval n-6 : n-3 PUFA ratio is favourable for animal feed. However, the fate of glucosinolates and their derivatives remains to be determined to warrant chemical safety of the larvae for use in animal feed.

BSF larvae interact with a rich microbial community of bacteria and fungi, which strongly depends on the type of substrate. These microorganisms impact the larval microbiota, but the larvae can also alter substrate microbiota. **Chapter 3** aimed to investigate the relative importance of substrate type and larval density on bacterial community dynamics. Four larval densities (0 (control), 50, 100, or 200 larvae per container) and three feed substrates (chicken feed, chicken manure, and camelina substrate (50% chicken feed, 50% camelina oilseed press cake) were investigated and bacterial communities of substrates and larvae were sampled at three time points over 15 days. Black soldier fly larvae altered bacterial community composition over time in all three feed substrates and substrate type was the strongest driver of bacterial community composition. The impact of the larvae depended on substrate and larval density, which was possibly related to substrate nutritional value, foraging behaviour, and larval performance. Larval and substrate microbiota differed for chicken manure and camelina, whereas they overlapped in chicken feed. These findings demonstrate the flexibility of the association between black soldier fly larvae and bacteria and support the substrate-dependent impact of black soldier fly larvae on bacteria both within the larvae and in the substrate. This study indicates that substrate composition and larval density can alter bacterial community composition and possibly be used to improve insect microbiological safety.

In **Chapter 4**, I aimed to quantify the relative importance of substrate-associated and egg-associated microorganisms on BSF larval performance, bacterial abundance, and microbiota composition, when larvae were fed with chicken feed or chicken manure. For this we inactivated substrate-associated microorganisms by autoclaving, or disinfected BSF eggs. Larval survival, biomass, and proportion of prepupae were determined on day 15. We collected substrate and larval samples on days 0 and 15 and performed

16S rRNA gene-targeted qPCR and amplicon sequencing. In both chicken feed and chicken manure, egg disinfection did not cause any difference in larval performance or overall microbiota composition. In contrast, in chicken manure, substrate-associated microorganisms increased larval biomass and autoclaving caused major shifts in microbiota. Thus, substrate-associated microorganisms not only impact the larval microbiota but also larval performance, whereas egg-associated microorganisms have a minor role at the densities present.

The findings of this thesis were discussed in a community ecology context in **Chapter 5**. This approach facilitates future identification of key players and processes in the community dynamics and functions of the microbiota of the BSF gut and feed substrate during bioconversion. Both the impact of larval density on substrate microbiota and the impact of egg-associated microorganisms on gut microbiota were subordinate to the contribution of the feed substrate microorganisms. This suggests that potentially vertically transmitted microorganisms play a minor role in larval gut microbiota and performance, compared to environmentally acquired microorganisms. Intrinsic and extrinsic disturbances during larval development suggest it is unlikely that the BSF larval gut microbiota reaches a stable composition. The most drastic disturbance of larval microbiota may be the common practice of transferring larvae from a nursery diet to a biowaste substrate. Substrate-associated microorganisms and chemicals may exert additional selection pressures on the host and resident gut microbiota. Microbial community recovery after disturbance depends on the competitive strength of the resident microbiota relative to ingested microbiota, the host immune response, the combined digestive capabilities of host and microbiota, and the transmission of microorganisms between larvae. Although current research is biased towards bacteria, other microbial groups such as fungi, viruses, and protists may play significant roles in the BSF larval gut microbiome. In order to predict and control host health, microbial safety, and successful application of microbial inocula, future research should prioritize quantifying the resilience of the larval gut microbiome to disturbances and identifying key contributors and conserved functions.

