Development of Sustainable Strategies for the Production of Insect Protein by *Hermetia illucens*

Cumulative Dissertation

Presented by

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Submitted to the

Faculty of Biology and Chemistry

Prepared in the

Institute of Food Chemistry and Food Biotechnology

&

Fraunhofer Institute for Molecular Biology and Applied Ecology IME Branch Bioresources

For the degree of

Doctor rerum naturalium (*Dr. rer. nat.*)

Justus Liebig University Giessen, Germany

This thesis is submitted as a doctoral dissertation in fulfilment of the requirements for the degree of *Doctor rerum naturalium* to the Faculty of Biology and Chemistry, Justus Liebig University Giessen, Germany.

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I declare that I have completed this dissertation single-handedly without the unauthorized help of a second party and only with the assistance acknowledged therein. I have appropriately acknowledged and cited all text passages that are derived verbatim from or are based on the content of published work of others, and all information relating to verbal communications. I consent to the use of an anti-plagiarism software to check my thesis. I have abided by the principles of good scientific conduct laid down in the charter of the Justus Liebig University Giessen "Satzung der Justus-Liebig-Universität Giessen zur Sicherung guter wissenschaftlicher Praxis" in carrying out the investigations described in the dissertation.

Date, Place Signature

This work was carried out at the Institute of Food Chemistry and Food Biotechnology, Justus Liebig University, Giessen, and the Fraunhofer Institute for Molecular Biology and Applied Ecology IME (Branch Bioresources), Giessen, between September 2018 and September 2021. I would like to kindly acknowledge the cooperation with PT Alternative Protein Indonesia and especially the financial support of the Hessian Ministry of Science and Art in the course of the LOEWE Center for Insect Biotechnology & Bioresources.

The practical and theoretical part of my dissertation would not have been possible without the participation and support of many people. First of all, I would like to express my deep gratitude to **Prof. Dr. Holger Zorn** for providing such an interesting research project and for giving me the opportunity to spend my doctoral time in his interdisciplinary institute. I appreciate his scientific expertise and his always-open ear for questions and discourses. I would also like to thank him for the trust he has placed in me and for the freedom that has made a significant contribution on the development of my own ideas. In addition, I would like to sincerely thank **Prof. Dr. Martin Rühl** for his outstanding advice, guidance and continuous motivation over the past years, without which this work would not have been possible. It was my great pleasure to work under the supervision of both of you.

I would also like to show gratitude to **Prof. Dr. Marc Schetelig** and **Prof. Dr. Steffen Pauls** as members of my thesis committee.

I highly appreciate the advice and microbiological expertise of **Prof. Dr. Eugen Domann**. In particular, I am grateful for the opportunity to perform antimicrobial susceptibility tests and infection experiments in the laboratories of the Institute of Medical Microbiology, Faculty Medicine, Justus Liebig University, Giessen.

Many thanks to the company Bio.S Biogas (especially **Dipl.-Ing. Kai Kreller** and **Dennis Hluschi**) for initial information on breeding and for providing black soldier fly larvae in early 2018. I also want to acknowledge Lence Technology, especially Mr. **Ziming Yang**, for providing LED panels and technical product specifications.

In addition, I would like to thank all employees and previously unnamed colleagues from the Institute of Food Chemistry and Food Biotechnology and the Fraunhofer Institute for Molecular Biology and Applied Ecology IME for their support, which made this work possible. My special thanks go to **Bernhard Hellmann**, **Victoria Klis**, **Wendell Albuquerque**, **Fabio Brescia**, **Suzan Yalman**, **Nadine Sella**, **Philipp Honold**, **Svenja Sommer**, **Dominik Karrer**, **Axel Orban**, **Katharina Happel**, **Darleen Münch**,

Weronika Michalak, **Bianka Daubertshäuser**, **Peter Seum**, **Carolin Mewe**, **Dr. Garima Maheshwari**, **Dr. Miriam Sowa**, **Dr. Martin Gand**, **Dr. Tatyana Zhuk**, **Dr. Alejandra Omarini**, **Dr. Marco Fraatz**, **Dr. Kwang-Zin Lee** and **Tobias Kessel**.

I would especially like to thank **Dr. Daniel Bakonyi**, who not only supported me technically in the laboratory and greenhouse, but also managed to cheer me up with his pleasantly relaxed manner. Our breaks together were always very amusing and often led to constructive discussions and powerful ideas. Thank you for the good time, you are like the big brother I never had.

In addition, I would like to thank **Dr. Dorothee Tegtmeier** for her help in evaluating and interpreting microbiome data. Our comprehensive phone calls were always very enriching and gave me new perspectives.

Many thanks also to **Sabine Hurka** for her outstanding bioinformatic advice and evaluation. Despite the fact that a large part of our meetings took place exclusively digital due to the Corona situation, they were very amusing and exhilarating.

Many thanks to **Janin Pfeiffer** for her ongoing support in food chemistry methodology. I appreciate you not only as an office partner, but also as a good friend.

I also wish to thank **Dr. Andreas Hammer** for his help with GC-MS related experiments and our daily conversations on the way to work, which I always enjoyed.

Further, I would also like to express my appreciation to **Christopher Back** and **Maren Bodenschatz** for their independent and dedicated work in the laboratory.

Special thanks go to **Dr. Till Röthig** for his contribution to the preparation of the patent application.

During my time as a PhD student, I was able to supervise several students through their degree theses. I would like to thank **Jonas Schmidt**, **Melina Redin**, **Giuliano Schneider** and **Lea Zeller** for their great performance during their B.Sc. theses. To **Stephanie Müller**, **Emna Arous** and **Niclas Weber** I want to express my gratitude for their highly motivated and persistent work during their M.Sc. theses.

Some special words of gratitude go to my friends **Dieter**, **Larissa**, **Julian**, **Lara**, **Luca**, **Connor**, **Diego** and **Sebastian**: Thank you for making every evening and every weekend a highlight.

I would like to express my deep gratitude to **Bianca Härteis** for her overwhelming effort and being my emotional support. Thank you for all your empathy and love in these years.

Finally, I would like to thank my parents **Roland** and **Manuela** and my grandparents **Anita**, **Winfried** and **Elvira** from the bottom of my heart for their unconditional support, encouragement and belief in me.

Insects are not only on the menu of wild animals, but have been consumed by humans, their ancestors and related primates for thousands of years. Insects are suitable for sustainable commercial mass production due to their ability to convert side streams of agriculture and food industry with low nutritional properties into valuable insect protein in a short period. With regard to projections of global population growth and the associated rapid increase in protein demand, insects are coming into the focus of science as an alternative protein source for food and feed.

Although previous studies have focused on the optimization of black soldier fly (*Hermetia illucens*, BSF) larval breeding conditions, little is known about light-dependent adult development. Since the mating only takes place under adequate lighting, artificial illumination systems are indispensable especially at latitudes with short days in autumn and winter months. In this thesis, the effect of broad spectrum (350–800 nm) light-emitting diode panels with color temperatures of 3,000, 4,000, and 6,500 K on the oviposition was examined. Mating occurred under all light panels, resulting in comparable preoviposition periods (16.8 ± 0.3 d) and the subsequent deposition of fertilized egg clutches. The oviposition period and performance were not affected by color temperature, ranging between 2–15 d and 4.7 ± 0.5 mg eggs per female, respectively. In contrast, oviposition peaked after 1–7 d and was positively correlated with increasing color temperature (r = 0.61).

The intestinal microbiota of BSF larvae contributes heavily to dietary breakdown and enables the larvae to utilize a variety of organic substrates including fiber-rich plant-derived side streams. Therefore, BSF larvae were reared on palm kernel meal (PKM) and their bacterial and fungal gut communities were characterized in a culture-dependent approach. A total of 93 isolates could be generated, of which 74% belong to the bacterial phyla *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, and *Proteobacteria*. Consistent with further studies and regardless of the diet composition and the rearing conditions, *Klebsiella*, *Enterococcus* and *Sphingobacterium* were identified as part of the larval core microbiome. With 75%, a majority of fungal isolates belonged to the phylum Ascomycota, followed by Basidiomycota, and Mucoromycota. Furthermore, representatives of the taxa *Cellulomonas*, *Enterococcus*, *Pichia*, and *Fusarium* have frequently been described to degrade lignocelluloses and they could accordingly have contributed to increase the digestibility of PKM. All isolates were subsequently screened for entomopathogenicity and putative candidates injected intracoelomally. The injection

assay revealed that *Alcaligenes faecalis* caused no, *Diutina rugosa* weak, *Microbacterium thalassium* moderate, and *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* high lethality. *Fusarium solani* injection resulted in 100% lethality one day post-infection.

In addition, a combination of PKM with empty fruit bunches (EFB) – another side stream of palm oil production – was evaluated for its suitability as feed for BSF larvae. It was shown that larvae whose feed was fermentatively pretreated with *Bjerkandera adusta* (BAD) developed significantly faster and reached a higher final weight than those reared on the non-fermented reference (NFR) feed or on feed fermented with *Irpex consors* or *Marasmius palmivorus*. Amplicon sequencing revealed major differences in the larval gut microbiomes. While the NFR group was dominated by facultatively anaerobic, cellulolytic *Enterobacteriaceae*, BAD guts favored obligate anaerobic, cellulolytic taxa like *Ruminococcaceae* and *Lachnospiraceae*. Thus, *B. adusta* pretreatment led to lignin degradation and mycelia accumulation, resulting in a microbial adaptation that may have further enhanced substrate digestibility through cellulolytic breakdown of fiber residues.

In summary, the results of this thesis underline the tremendous adaptability and bioconversion capacity of BSF larvae. The optimization of artificial lighting systems enables year-round breeding of BSF outside of (sub-) tropical regions with heavily fluctuating day lengths. Due to the fermentative pretreatment, not only PKM, but also the less nutritious EFB could be utilized by BSF larvae and upcycled into valuable insect protein. The intestinal microbiome seems to play an essential role in this process and general aspects of insect health.

Insekten stehen nicht nur auf dem Speiseplan von Wildtieren, sondern werden bereits seit Jahrtausenden von Menschen, deren Vorfahren, sowie verwandten Primaten konsumiert. Auf Grund ihrer Fähigkeit, Nebenströme der Landwirtschaft und Lebensmittelindustrie mit geringen ernährungsphysiologischen Eigenschaften in kurzer Zeit in wertvolles Insektenprotein aufzuwerten, eignen sich Insekten für eine nachhaltige kommerzielle Massenproduktion. Im Hinblick auf Prognosen des globalen Bevölkerungswachstums und der damit einhergehenden rapiden Zunahme des Proteinbedarfs, geraten Insekten als alternative Proteinquelle für Lebens- und Futtermittel in den Fokus der Wissenschaft.

Obwohl sich frühere Studien auf die Optimierung der Zuchtbedingungen der Schwarzen Soldatenfliegenlarven (*Hermetia illucens*, BSF) konzentrierten, ist wenig über die lichtabhängige Entwicklung adulter Tiere bekannt. Da die Paarung ausschließlich bei adäquater Belichtung stattfindet, sind artifizielle Beleuchtungssysteme gerade in Breitengraden mit kurzen Tagen während der Herbst- und Wintermonate unentbehrlich. In dieser Arbeit wurde der Effekt von Breitspektrum-Leuchtdioden (350–800 nm) mit Farbtemperaturen von 3.000, 4.000 und 6.500 K auf die Eiablage untersucht. Die Paarung konnte unter allen Lichtplatten beobachtet werden, was in einer vergleichbaren Präovipositionszeit (16,8 ± 0,3 d) und anschließenden Ablage befruchteter Eigelege resultierte. Der Eiablage-Zeitraum sowie die -Leistung wurden nicht von der Farbtemperatur beeinflusst und lagen zwischen 2–15 d bzw. 4,7 ± 0,5 mg Eiern je Weibchen. Im Gegensatz dazu erreichte die Anzahl der Eigelege nach 1–7 d ihr Maximum und war positiv mit steigender Farbtemperatur korreliert (r = 0,61).

Die Darmmikrobiota der BSF Larven trägt entscheidend zum Aufschluss der Nahrung bei und ermöglicht es den ihnen, eine Vielzahl organischer Substrate zu verwerten, insbesondere faserreiche Nebenströme pflanzlichen Ursprungs. Daher wurden BSF Larven auf Palmkernmehl (PKM) kultiviert und ihre bakteriellen und fungalen Darmgemeinschaften in einem kulturabhängigen Ansatz charakterisiert. Insgesamt konnten 93 Isolate generiert werden, von denen 74% zu den bakteriellen Phyla *Actinobacteria*, *Bacteroidetes*, *Firmicutes* und *Proteobacteria* zählen. In Übereinstimmung mit weiteren Studien und unabhängig von der Futtermittelkomposition und den Aufzuchtbedingungen wurden Vertreter der Gattungen *Klebsiella*, *Enterococcus* und *Sphingobacterium* als Teil des larvalen Kernmikrobioms identifiziert. Mit 75% gehörte ein Großteil der Pilzisolate zum Phylum Ascomycota, gefolgt von Basidiomycota und Mucoromycota. Weiterhin wird häufig beschrieben, dass Vertreter der Taxa

Cellulomonas, *Enterococcus*, *Pichia* und *Fusarium* Lignocellulosen degradieren können, was zur Verbesserung der Verdaulichkeit von PKM beigetragen haben könnte. Alle Isolate wurden anschließend auf Entomopathogenität untersucht und putative Kandidaten intracoelomal injiziert. Die Infektionsexperimente ergaben, dass *Alcaligenes faecalis* keine, *Diutina rugosa* eine schwache, *Microbacterium thalassium* eine mäßige und *Pseudomonas aeruginosa* sowie *Klebsiella pneumoniae* eine hohe Letalität verursachten. Die Injektion von *Fusarium solani* führte bereits nach einem Tag zu einer Letalität von 100%.

Darüber hinaus wurde eine Kombination von PKM mit leeren Fruchtständen (EFB) – ein weiterer Nebenstrom der Palmölproduktion – auf ihre Eignung als Futtermittel für BSF Larven evaluiert. Larven, deren Futter fermentativ mit *Bjerkandera adusta* (BAD) vorbehandelt wurde, entwickelten sich deutlich schneller und erreichten ein höheres Endgewicht als Larven, die mit anderen Pilzen vorbehandelten Diäten (*Irpex consors*, *Marasmius palmivorus*) oder der nicht fermentierten Referenz (NFR) gefüttert wurden. Die Amplikon-Sequenzierung zeigte auffällige Unterschiede im intestinalen Mikrobiom der Larven. Während die NFR-Gruppe von fakultativ anaeroben, cellulolytischen *Enterobacteriaceae* dominiert wurde, waren Därme der BAD Larven von obligat anaeroben, cellulolytischen Taxa wie *Ruminococcaceae* und *Lachnospiraceae* geprägt. Somit führte die Vorbehandlung mit *B. adusta* zum Ligninabbau und der Akkumulation von Pilzmyzel, was in einer Adaptation des larvalen Darmmikrobioms resultierte und die Substratverdaulichkeit durch cellulolytischen Abbau von Faserrückständen verbessert haben könnte.

Zusammenfassend unterstreichen die Ergebnisse dieser Arbeit die enorme Anpassungsfähigkeit und Biokonversionskapazität von BSF Larven. Die Optimierung artifizieller Beleuchtungssysteme ermöglicht eine ganzjährige Zucht der BSF außerhalb (sub-) tropischer Regionen mit stark schwankenden Tageslängen. Durch die fermentative Vorbehandlung konnten nicht nur PKM, sondern auch das weniger nahrhafte EFB an Larven der BSF verfüttert und in hochwertiges Insektenprotein konvertiert werden. Das Darmmikrobiom scheint bei diesem Prozess sowie allgemeinen Aspekten der Insektengesundheit eine maßgebliche Funktion innezuhaben.

Peer-reviewed publications

1. **Klüber P**, Bakonyi D, Zorn H, and Rühl M. Does light color temperature influence aspects of oviposition by the black soldier fly (Diptera: Stratiomyidae)? *J. Econ. Entomol.* 2020; 113(5): 2549-2552.
2. Tegtmeier D, Hurka S, **Klüber P**, Brinkrolf K, Heise P, and Vilcinskas A. Cottonseed press cake as a potential diet for industrially farmed black soldier fly larvae triggers adaptations of their bacterial and fungal gut microbiota. *Front. Microbiol.* 2021; 12:634503.
3. Pecina A, Schwan M, Blagotinsek V, Rick T, **Klüber P**, Leonhard T, Bange G, and Thormann KM. The stand-alone PilZ-domain protein MotL specifically regulates the activity of the secondary lateral flagellar system in *Shewanella putrefaciens*. *Front. Microbiol.* 2021; 12:668892.
4. **Klüber P**, Meurer SK, Lambertz J, Schwarz R, Zechel-Gran S, Braunschweig T, Hurka S, Domann E, and Weiskirchen R. Depletion of Lipocalin 2 (LCN2) in mice leads to dysbiosis and persistent colonization with segmented filamentous bacteria. *Int. J. Mol. Sci.* 2021; 22(23):13156.
5. **Klüber P**, Müller S, Schmidt J, Zorn H, and Rühl M. Isolation of bacterial and fungal microbiota associated with *Hermetia illucens* larvae reveals novel insights into entomopathogenicity. *Microorganisms* 2022; 10(2):319.
6. **Klüber P**, Tegtmeier D, Hurka S, Pfeiffer J, Vilcinskas A, Rühl M, and Zorn H. Diet fermentation leads to microbial adaptation in black soldier fly (*Hermetia illucens*; Linnaeus, 1758) larvae reared on palm oil side streams. *Sustainability* 2022; 14(9):5626.

Patents

1. **Klüber P**, Zorn H, Rühl M, Bakonyi D, Pfeiffer J, and Vilcinskas A. Process for the production of an insect substrate, insect substrate and uses thereof. Application number EP22152265.9.

Conference contributions

1. **Klüber P**, Pfeiffer J, Bakonyi D, Rühl M, and Zorn H. Upcycling von Nebenstoffströmen der Palmölindustrie zu Insektenprotein. LOEWE-ZIB Evaluierung 2019, 08.-09.08.2019, poster presentation.

2. **Klüber P**, Nayak A, Rühl M, and Zorn H. Strategies and suggestions for optimizing *Hermetia illucens* rearing. INSECTA Conference 2021, 08.-09.09.2021, poster presentation.
3. **Klüber P**, Vilcinskas A, and Rühl M. Sustainable rearing of *Hermetia illucens*. Jahreskonferenz des Innovationsbündnisses BioBall 2021, 27.09.2021, poster presentation.