Samenvatting

Over maden en microben

De zwarte soldaatvlieg, *Hermetia illucens* (L.) (Diptera: Stratiomyidae) is een van de meest veelbelovende insectensoorten die kan dienen als alternatieve eiwitbron voor diervoeders. Door een groeiende wereldbevolking en stijgende inkomensniveaus, zal de wereldwijde voedselproductie tot 2050 moeten toenemen met tenminste 70% om aan de voorspelde vraag te voldoen. Volgens prognoses zal de vleesproductie jaarlijks toenemen met 200 miljoen ton. De eiwitcomponent van de diervoeders nodig voor die vleesproductie bestaat momenteel voornamelijk uit soja- en vismeel, en een grotere vraag naar deze twee bronnen zal milieuproblemen doen toenemen en voederprijzen doen oplopen. Insecten die gekweekt zijn op biologische reststromen kunnen een duurzamere alternatieve eiwitbron zijn dan soja- of vismeel. Larven van de zwarte soldaatvlieg kunnen overleven en groeien op een divers assortiment van biologische reststromen en bijproducten. De nutritieve kwaliteit en groeisnelheid van de larven hangt echter af van het type voedingssubstraat. Naast de voedingssamenstelling van het substraat kunnen substraat-gebonden micro-organismen en chemische verontreinigingen de groeisnelheid en nutritieve kwaliteit van larven beïnvloeden, en microbiologische en chemische veiligheidsrisico's met zich meebrengen in het gebruik van de larven als veevoeringrediënt. Micro-organismen kunnen de darm van de larven koloniseren vanuit de omgeving (inclusief voedingssubstraat) en de eitjes van het insect. De larvale darm oefent selectiedruk uit op de inwendige microbiota en scheidt microbiota van veranderde soortensamenstelling uit in het substraat. De larven kunnen zodoende de microbiota en fysisch-chemische eigenschappen van het substraat veranderen. De relatieve bijdragen van substraat, eitjes, en dichtheid van larven op de groeisnelheid en microbiota van larven zijn echter tot dusver niet onderzocht. Dit thesisproject richtte zich op het bestuderen en begrijpen van de groeisnelheid van de vliegenlarven en de interacties tussen de larven en microbiële gemeenschappen in biologische reststromen.

Hoofdstuk 2 presenteert de resultaten over de groeisnelheid van zwarte soldaatvliegenlarven op diëten van kippenvoer met gedeeltelijke vervanging door bijproducten van oliezaden. De zaden van crambe en camelina bevatten olie die rijk is aan respectievelijk erucazuur en n-3 meervoudig onverzadigde vetzuren. Na het persen van de zaden resteert een eiwitrijke koek, die chemisch ontvet kan worden tot zaadmeel. Deze bijproducten hebben beperkte waarde als veevoeders omdat ze mosterdolieglycosiden (glucosinolaten) bevatten die enzymatisch afgebroken worden

tot giftige isothiocyanaten en nitrillen. De larven zouden last kunnen hebben van deze toxines of van een overmaat aan eiwitten. Ik onderzocht de overleving, ontwikkeling, biomassaproductie en vetzuursamenstelling van de larven op effecten van 25%, 50% en 100% oliezaad bijproduct in het dieet. Larven op een dieet met maximaal 50% camelina bijproduct of 25% crambe bijproduct, groeiden even goed als larven op controledieet (kippenvoer), en de biomassa van larven nam af met hogere inclusiepercentages. Larven op camelina-perskoek bevatten meer linoleenzuur (C18:3 n-3), terwijl larven op crambe-bijproducten meer oleïne (C18:1 cis-9) bevatten. De verhouding n-6 : n-3 nam af met een toenemend aandeel bijproduct, vooral in larven op camelinadiëten. Het laurinezuurgehalte (C12:0) was het hoogst in larven gekweekt op diëten met 100% camelina-zaadmeel of 50% crambe-zaadmeel. Deze resultaten wijzen erop dat BSF larven succesvol kunnen groeien op diëten met camelina of crambe oliezaadbijproducten en dat de resulterende larven een n-6 : n-3 ratio van meervoudig onverzadigde vetten hebben die gunstig is voor diervoeders. De volgende stap zal zijn de concentratie van glucosinolaten en hun afbraakproducten in larven te bepalen om veilig gebruik van larven in diervoeders te waarborgen.