BSF	black soldier fly
EFB	empty fruit bunches
EC	European Commission
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
IPIFF	International Platform of Insects for Food and Feed
OECD	Organization for Economic Cooperation and Development
PKM	palm kernel meal
UN	United Nations

1. Synopsis

According to estimations of the United Nations (UN), the world population will reach between 9.4 and 10.2 billion by 2050 (UN, 2017). Driven by global population growth and other socio-economic factors such as educational level, income and increasing urbanization, not only the amount of food required will change, but also consumption patterns (Henchion et al. 2017). In particular, the demand for animal-derived protein is projected to increase by 70% until 2050, which has far-reaching environmental impacts on land use, water consumption and the emission of climate-relevant greenhouse gases (OECD and FAO, 2018). Consequently, natural ecosystems are converted into agricultural land, so that ecosystem services are lost and biodiversity is endangered on a global scale. However, due to sustainability or health aspects, consumer behavior is beginning to change and the demand for and acceptance of meat substitutes is steadily increasing. Although the alternative protein sources segment is currently less than 5% compared to the conventional meat and poultry market, its average growth rate is twice as high (Joseph et al. 2020). Some of the emerging and most promising non-meat sources include plant-derived proteins, seaweed and microalgae, fungal mycelium, as well as insects.

With around 2000 edible species, insects play a prominent role because entomophagy – the consumption of insects in their different developmental stages (eggs, larvae, pupae, adults) by humans – has been historically documented for thousands of years and even in our modern society more than 2 billion people regularly have insects on their menu (van Huis et al. 2013). In fact, they stand out from other livestock because of their comparatively low land and water requirements (50-500% reduction), lower greenhouse gas (CO_2, CH_4, N_2O) and NH_3 emissions, shorter generation times, higher reproductive capacities and highly efficient feed conversion rates (Oonincx and de Boer, 2012; Smetana et al. 2016; Henchion et al. 2017). The black soldier fly, *Hermetia illucens* (BSF; Diptera: Stratiomyidae), comes with big promises for industrial purposes since its larvae feed polyphagously on a broad spectrum of organic substrates of animal and plant origin, including waste and side streams (Lalander et al. 2015). In view of the resource limitations of the food and feed producing industry, it makes sense to use BSF larvae as bioconversion agent to upcycle side streams into high-quality insect biomass in order to conceptualize the entire production chain more efficiently and sustainably.

This economic development is reflected in the legislation of the European Union (EU) since 2017. As a result, BSF larvae and six other insect species, including the protein fractions obtained from them, have been permitted for feeding aquaculture animals (Regulation (EU) No 893/2017). In addition, the approval was extended to pig and poultry farming in 2021 (Regulation (EU) No 1372/2021). An application for the authorization of BSF larvae for human consumption is currently being assessed by the European Food Safety Authority, taking into account the novel food regulation.

In general, further questions arise that are closely related to the sustainability aspect of BSF as food and feed. Which organic side streams are suitable for competitive larval rearing on an industrial scale, whether pretreatments are necessary due to their physicochemical properties, the influence of the gut microbiota on animal health and the digestion of the feed, as well as basic circumstances of mating and oviposition, are barely understood. In addition and due to the steadily increasing production output of the BSF, although no major outbreak of a pathogen has been documented in a rearing facility (Joosten et al. 2020), there is a legitimate interest in identifying putative entomopathogens.

1.1 Life cycle of the black soldier fly

The BSF is originally endemic to the tropics and subtropics of America. Predominantly anthropogenic influences led to the species being spread to other tropical and subtropical areas such as Africa, Australasia and the Orient, but also to temperate and subpolar zones such as North America, Europe and large parts of Asia (Rozkošný, 1983). In addition, the wide temperature tolerance (12 – 40 °C) and the saprobiotic polyphagous diet of the larvae favor the spread of the species (Rozkošný, 1983; Chia et al. 2018).

The life cycle of the BSF is relatively short at 40 – 45 days and can be categorized into four major developmental stages: egg, larva, pupa, and adult fly. Female adults usually lay between 300 – 1000 eggs, with the quantity of eggs strongly depending on the rearing substrate (Tomberlin and Sheppard, 2002; Georgescu et al. 2021a). The eggs are elliptical in shape, rounded at both ends, and are 1 – 1.4 mm long with a 0.4 – 0.6 mm diameter (Figure 1a). Typically, the eggs are deposited as clusters in the form of overlapping layers (clutches) and covered with mucus, which ensures adhesion within the clutches, but especially to the substrate (Barros et al. 2018). The exact chemosensory perception of oviposition attractants has not yet been clarified, but odorant binding proteins are

increasingly being identified, which could be part of the molecular machinery underlying odor recognition (Nardiello et al. 2022). Related to this, decaying organic matter is currently used as an artificial attractant to promote directional oviposition. In general, however, females lay their eggs in protected, hidden places (Tomberlin and Sheppard, 2002; Georgescu et al. 2021a). The larvae hatch after about four days and have basically the same morphology, which differs only slightly during juvenile development. The larval instars (L1-L6; Figure 1b – g) can be differentiated from each other based on the diameter of the head capsule, as this is more sclerotized and only increases after moulting (L1: 0.05 – 0.08 mm; L2: 0.09 – 0.24 mm; L3: 0.25 – 0.45 mm; L4: 0.46 – 0.57 mm; L5: 0.58 – 1.04 mm; L6: 1.05 – 1.14 mm) (Barros et al. 2018). Concurrently, the weight is steadily increasing, so that by the end of the fifth instar, an individual may weigh up to 300 mg. During the first two instars, the larvae are white, which changes to creamy white or yellow by the fifth instar and finally turning to distinctive shades of brown by the sixth instar (prepupa) (Barros et al. 2018).

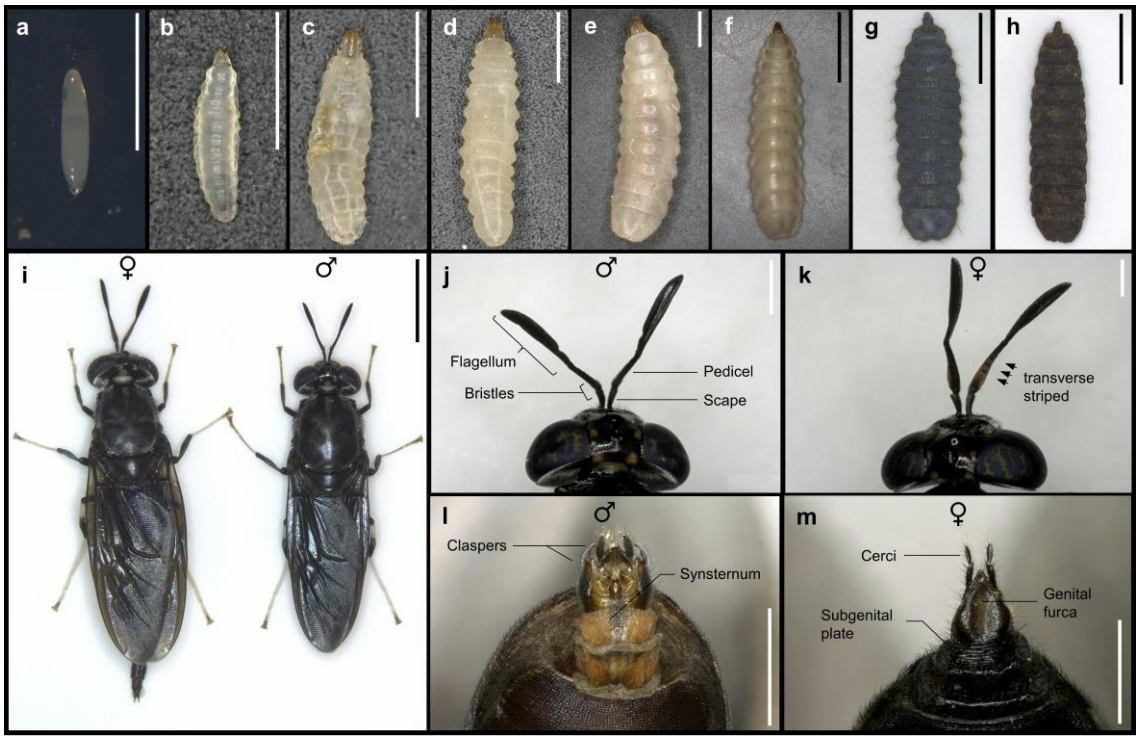

Figure 1: Life cycle and sexual dimorphisms of the antennae and abdominal genitalia of adult BSF. a) single deposited egg; b–g) temporal sequence of larval instars L1–L6 (prepupa); h) pupa; i) full body view of adult female (left) and male (right) BSF; j,k) dorsocranial view of male and female antennae; l,m) ventrocaudal view of male and female genital structures; scale bars: white = 1 mm, black = 5 mm; pictures by Patrick Klüber.

The larval development (L1-L5) lasts 13 – 18 days on average and is of exceptional importance for the entire life cycle, since only in this stage feed is used for the building of body mass, especially that of the fat body, serving as energy reserve for subsequent

non-feeding stages, namely prepupa, pupa, and adult fly (de Smet et al. 2018). During the transition from L5 to prepupa, strong morphological and, as a result, behavioral adaptations take place. While L5 larvae still have movable mouthparts, those of the prepupa have receded. Consequently, they can no longer feed and tend to leave the substrate (Schremmer, 1984). This property is used industrially as the so-called "self-harvesting". After about seven days, the prepupa successively sclerotizes the cuticle, thus forming the immobile pupa (Figure 1h). In the pupal stage, metamorphosis takes place in the puparium for approximately ten days, followed by the emergence of adult flies through a T-shaped dorsal thoracic suture (Schremmer, 1984). Adults have pronounced external sexual dimorphisms including the antennae and genital structures. In contrast to the males, females have a voluminous pedicel occupied by light horizontal stripes, whereas the second antennomer of the male imagines are markedly darker and transverse stripes are only schematically recognizable. At the basal junction of pedicel and scape, males show stronger bristles (Figure 1i – k). Abdominal male genitalia have claspers and the synsternum is flanked on both sides by two posterolateral lobes (Oliveira et al. 2015). Characteristics of the female terminalia are the tapered, distally elongated subgenital plate and the subtriangular genital furca (Figure 1l – m). The cerci are elongated and have two segments (Rozkošný, 1983; Oliveira et al. 2015). The reported adult lifespan differs greatly in the literature, but is usually 5 – 15 days in which they mate (de Smet et al. 2018). Reproduction is light dependent and naturally stimulated by sunlight (Tomberlin and Sheppard, 2002). Successful copulation marks the beginning of a new life cycle.

In general, BSF life-history traits are modulated by various biotic and abiotic factors, e.g. stocking density, quality of the feed substrate (Meneguz et al. 2018; Lalander et al. 2019; Li et al. 2022), temperature (Chia et al. 2018), feed moisture (Cammack and Tomberlin, 2017) and the microbial load (Čičková et al. 2015).

1.2 Factors influencing mating and oviposition

Reproduction of the adult BSF is regulated multifactorially and represents still one of the biggest bottlenecks in industrial mass rearing (Oonincx et al. 2016). Especially volumetric factors like cage size and fly density are supposed to play a critical role in egg productivity (Park et al. 2016; Hoc et al. 2019). BSF can be cultivated successfully in small-scale indoor systems (27 x 27 x 27 cm) in addition to conventional procedures (> 100 x 100 x 100 cm; Nakamura et al. 2016). Park et al. (2016) showed that egg numbers and total egg weight increased at higher population densities (4, 8, and 10 kg

prepupae per cage). In addition, fecundity is influenced by the ambient temperature. While a female deposited 475–516 eggs at 30 °C, egg clutch size decreased to about 300 eggs at 25 °C (Chia et al. 2018). Relative humidity effects on fecundity of several arthropods have been investigated in detail, whereas it is still unclear whether mating and oviposition of the BSF are affected (Holmes et al. 2012). In general, quality and availability of feed during larval development as well as adult water supplements like sugar, milk powder and peptone were shown to enhance egg production and extend oviposition period and adult longevity (Bertinetti et al. 2019; Macavei et al. 2020).

Differences in reproductive success may also reflect microbe–insect interactions. In BSF, oviposition was shown to be mediated by bacteria in a conspecific manner. For example, *Bacillus* species isolated from BSF eggs reduced the ovipositional response of females by more than 50% when added to artificial egg traps (Zheng et al. 2013). Other dipteran species, including *Aedes aegypti* (Diptera: Culicidae), also recognize specific bacterial compositions, indicating appropriate conditions for egg development and deposition (Ponnusamy et al. 2010).

The sex ratio represents one of the key regulatory parameters of reproduction, although the ratio that ensures efficient reproduction is still unknown. Theoretically, a 1:1 ratio would be optimal given that both female and male flies mate only once. An unbalanced sex ratio raises several problems: If the males dominate the population, a competitive behavior might occur. Furthermore, same-sex courtship was observed using virgin males (Giunti et al. 2018). The probability of successful fertilization would also decrease if the population were predominantly female. According to Hoc and colleagues, a sex ratio slightly shifted in favor of males could promote efficient egg fertilization (Hoc et al. 2019).

Mating behavior and the associated reproductive success of BSF are light-dependent and most efficient under direct sunlight (Tomberlin and Sheppard, 2002; Zhang et al. 2010). Oonincx et al. (2016) demonstrated that the ommatidia photoreceptors of adults perceive ultraviolet, blue, and green light. Based on the spectral sensitivity of their compound eyes, it is assumed that wavelengths between 332 nm and 535 nm influence mating behavior, whereas other studies suggest a spectrum of 450–700 nm (Zhang et al. 2010). Various lighting systems of different construction, including fluorescence lamps, quartz iodine lamps, metal halide lamps, and light-emitting diodes (LEDs), have already been

investigated for the artificial illumination of adult BSF, with LEDs in particular offering advantages for commercial breeding due to their outstanding energy efficiency (Heussler et al. 2018; Schneider, 2019; Liu et al. 2020; Macavei et al. 2020). Moreover, LED illumination resulted in a higher hatching rate within the same number of egg clutches compared to fluorescent tubes (Oonincx et al. 2016). In contrast, no mating occurred under rare earth lamp illumination (Zhang et al. 2010). A recent study postulates that increasing the irradiance from 0.92 W/m^2 to 431 W/m^2 improved the mating probability from 23% to 70% and simultaneously halved the time of mating initiation. Thus, optimization of irradiance level can maximize the mating success in indoor breeding systems (Schneider, 2019). Increasing the light duration from 6 to 18 h led to changes in the nycthemeral cycle of adults, which resulted in a reduction of the oviposition period by 3 d (Hoc et al. 2019). Modulation of the oviposition period by changes in light duration was already demonstrated in several studies (Zhang et al. 2010; Nakamura et al. 2016; Heussler et al. 2018; Hoc et al. 2019).

Altogether, BSF reproduction is subject to a complex regulatory network of various biotic and abiotic factors, which are not yet fully understood. However, the interaction of these factors and the application of knowledge for mass rearing is of particular interest.

1.3 The black soldier fly as bioconversion agent

As both human and animal populations are predicted to grow, the agricultural and livestock associated production of wastes and side streams will also increase (Kooienga et al. 2020). To counteract the further intensification of the food and feed sector, an innovative approach is necessary to re-integrate resources that are currently lost along the production chain (Peguero et al. 2022). BSF larvae were shown to be voracious feeders that convert a broad range of organic matter highly efficiently into valuable biomolecules like proteins, lipids, and chitin, which in turn serve as raw materials for the production of animal feed (Tschirner and Simon, 2015; Smetana et al. 2016; Mujahid et al. 2017; Hudson et al. 2019), lubricants (Xiong et al. 2020) or biodiesel (Surendra et al. 2016), chitosan (Soetemans et al. 2020), pharmaceuticals and cosmetics (Almeida et al. 2020). In addition, the waste dry mass is reduced, making an important contribution to the circular economy concept. Although BSF larvae do not appear to have certain dietary restrictions and are even capable of valorizing low-value organic material into insect biomass (Gorrens et al. 2021; Table 1), feeding agri-food wastes with high lignocellulose contents resulted in low growth performance (Peguero et al. 2022). As with other

livestock, it is assumed that the nutritional value of the diet has the greatest impact on performance. Accordingly, underfeeding or insufficient nutrient composition lead to a reduction in growth and waste conversion, and consequently prolongs larval development (Danielsen et al. 2013). To enhance the digestibility and nutrient accessibility for BSF larvae, various pretreatment procedures may be implemented depending on the substrate properties. These include physical (mechanical, thermal, electromagnetic), chemical (oxidizing agents, acids, alkalis) as well as microbiological (bacteria, fungi, yeasts) approaches and combinations thereof (summarized in Peguero et al. 2022).

In general, the great variability of waste types and their nutritional values represents a major challenge when used as feed for BSF. However, it was shown that the larval performance can be improved by co-conversion of mixed waste streams compared to individual biowastes (Gold et al. 2020). Using mixed formulations designed on the quantity and compositional data of individual wastes offer a more predictable waste management in BSF facilities (Gold et al. 2020). The suitability of various organic matter as feeding substrate for BSF larvae has been studied extensively over the past two decades. These include waste and side streams of plant, animal and human origin that are generated during the production of food and feed, or that are disposed by consumers. In order to provide a comprehensive overview, the major findings of 67 publications have been summarized in Table 1.

Despite the promising and versatile application possibilities of BSF larvae in waste management, feeding of insects is regulated within the legislative framework of the EU. Since industrially reared insects are classified as "farmed animals", the same general rules and restrictions apply as for conventional livestock. Thus, farmed insects are subject to the feed ban rules laid down in Article 7 and Annex IV to Regulation (EC) No 999/2001 and, additionally, to animal feeding rules laid down in Regulation (EC) No 1069/2009. According to the abovementioned regulations, insects are not allowed to be fed with slaughterhouse or rendering-derived materials, faeces, catering waste, and unsold supermarket or industrial products that are containing fish or meat (Veldkamp et al. 2022).

Table 1: Comprehensive categorization and description of the waste and side streams that have been investigated in the literature as feeding substrates for BSF larvae. Poultry/ chicken feed or Gainsville formulation are included as well, since these diets are used frequently as high-quality reference in many studies. The various mixtures and ratios of individual components are not specified. Superscript numbers refer to the corresponding references.