De vliegenlarven leven samen met een soortenrijke microbiële gemeenschap van bacteriën en schimmels, die sterk afhangt van het type substraat. Deze micro-organismen beïnvloeden de larvenmicrobiota, maar de larven kunnen ook de substraatmicrobiota veranderen. **Hoofdstuk 3** richt zich op het relatieve belang van substraattype en larvendichtheid op de dynamiek van bacteriegemeenschappen. Vier larvendichtheden (0 (controle), 50, 100, of 200 larven per container) en drie voedingssubstraten (kippenvoer, kippenmest en camelina-substraat (50% kippenvoer en 50% camelina-perskoek)) werden onderzocht en de bacteriegemeenschappen in substraten en larven werden bemonsterd op drie tijdspunten verspreid over 15 dagen. De larven veranderden de samenstelling van bacteriegemeenschappen over de tijd in alle voedingssubstraten en het substraattype had de grootste invloed op de samenstelling van de bacteriële gemeenschap. De invloed van larven hing af van het substraat en de larvendichtheid, en hield mogelijk verband met de voedingswaarde van het substraat, het foerageergedrag van larven, en hun groeisnelheid. Microbiota in larven en substraat verschilden van elkaar in kippenmest en camelina, maar waren vergelijkbaar in kippenvoer. Deze resultaten tonen de flexibiliteit aan van de associatie tussen larven van de zwarte soldaatvlieg en bacteriën, zowel in de larven als in het substraat. Dit onderzoek laat zien dat de substraatsamenstelling en larvendichtheid de samenstelling van bacteriegemeenschappen kunnen veranderen en

mogelijk gebruikt kunnen worden om de microbiologische veiligheid van dit insect te verbeteren.

In **Hoofdstuk 4** wordt de relatieve bijdrage bepaald van micro-organismen in het substraat of de insecteneitjes aan de groeisnelheid van de vliegenlarven, de talrijkheid van bacteriën, en de samenstelling van bacteriegemeenschappen, als larven gevoerd werden met kippenvoer of kippenmest. Hiervoor inactiveerden we micro-organismen uit het substraat door autoclaveren, of desinfecteerden we de vliegeneitjes. Overleving, biomassa en het aandeel prepoppen van de larven werden bepaald op dag 15. We verzamelden substraat- en larvenmonsters op dag 0 en 15 en voerden qPCR en amplicon-sequencing uit op het 16S rRNA gen. In zowel kippenvoer als kippenmest veroorzaakte desinfectie van de eitjes geen verschil in de groeisnelheid van larven of de algehele samenstelling van de microbiota. Daartegenover verhoogden micro-organismen in kippenmest de biomassa van larven en veroorzaakte de sterilisatie van mest een aanzienlijke verschuiving in samenstelling van microbiota in de larven en het substraat. Zodoende beïnvloedden micro-organismen uit het substraat niet alleen de larvenmicrobiota maar ook de groeisnelheid van larven, terwijl micro-organismen van de insecteneitjes in de aanwezige dichtheden een ondergeschikte rol hadden.

In **Hoofdstuk 5** worden de bevindingen uit deze thesis besproken binnen de context van gemeenschapsecologie. Deze benadering vergemakkelijkt toekomstige identificatie van sleutelspelers en -processen in de dynamiek van gemeenschappen en functies van de microbiota in de larven en het voedingssubstraat tijdens bioconversie. Zowel het effect van de larvendichtheid op substraatmicrobiota als de impact van micro-organismen van de eitjes op darmmicrobiota waren ondergeschikt aan de bijdrage van micro-organismen uit het voedingssubstraat. Dit doet vermoeden dat potentieel verticaal overgedragen micro-organismen slechts een beperkte rol spelen in de darmmicrobiota en groeisnelheid van larven in vergelijking met micro-organismen afkomstig uit de omgeving. Intrinsieke en extrinsieke verstoringen tijdens de larvale ontwikkeling suggereren dat het onwaarschijnlijk is dat de larvale darmmicrobiota een stabiele samenstelling bereikt. De meest drastische verstoring van de larvenmicrobiota is mogelijk de algemene praktijk van het overzetten van larven vanaf een speciaal kweekdieet voor de jongste larfjes naar een reststroomsubstraat. Micro-organismen en chemische stoffen in het substraat oefenen mogelijk aanvullende selectiedruk uit op de gastheer (larve) en diens gevestigde darmmicrobiota. Het herstel van de microbiële gemeenschap in de