Category	Waste/ side stream description	References
Milling/ alcohol production side streams	*Milling products and side streams:* wheat by-products (not specified)[1], wheat bran[2], processed wheat[3,4], corn meal[5-7], feed mill middlings (broken pellets, spilled grains, grinding dust)[8] *Brewery/ winery/ distillery side streams:* brewers' spent grains[7,9-15], beer yeast[9,15], millet brew waste[16], brewery trub[13], winery side streams (grape seeds, pulps, skins, stems, leaves)[11], dried distillers' grains with solubles (barley, corn, wheat, sugar syrup)[8], corn distillers' grains[12], waragi (Ugandan homemade gin) brewing waste[17]	Gold et al. 2020[1]; Gao et al. 2019[2]; Biancarosa et al. 2017[3]; Liland et al. 2017[4]; Georgescu et al. 2021[5]; Barbi et al. 2020[6]; Broeckx et al. 2021[7]; Tschirner and Simon, 2015[8]; Oonincx et al. 2015[9]; Chia et al. 2018a[10]; Meneguz et al. 2018[11]; Bava et al. 2019[12]; Jucker et al. 2019[13]; Shumo et al. 2019[14]; Magee et al. 2021[15]; Hudson et al. 2019[16]; Dobermann et al. 2019[17]; Ewald et al. 2020[18]; Lindberg et al. 2022[19]; Sarpong et al. 2019[20]; Roberts et al. 2019[21]; Srikanth and Deshmukh, 2021[22]; Diener et al. 2011[23]; Spranghers et al. 2016[24]; Lalander et al. 2019[25]; Surendra et al. 2016[26]; Matheka et al. 2021[27]; Veldkamp et al. 2021[28]; Romano et al. 2022[29]; Harnden and Tomberlin, 2016[30]; Kawasaki et al. 2022[31]; Ong et al. 2017[32]; Gabler, 2014[33]; Lalander et al. 2013[34]; Lalander et al. 2015[35]; Peguero et al. 2021[36]; Popa and Green, 2012[37]; Jucker et al. 2017[38]; Cappellozza et al. 2019[39]; Isibika et al. 2021[40]; Rahmi et al. 2020[41]; Putra et al. 2020[42]; Rahman et al. 2021[43]; Lardé, 1990[44]; Pliantiangtam et al. 2021[45]; Palma et al. 2020[46]; Tinder et al. 2017[47]; Li et al. 2022[48]; Rehman et al. 2017[49]; Permana et al. 2021[50]; Cai et al. 2019[51]; Klüber et al. 2022[53]; Klüber et al. 2022a[54]; Mujahid et al. 2017[55]; Abduh et al. 2017[56]; Abduh et al. 2017a[57]; Tegtmeier et al. 2021[58]; Manurung et al. 2016[59]; Rodrigues et al. 2022[60]; Sheppard et al. 1994[61]; Miranda et al. 2019[62]; Oonincx et al. 2015a[63]; Zhou et al. 2013[64]; Jucker et al. 2020[65]; Schmitt et al. 2019[66]; Wu et al. 2022[67]
Municipal organic wastes	*Food and organic wastes:* general household food[7,18-20] and kitchen waste[21,22], specific kitchen waste (potato peelings, carrots and their peelings, rice, bread debris[18])[14], municipal organic waste from university[23], supermarket[7,20], restaurant[7,20,24,25], canteen (as well as vegetable canteen)[1], cafeteria[26,27] and catering swill[28], spent coffee[29], specific meat (pork[30], minced pork[31], beef[30]) and rice dishes (pure, curry, coconut milk, fried)[32] *Manure and wastewater:* human faeces (freshly collected)[25,33-35], faecal sludge from urine-diverting dry toilets[1,27,36], wastewater sludge (primary, undigested, anaerobically digested) from sewage treatment plants[25], compost leachates[37]	
Agro- and food industry wastes	*Fruit and seed wastes:* general fruit waste (not specified)[5,7,15], exotic fruit waste[6], pomace[6], apple[11,22,25,38,39] (leftovers[11] and pulp[6,7]), orange[11,38,39], orange peel[19,40], mandarin and lemon[11], banana[11,22], banana peel[40-42], pineapple[6,22], kiwi[6,11,39], pear[11,38], (water)melon[6,22], pepper[11,39], mango[22], peach[6], strawberry[11], plum, eggplant, zucchini[39], cocoa pod husk[43], olive pulp[28], coffee pulp[44], avocado[16], tomato leaves and stalk-leftovers[6,7], coconut endosperm[45] and testa[42], almond hulls and shells[46] *Vegetable and grain wastes:* general vegetable waste (not specified)[5,7,15], legumes[6] like pea (not specified)[24], cowpea[47], soybean meal[48], okara (soybean curd residue)[12,45,49], green beans[38,39], sorghum[47], fermented barley (husks, seeds)[50], cucumber[22], broccoli/cauliflower trimmings[19], cabbage[22,38], celery[11,24,39], matured chicory roots[7], lettuce[25,38], carrot[22,24,39], (dried) sugar beet pulp[8,15], beet molasses[9], potato[25,39] (steam peelings[9]), sweet potato[29], sweet potato root silage[21], cassava peels[41], salsify[24], mushroom waste (*Flammulina velutipes*)[51], seaweed biomass(*Ascophyllum nodosum*)[3,4] *Processed wastes:* bakery waste (not specified)[15], bread dough[29], bread and cookie remains[9], cheese waste[15] *Other wastes:* fermented[41,53]/ not fermented palm kernel meal[54], fermented empty fruit bunches (*Trichoderma* sp.[55], *Bjerkandera adusta*[53]), rubber seeds (defatted, fermented)[56], *Pandanus tectorius* fruit[57], cottonseed press cake[58], rice straw[59], fermented corn straw (*Aspergillus oryzae*)[2], biogas digestate[24] (beet pulp, leaves, root tips[28]), silage grass[28]	
Lifestock wastes	*Animal feed:* Gainsville diet[5,7,30,45,47], chicken/poultry feed[1,24,25,28,33,45,60] (chicken start mash[7,17], laying hens[12], broilers[28]), rabbit feed[23], dog food[25,35], fish feed waste[15], expired fish feed[60] *Rearing wastes:* chicken/poultry manure[14,25,61-64], feathers[16], bedding materials (sawdust[16], wood-pulp[7]), pig manure[16,28,35,62-64], cow/dairy cattle manure[1,16,49,62-64], insect rearing waste (frass, exuviae, dead insects, uneaten feed) from locusts (*Schistocera gregaria*) and crickets (*Gryllus bimaculatus, Gryllodes sigillatus*)[65], aquaculture sludge[66] *Abattoir wastes:* slaughterhouse wastes from chicken (meat and bone meal)[67], poultry (feet, head, liver, stomach, intestine)[1], sheep (stomach contents, manure, meat, lungs, heart) and cattle (blood)[25], rainbow trout[18], fish trimmings (not specified)[15], perch and roach trimmings (fins, internal organs)[40], blue mussel (fresh, ensiled, rotten)[18]	

Feeding substrates for insects are restricted to products of vegetal origin, but also products of animal origin of Category 3 are authorized: fishmeal, blood products from non-ruminants, di- and tricalcium phosphate of animal origin, hydrolyzed proteins from non-ruminants or from hides and skins of ruminants, gelatin and collagen from non-ruminants, eggs and their products, milk, milk-based or -derived products, honey, and rendered fats (Regulation (EU) No 893/2017).

1.4 The larval gut microbiota – a multifunctional key mediator

Larvae of the BSF occupy an outstanding position within the order of Diptera, since they have a low diet specialization and are more likely to be regarded as generalists. Similar to many other animals, the BSF relies on a beneficial gut microbiota, which includes viruses, bacteria, archaea, protists, and fungi colonizing its digestive tract (Blyton et al. 2019; Sontowski and van Dam, 2020). These insect-associated microbes are involved in critical processes including (i) development of the gastrointestinal tract, (ii) modulation of the immune system, (iii) digestion of stodgy and detoxification of noxious compounds, (iv) provision of essential amino acids and vitamins, (v) as well as the synthesis of pheromones and kairomones required for inter- and intraspecific communication (Engel and Moran, 2013; Broderick et al. 2014; de Smet et al. 2018; Vogel et al. 2018; Ao et al. 2020; Tegtmeier et al. 2021). Moreover, beneficial microbiota occupy niches in the host's gut providing colonization resistance against pathogens and parasites through direct competition or by stimulating the synthesis of insect antimicrobial peptides (AMPs) (Koch and Schmid-Hempel, 2011; Sontowski and van Dam, 2020). These are part of the humoral immunity produced especially in the larval fat bodies. Recently, articles investigating extracts of BSF larvae as well as specific AMPs are increasing and report strong antimicrobial effects against bacterial pathogens such as *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas fluorescens*, multi-drug resistant *Pseusomonas aeruginosa*, *Salmonella* spp., and *Micrococcus luteus* (Vogel et al. 2018; Lee et al. 2020; van Moll et al. 2022). On the other hand, no antifungal activity could be observed in a study evaluating 36 AMPs against *Aspergillus fumigatus* and *Candida albicans* (van Moll et al. 2022).

Particularly, the ability to grow on substrates with a high microbial load, such as faeces, implies a highly adaptable immune response. In fact, BSF larvae are able to change the microbial composition of the substrate (Zhang et al. 2020; Cifuentes et al. 2020) and reduce the amount of pathogens such as *Salmonella* spp. and enterohemorrhagic *E. coli*

O157:H7 in manures and aquaculture waste, whereas no reduction of *Enterococcus* spp. was reported throughout the rearing cycle (Erickson et al. 2004; Liu et al. 2008; Lopes et al. 2020). Concurrently, Erickson and colleagues isolated viable *Salmonella enterica* serovar enteritidis after six days of exposure to contaminated manure in the larval gut (Erickson et al. 2004). Other potential foodborne pathogens such as *Bacillus cereus* were also found to be accumulating in the BSF gut, emphasizing the requirement of a decontamination step in terms of food and feed safety (Wynants et al. 2019).

Several bacterial and fungal isolates from the gut of BSF larvae were examined for their antimicrobial activity against common pathogens. Six *Providencia rettgeri* isolates demonstrated substantial inhibitory effects against *P. aeruginosa in vitro* while three *Alcaligenes faecalis* isolates inhibited the growth of *Staphylococcus aureus* or *E. coli* K12 strains (Tegtmeier et al. 2021a). Representatives of the genus *Providencia* are frequently detected in guts of BSF larvae fed with livestock manure or kitchen wastes, with the relative abundance varying between studies (Ao et al. 2020; Shumo et al. 2021). Until now, only two fungal species were isolated from the digestive tract of BSF larvae that are known for the production of antimicrobials: *Trichosporon asahii* was shown to inhibit *Candida* yeasts such as *C. glabrata* and *C. lusitaniae* and *Chrysosporium multifidum* was found to have moderate antimicrobial activity against *S. aureus* (Varotto Boccazzi et al. 2017; Correa et al. 2019). *Trichosporon* spp. has been associated with BSF larvae reared on different diets and was detected as a predominant member (up to 90.4%) of their gut mycobiome (Varotto Boccazzi et al. 2017; Tegtmeier et al. 2021). Interestingly, a study by Gorrens and colleagues revealed that six *Trichosporon* spp. isolates exhibited pronounced antistaphylococcal activity (Gorrens et al. 2021).

Until now, most of the studies on microbes were culture-independent using high-throughput next-generation sequencing methodologies and have mainly focused on the bacterial composition, while little is known about the dynamics and functional relationships of mycobiota. Since many microbes associated with insect guts are obligate anaerobes or have unique requirements, they remain uncultivated and their exploitation potential for biotechnological applications is not yet realized (Varotto Boccazzi et al. 2017; Callegari et al. 2020; Gorrens et al. 2021a; Tegtmeier et al. 2021a). Nevertheless, functional parallels were drawn between the gut microbiome of insects and conventional farm animals, suggesting a significant contribution to productivity and health status. The utilization of versatile and often recalcitrant feeding substrates by larvae of the BSF is

strongly depending on their microbiota (Jeon et al. 2011; Klammsteiner et al. 2020; Klammsteiner et al. 2021; Tanga et al. 2021). For example, the degradation of complex plant biopolymers such as cellulose and lignin would not be possible without associated microbes. For *Monochamus marmorator* (Coleoptera: Cerambycidae), it could be shown that the ascomycete *Trichoderma harzianum* enables the larvae to degrade cellulose (Kukor and Martin, 1986). Conversely, the gut microbial composition and diversity adapt dynamically and reflect the type of nutrients contained in the feed (Jeon et al. 2011; Bruno et al. 2019; Ao et al. 2020; Shelomi et al. 2020; Galassi et al. 2021; Tanga et al. 2021; Gold et al. 2022), as well as the instar of BSF larvae (Zheng et al. 2013a; Cifuentes et al. 2020). In this context, the diet-dependent expression of more than 50 putative AMPs plays a key role in the regulation of gut microbial composition (Vogel et al. 2018) although there is also an omni-present core community (Klammsteiner et al. 2020; Tegtmeier et al. 2021). Based on the intersection of various articles, representatives of the genera *Enterococcus*, *Morganella*, *Providencia*, *Klebsiella*, *Scrofimicrobium*, and *Actinomyces* can be considered as an integral part of the bacterial core gut microbiome of BSF larvae (Wynants et al. 2019; Ao et al. 2020; Cifuentes et al. 2020; Klammsteiner et al. 2020; Liu et al. 2020a; Khamis et al. 2020; Zhang et al. 2020; Klammsteiner et al. 2021; Shumo et al. 2021; Tegtmeier et al. 2021; Tegtmeier et al. 2021a; Gold et al. 2022; Gorrens et al. 2022). It can be assumed that yeasts and filamentous fungi are selected to a much greater extent by the diet than bacterial representatives, since Varotto Boccazzi and colleagues were not able to detect even one common operational taxonomic unit between different feeding groups (Varotto Boccazzi et al. 2017). Besides intrinsic substrate properties, it was shown that external factors such as temperature also affect the structure of the bacterial community. While higher temperatures (between 20–33 °C) led to faster larval growth and a higher mortality, counts of viable *Campylobacter* and *Listeriaceae* in corresponding prepupae increased (Raimondi et al. 2020). The inoculation of the substrate with companion bacteria such as *Bacillus subtilis* or *Bacillus licheniformis* had positive effects on growth and developmental parameters of BSF larvae (Yu et al. 2011; Callegari et al. 2020). The feeding status (especially periods of starvation) also influence the microbiome and its associated metabolic functions, thus potentially disrupting larval growth and feed conversion efficiency (Yang et al. 2021). New findings suggest that host genetics also alters the diversity and metabolic function of the gut microbiome (Greenwood et al. 2021).

In conclusion, homeostasis of the BSF larval gut microbiota represents an indispensable key regulator for feed degradation capacity and insect health, without which the larvae would be less adaptable and have a more restricted feed spectrum.

1.5 Entomopathogenicity in insect rearing

The commercialized insect rearing has been steadily increased internationally over the last two decades, resulting in both, more insect farms being started and an increase in their scale of production, leading to better price competitiveness with other plant- or animal-derived protein sources. In Europe, ~6,000 tons of insect protein were produced annually by 2019. According to the International Platform of Insects for Food and Feed (IPIFF), the production volume will grow to an estimated 2 – 5 million tons of insect protein per year by 2030, depending on the legislative framework within the EU. This trend is reflected in the investments made; over 600 million € had been raised from European insect farmers by the end of 2019 (IPIFF, 2019).

The rapid growth of the production output and upscaling of the facilities entail health risks for the insects (Joosten et al. 2020; Maciel-Vergara et al. 2021). Entomopathogenicity can generally be defined as the property of viruses or microorganisms – including bacteria, fungi, protozoa, and nematodes – to colonize insects, outwit their immune response with the help of varying virulence factors, and thus harm or kill the host. Indeed, insect pathogens and diseases in mass-rearing facilities are not a new phenomenon, particularly in traditional husbandry systems such as beekeeping or silk production (Maciel-Vergara et al. 2021). In addition, various disease outbreaks associated with entomopathogens have recently been reported in insect species produced for food and feed, such as the mealworm, *Tenebrio molitor* (Coleoptera: Tenebrionidae), and the house cricket, *Acheta domesticus* (Orthoptera: Gryllidae) (Eilenberg et al. 2015). BSF larvae, on the other hand, occupy a special position. They are almost anecdotally considered highly resistant to colonization by pathogens, as no major outbreaks have been documented to date and because of their ability to survive even on microbially contaminated substrates such as faeces (Erickson et al. 2004; Lalander et al. 2013; Eilenberg et al. 2015). However, it should not be mistakenly assumed that the BSF is immune to any microbial attack. For example, representatives of the fungal orders Entomophthorales and Hypocreales, as well as bacterial genera *Bacillus*, *Lysinibacillus*, and *Pseudomonas*, are prominent taxa, which were shown to infect various dipterans (Leatherdale, 1970; Keller, 2007; Berry, 2012; Ben-Dov, 2014; Panayidou et al. 2014).

On the other hand, some entomopathogens represent powerful resources that can be used as biological control agents in integrated pest management (Ruiu, 2015).

Due to the tremendous biodiversity of insects and microorganisms, as well as the complexity of interkingdom interactions, our knowledge is still very limited. Accordingly, identifying putative pathogens of economically relevant insects at an early stage is of particular interest in order to be able to react quickly with appropriate zoohygienic measures in the event of an outbreak. Aquaculture is suitable for assessing future economic risks in insect farming, as it also keeps poikilothermic animals in a controlled environment under high-density conditions (Joosten et al. 2020). According to the FAO world fisheries and aquaculture report, Chinese aquaculture alone recorded disease-related production losses of 205,000 tons, worth 375 million €, in 2018 (FAO, 2020).

1.6 Research objectives

The food sector faces the difficult challenge of meeting the protein demand of a steadily growing world population. In addition to overfishing and the inefficient use of fishmeal in aquaculture, the arable land used for the cultivation of plant-based feedstuffs such as soybeans or corn consumes an enormous amount of land. Large quantities of not used or underutilized side streams are generated during various stages of food and feed production, ranging from initial harvesting to subsequent processing. Insects are rapidly becoming recognized as a sustainable source of animal protein due to their excellent nutritional properties and their industrially scalable husbandry conditions. In particular, the black soldier fly (BSF; *Hermetia illucens*) has a high exploitation potential and was reported to be capable of acting as a bioconversion agent for a variety of such side streams, although many aspects of breeding still need to be optimized. The aim of this thesis was to valorize low-value side streams of the palm oil production by breaking them down through a fermentative pretreatment followed by an evaluation study as a cost-effective diet for BSF larvae. Furthermore, the role of the larval gut microbiome as well as diet-dependent changes thereof were examined by cultivation and Illumina high-throughput sequencing.