darm na verstoring hangt af van de concurrentiekracht van de gevestigde microbiota ten opzichte van microbiota in het geconsumeerde substraat, de immuunrespons van de gastheer, de gezamenlijke spijsvertering door gastheer en microbiota, en de overdracht van micro-organismen tussen larven. Hoewel het huidige onderzoek de nadruk legt op bacteriën, spelen andere microbiële groepen als schimmels, protisten en virussen mogelijk een significante rol in de groeisnelheid van de larven. Om de gezondheid van het insect, de microbiologische veiligheid en succesvolle toepassing van microbiële inocula te voorspellen en te beheersen, moet toekomstig onderzoek prioriteit geven aan het meten van de veerkracht van het larvale darmmicrobioom na verstoringen en het identificeren van de belangrijkste spelers en geconserveerde functies.

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Curriculum vitae & publication list

Stijn Schreven was born on 18 October 1988 in Groesbeek, the Netherlands. Since childhood he has been fascinated by nature. After secondary school at the Stedelijk Gymnasium Nijmegen, he completed a BSc and MSc Biology at Wageningen University. In 2012, he did an internship at the Borneo Nature Foundation in Central Kalimantan, to survey insect communities in a tropical peat-swamp forest. He worked as a research assistant at the Netherlands Institute of Ecology, before joining the European Union-funded COSMOS project as a junior researcher at the Laboratory of Entomology in 2015. Within this project he started his PhD research focusing on the performance and microbiology of black soldier fly larvae.

Stijn was involved in local nature education in Groesbeek and later joined the NJN, a youth association for natural history. Since 2010 he has surveyed wild bees and wasps in nature reserves. Last year he donated his collection of over 2500 specimens and 350 species to the Naturalis Biodiversity Centre in Leiden. He illustrated the Dutch identification keys for two wild bee genera, published in 2016.

Stijn values art-science crossovers and scientific outreach to the public. During the WUR centennial celebrations in 2018, he co-organised and participated in the cross-pollinations project between Wageningen University PhD candidates and students of the Gerrit Rietveld Academy and Design Academy Eindhoven. In 2020, he was a finalist in the national FameLab competition for science communication and was a member of the outreach team of the Dutch Deltaplan for Biodiversity Recovery.

Besides his PhD, Stijn has been exploring creative career paths. In 2018-2019, he followed the Orientation Year of the Gerrit Rietveld Academy of Fine Arts and Design, Amsterdam. He played in musical theatre association Sempre Sereno Wageningen from 2014-2017 and since 2017 he has played improvisation theatre. In the past two years, he followed workshops on voice acting and voice-over techniques at IAM Studios and did a preparatory training for acting at theatre school De Trap, Amsterdam.

Stijn's passion is in storytelling. His ambition is to use writing, fine arts, and theatre to share stories of science and the natural world. He is currently working as a freelance science writer.

List of publications

Published in peer-reviewed journals

Schreven SJJ, Frago E, Stens A, De Jong PW, Van Loon JJA (2017). Contrasting effects of heat pulses on different trophic levels, an experiment with a herbivore-parasitoid model system. *PLoS ONE* 12: e0176704.