In contrast to various feeding studies reporting larval development, literature describing adult life-history traits is rare. In the frame of the first research objective, the suitability of three light-emitting diode (LED) panels with different color temperatures as an

artificial illumination system for BSF breeding was examined. Given that LEDs have a comparatively low energy requirement and are therefore cost-effective, it was postulated that they might represent an adjustable alternative to fluctuating sunlight supply, especially at latitudes with short days in autumn and winter months, as well as for other types of lamps. It was further suggested that the color temperature of the light source could affect mating and thus the general reproductive success of BSF adults. To test this hypothesis, opaque chambers were constructed in which randomized populations of 200 adults were illuminated with the respective LEDs (3,000, 4,000, 6,500 K) as the sole light source. To assess the effect of color temperature on reproductive performance, parameters including mating, preoviposition period, oviposition period and peak, egg weight, as well as the egg viability were documented. Detailed information on BSF breeding and the experimental design described above are presented in the first publication of this thesis:

Klüber P, Bakonyi D, Zorn H, and Rühl M. Does light color temperature influence aspects of oviposition by the black soldier fly (Diptera: Stratiomyidae)? *J. Econ. Entomol.* **2020**; 113(5): 2549-2552.

Larvae of the BSF grow even on substrates with a high microbial load and have been shown to alter the microbial composition of the feed. They probably owe this property to the synthesis of antimicrobial peptides (AMPs) and associated microbiota, which mediate colonization resistance against pathogens. However, large-scale industrial projects and the production volume of BSF larvae have increased significantly in recent years, as has the risk of contamination of such facilities by entomopathogenic microbes. Although there has not yet been a proven outbreak, there is still a legitimate interest in identifying putatively harmful germs. As part of the second research objective, the gut microbiota of BSF larvae fed with untreated palm kernel meal (PKM) – a side stream of palm oil production – was characterized culture-dependently in order to discuss their functional roles, along with possibilities for biotechnological application. The entomopathogenic potential of the isolates was assessed on the basis of a literature review and subsequently quantified *in vivo* by intracoelomal injection. A comprehensive description of the methodology employed for the isolation of gut microbiota, along with the discussion of infection mechanisms can be found in the second research article:

Klüber P, Müller S, Schmidt J, Zorn H, and Rühl M. Isolation of bacterial and fungal microbiota associated with *Hermetia illucens* larvae reveals novel insights into entomopathogenicity. *Microorganisms* **2022**; 10(2):319.

Side streams of the palm oil production, especially empty fruit bunches (EFB) and PKM, are characterized by a high lignocellulose content, which makes feeding of BSF larvae challenging. Unlike termites or cerambycid beetles, the BSF does not naturally have an exclusively phyto- or xylophagous diet, resulting in a lack of physiological prerequisites that would be necessary for this. The degradation of lignocelluloses requires special enzyme systems that are found in wood-degrading fungi, such as representatives of the Basidiomycota. In order to improve the digestibility, a fungal screening and subsequent solid-state fermentation was established as pretreatment of lignocellulosic side streams. The extent to which this procedure influences BSF life-history traits was tested in several feeding trials. Since the intestinal microbiota is considered to play a key role in dietary breakdown, but at the same time is strongly affected by the diet and its properties, adaptations of the bacterial and fungal communities were hypothesized. The microbial composition was identified by Illumina high-throughput sequencing of the 16S rRNA gene and the fungal internal transcribed spacer (ITS) regions. A thorough description of the implemented methodology is presented in the third publication:

Klüber P, Tegtmeier D, Hurka S, Pfeiffer J, Vilcinskas A, Rühl M, and Zorn H. Diet fermentation leads to microbial adaptation in black soldier fly (*Hermetia illucens*; Linnaeus, 1758) larvae reared on palm oil side streams. *Sustainability* **2022**; 14(9):5626.

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Research Articles

Research Article 1 – Does light color temperature influence aspects of oviposition by the black soldier fly (Diptera: Stratiomyidae)?

Klüber P, Bakonyi D, Zorn H, Rühl M. Does light color temperature influence aspects of oviposition by the black soldier fly (Diptera: Stratiomyidae)? *J. Econ. Entomol.* 2020; 113(5): 2549-2552.

DOI: https://doi.org/10.1093/jee/toaa182

Journal of Economic Entomology, 113(5), 2020, 2549–2552
doi: 10.1093/jee/toaa182
Advance Access Publication Date: 5 September 2020
Short Communication

Short Communication

Does Light Color Temperature Influence Aspects of Oviposition by the Black Soldier Fly (Diptera: Stratiomyidae)?

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Subject Editor: Allan Showler

Received 27 February 2020; Editorial decision 15 July 2020

Abstract

In recent years, black soldier fly, *Hermetia illucens* (L.), larvae have attracted increasing attention because of their high capacity for bioconversion of diverse organic material into high-quality protein and lipids. Although previous studies have focused on optimization of breeding conditions, such as the acceptance of substrates, and temperatures and moisture contents, little is known about light-dependent adult development. Artificial light sources are important to commercial *H. illucens* breeding, especially at latitudes with short days in autumn and winter months. We examined how 3,000, 4,000, and 6,500 K color temperatures affect aspects of oviposition. Mating occurred under all of the broad spectrum light-emitting diode panels, resulting in fertilized egg clusters. Oviposition lasted up to 15 d, while the shortest oviposition period, in the 3,000 K light treatment, was 2 d. Total oviposition performance and oviposition period were not affected by the light treatments. Oviposition peaked 1–7 d after eggs were first deposited. The time until oviposition peaked was positively correlated with increasing color temperature.

Key words: reproduction, mating, insect farming, light-emitting diode, artificial illumination

The black soldier fly, *Hermetia illucens* (L.), originally endemic to the Americas, has spread globally across subtropical and tropical regions worldwide (Makkar et al. 2014). As the larvae are saprobionts with a polyphagous diet, they have great potential for upcycling biogenic byproducts into high-quality insect protein and lipids (Nguyen et al. 2015, Barragan-Fonseca et al. 2018a), which can be used as feed for livestock (Allegretti et al. 2018, Lei et al. 2019, Yu et al. 2019, Zarantoniello et al. 2019).

The identification of suitable artificial light sources has a high priority because the mating of adult flies is light dependent and most successful in sunlight (Tomberlin et al. 2002, Zhang et al. 2010). Particularly in climatic zones outside the subtropics and tropics, sunlight is a limiting factor for commercial breeding due to seasonal fluctuations in autumn and winter months (Heussler et al. 2018). Oonincx et al. (2016) demonstrated that the ommatidia photoreceptors of imagines perceive ultraviolet, blue, and green light. Based on the spectral sensitivity of compound eyes, it is assumed that wavelengths between 332 and 535 nm influence mating behavior, whereas other studies postulate a spectrum of 450–700 nm (Zhang et al. 2010). Light sources of various designs have already been examined, including light-emitting diodes (LEDs), and fluorescence and quartz iodine lamps, in which successful reproduction occurred without differences in oviposition performance (Heussler et al. 2018). Other substitutes, such as rare earth lamps, were not suitable for illumination (Zhang et al. 2010). However, little attention has been given to the color temperature of artificial illumination systems. This study was conducted to investigate the effects of color temperature on oviposition by *H. illucens*.

Materials and Methods

Rearing *H. illucens*

Hermetia illucens larvae were obtained from Bio.S Biogas (Grimma, Germany). Three colonies were fed ad libitum with chicken feed composed of 16% crude protein, 3.5% crude fat, 5% crude fiber, and 12.5% crude ash (GoldDott Eierglück, DERBY Spezialfutter, Muenster, Germany; Diener et al. 2009, Ma et al. 2018, Lalander et al. 2019). Larvae were maintained in 19.5 × 16.5 × 9.5 cm (l × w × h) polypropylene containers with 150 mg of eggs per container at 27 ± 1°C and 60 ± 10% RH in darkness (Nakamura et al. 2016, Samayoa et al. 2016, Giunti et al. 2018). Once ≥50% of the

population had reached the sixth instar, prepupae were separated from the substrate by sieving with a 5-mm mesh gauge (AS 200, Retsch, Haan, Germany) and then pooled in a 25 × 18 × 13 cm (l × w × h) polypropylene container. Once they became pupae, twelve 60 × 60 × 90 cm (l × w × h) mesh cages (Bioform, Nuremberg, Germany), each with a mesh size of 0.6 mm, were stocked with 200 randomly selected adult *H. illucens* of the same age. Pupae were weighed (AT261 DeltaRange, Mettler, Giessen, Germany), and lengths were measured (Keyence VHX-2000 digital microscope, Keyence, Osaka, Japan) in order to exclude pupae that were below the critical weight for further development (Barragan-Fonseca et al. 2018b). Synchronization of pupal development was achieved by stocking cages exclusively with pupae that were ≤24 h old.

The Experiment

The experiments were conducted in 3,000, 4,000, and 6,500 K light chambers (L-PL-ECO623330, L-PL-ECO623340, L-PL-ECO623365, Lence Technology, Langen, Germany) in a greenhouse at 25 ± 1°C, 40 ± 10% RH, and 12:12 (L:D) h (Fig. 1). The front was covered with a movable opaque fabric that permitted gas exchange and collection of eggs. Water was available ad libitum throughout the experiment by water-soaked paper towels in a 17 × 11 × 6.5 cm (l × w × h) polypropylene container (Fig. 1). Three 20 × 5.5 × 0.9 cm (l × w × h) wooden board stacks held in place with rubber bands served as an artificial oviposition substrate. In each stack, three boards were spaced 2 mm apart using metal washers. Adult emergence was observed on a daily basis and considered completed when ≥50% of the population reached the imaginal stage. Beginning with the first occurrence of *H. illucens* imagines, mating behavior was observed. The preoviposition period lasted from initial stocking of the cages with *H. illucens* pupae until the first egg clusters were observed. Eggs were collected daily from the spaces between the wood stacks using a plastic spatula (Cole-Parmer Smart Spatula, Thermo Fisher Scientific, Waltham, MA). In addition, eggs that were deposited on the cage walls were collected. All of the eggs from each cage were then transferred to weighing paper (MN226, Macherey-Nagel, Düren, Germany) and weighed. The oviposition period was the time during which female *H. illucens* deposited eggs, whereas the oviposition peak was a specific day on which most eggs were deposited within the oviposition period. The weight of the eggs within a cage was summed at the end of the bioassay and normalized by the number of dead females to determine the average total weight of eggs deposited by individual females, a representation of fecundity. Collected egg clusters were cultivated in polypropylene containers as described above to verify their viability by observing larval hatching. To ensure that all pupae had enough time for metamorphosis, evaluation of the treatments was conducted 3 mo after the first adult had emerged. All experiments were conducted using four replicates.