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Schreven SJJ, Yener S, Van Valenberg HJF, Dicke, M, Van Loon JJA (2021). Life on a piece of cake: performance and fatty acid profiles of black soldier fly larvae fed oilseed by-products. *J Insects Food Feed* 7(1): 35-49. (Chapter 2 in this thesis)

Schreven SJJ, De Vries H, Hermes GDA, Smidt H, Dicke M, Van Loon JJA (2021). Relative contributions of egg-associated and substrate-associated microorganisms to black soldier fly larval performance and microbiota. *FEMS Microbiol Ecol* 97(5): fiab054. (Chapter 4 in this thesis)

Submitted

Schreven SJJ, De Vries H, Hermes GDA, Zeni G, Smidt H, Dicke M, Van Loon JJA. Substrate-dependent impact of black soldier fly larvae on bacterial community composition in substrate and larval body. (Chapter 3 in this thesis)

Education statement

PE&RC Training and Education Statement

With the training and education activities listed below the PhD candidate has complied with the requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities).

Review of literature (4.5 ECTS)

- Microbial ecology of detritivorous flies (2019)

Writing of project proposal (1.5 ECTS)

- Bioconversion of crop residues by insects (2016)

Post-graduate courses (6.2 ECTS)

- Grasping sustainability; SENSE (2018)
- New frontiers in microbial ecology: eco-evolutionary dynamics of microbial host interactions; RSEE, PE&RC, SENSE (2018)
- Introduction to R for statistical analysis; PE&RC, SENSE (2016)
- R and Big data; PE&RC, SENSE (2017)
- Open & reproducible microbiome data analysis spring school; VLAG (2018)

Competence strengthening / skills courses (7 ECTS)

- Coaching of groups; WUR ESC (2016)
- Scientific artwork with photoshop and illustrator; WGS (2016)
- Entrepreneurship in and outside science / get started; WGS (2017)
- Project and time management; WGS (2017)
- PhD Competence assessment; WGS (2017)
- Supervising BSc and MSc thesis students; WUR ESC (2017)
- Introduction to the art of science: scientific illustration; Transmitting Science (2019)

Scientific integrity/ethics in science activities (1.6 ECTS)

- Research ethics seminar; WGS (2017)
- Research integrity; WGS (2017)

PE&RC Annual meetings, seminars and the PE&RC weekend (3.3 ECTS)

- PE&RC Day (2015, 2016, 2018, 2019)
- PE&RC First years weekend (2016)
- PE&RC Midterm weekend (2017)
- PE&RC Last years weekend (2019)

Discussion groups / local seminars / other scientific meetings (15.2 ECTS)

- Entomologendag (2015-2019)
- COSMOS project meetings (2015-2019)
- Edible insects discussion group (2015-2020)
- Insect-plant interactions meeting (2015-2020)
- WEES seminars (2016-2018)
- Netherlands annual ecology meeting (2017, 2019, 2020)
- New ways of communicating science & collections; Museum für Naturkunde Berlin, Germany (2020)

International symposia, workshops and conferences (4.1 ECTS)

- The 2nd International conference "Insects to Feed the World" (IFW); Wuhan, China (2018)
- SDG-Conference "Towards Zero Hunger: partnerships for impact"; Wageningen, the Netherlands (2018)

Societally relevant exposure (4.1 ECTS)

- Art-science cross-pollinations project; collaboration between WUR, Gerrit Rietveld Art Academy and Design Academy Eindhoven, exhibitions in Stedelijk Museum Amsterdam and Impulse Wageningen (2018)
- Representative of NERN in the communication team of the Dutch Deltaplan for biodiversity recovery (2020)
- FameLab competition: Wageningen heat and national finals (2020)

BSc/MSc thesis supervision (3 ECTS)

- Feed for thought: bioconversion of seed oil by-products by black soldier fly larvae
- Impact of black soldier fly larvae density on substrate- and gut-microbiome composition
- The development of the gastrointestinal tract and mouth parts in the larvae of *Hermetia illucens*

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