Statistical Analyses

Statistical evaluation of oviposition period, oviposition peak, and total oviposition was performed using one-way ANOVA ($P < 0.05$; Excel 2016, Microsoft, Redmond, WA), and means were separated using the Bonferroni–Holmes test (Holmes 1979). The linear relationship between the time of oviposition peak and color temperature treatments was calculated using Pearson product-moment correlation (Hilgers et al. 2019).

Results

Pupal length and weight were 22.7 ± 0.1 mm and 169 ± 10.7 mg, respectively. After 10–11 d, ≥50% of the pupae had produced emerged adults. There were no differences in the female:male ratio between color temperature treatments. Incubation under light panels resulted in comparable preoviposition periods, averaging 16.8 ± 0.3 d, and subsequent deposition of fertile eggs. Eggs were deposited for a maximum of 14.3 ± 0.3 d, with 2 d, in the 3,000 K treatment, as the shortest observed oviposition period. The color temperature treatments did not affect oviposition period. Oviposition peaked 2.1 ± 0.4 d after the first eggs were deposited, and the time until the peak occurred was positively correlated with color temperature ($r = 0.61$, Fig. 2). At 6,500 K, the average peak occurred 2.3 d later than the 3,000 K treatment ($F = 1.02$; df = 2,7; $P = 0.009$), in which the oviposition peak occurred on day 1. Females deposited 4.7 ± 0.5 mg of eggs, unaffected by the color temperature treatments. Time of hatching, which occurred 2–5 d after oviposition, was also not affected by the color temperature treatments.

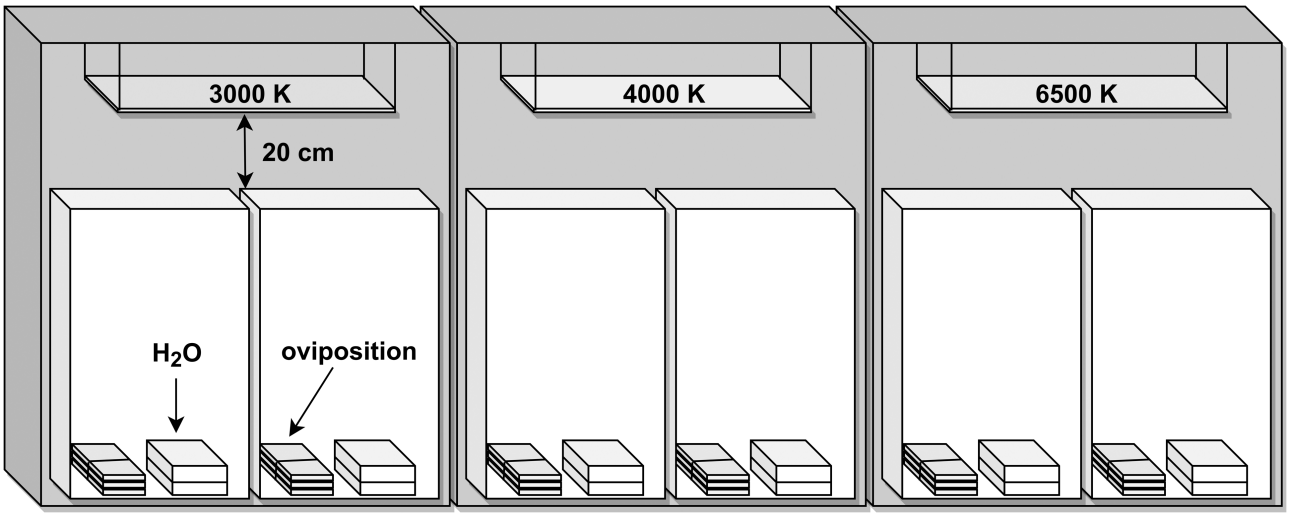

Fig. 1. Schematic diagram of light chambers with 3,000, 4,000, and 6,500 K color temperature LED panels installed in the center of 120 × 65 × 125 cm (l × w × h) wooden chambers with a vertical distance of 20 cm above two 60 × 60 × 90 cm (l × w × h) mesh cages; the front was covered with removable opaque fabric, and three wooden board stacks served as artificial oviposition substrate.

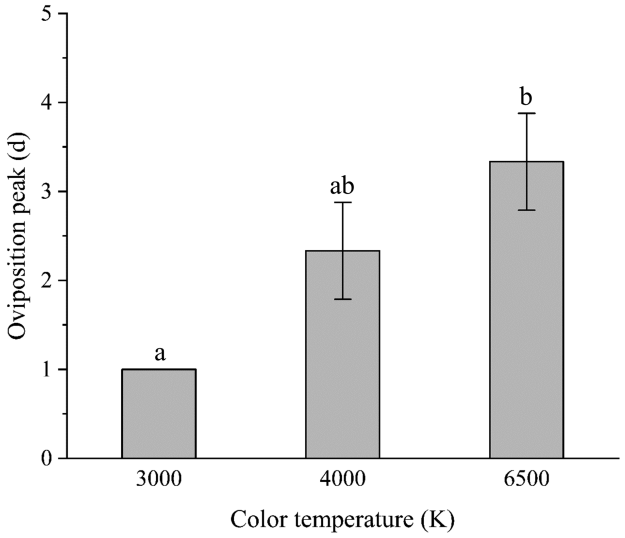

Fig. 2. Oviposition peak under different color temperature treatments; the bars represent means (±SE), treatment differences are designated with different letters (one-way ANOVA and Bonferroni–Holmes test [$P < 0.05$]).

Discussion

The mating behavior and reproductive success of *H. illucens* are light dependent and most efficient under direct sunlight (Booth and Sheppard 1984, Tomberlin and Sheppard 2002, Zhang et al. 2010). Artificial lighting systems have been investigated, with LEDs offering advantages for commercial breeding due to their energy efficiency (Heussler et al. 2018). The 350- to 800-nm wavelength spectrum of the LED panels covers the perceivable spectrum of *H. illucens* photoreceptors (Zhang et al. 2010, Oonincx et al. 2016, Heussler et al. 2018) and led to mating and successful oviposition at different color temperatures. The average oviposition period we observed is comparable with LED experiments using identical light durations showing oviposition periods of 13 ± 4.7 to 16 ± 5.3 d (Oonincx et al. 2016) and 11 ± 0.7 d (Hoc et al. 2019). Hoc et al. (2019) suggested that oviposition can be modulated depending on changes in the nycthemeral cycle. Increasing light duration from 6 to 18 h reduced the period by 3 d (Hoc et al. 2019); light duration effects on oviposition period were also demonstrated in other studies (Zhang et al. 2010, Nakamura et al. 2016, Heussler et al. 2018). Our findings are in accordance with the previous reports showing mean oviposition peaks at 1 d and 2–3 d using sunlight and LEDs, respectively (Tomberlin and Sheppard 2002, Heussler et al. 2018). In contrast, Zhang et al. (2010), using quartz iodine lamps, reported peaks at 8 d and, in sunlight, 12 d. It is still unclear as to whether the time of oviposition influences egg fertility. Oonincx et al. (2016) showed that *H. illucens* illuminated with LEDs generated more viable eggs at a later date than illumination with fluorescent tubes, and they suggested that early oviposited eggs might be infertile.

Egg production can be optimized for breeding *H. illucens* (Pastor et al. 2015, Oonincx et al. 2016). Hoc et al. (2019) reported fecundity at 17.4 ± 0.1 mg eggs per female at 26 ± 1°C and 60 ± 5% RH, using stocking densities of 500 adults per m^3. Park et al. (2016), using three adult population densities (4-, 8-, 10-kg pupae per nylon cage), showed that egg numbers and total egg weights increased at higher population densities independently of cage dimensions. Temperature also influences *H. illucens* fecundity; at 30°C, fecundity was 475–516 eggs per female, but fecundity decreased to ≈300 eggs per female at 25°C (Chia et al. 2018; effects of RH on mating and oviposition are not yet known; Holmes et al. 2012).

Our findings indicate a relationship between the time when oviposition peaks and the color temperature. The use of 3,000 K LED panels can increase *H. illucens* egg production, potentially increasing commercial yields of larvae for livestock feed.

Acknowledgments

This research was funded by the Hessian Ministry of Science and Art in the course of the LOEWE Centre for Insect Biotechnology and by PT Alternative Protein Indonesia. We thank Bio.S Biogas, especially K. Kreller and D. Hluschi, for information on breeding and for providing black soldier fly larvae. We also acknowledge Lence Technology for the provision of the LED panels and technical information. We highly appreciate the work of the anonymous reviewers and want to express our gratitude to the editor Allan Showler and his exquisite help.

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Research Article 2 – Isolation of bacterial and fungal microbiota associated with *Hermetia illucens* larvae reveals novel insights into entomopathogenicity.

Klüber P, Müller S, Schmidt J, Zorn H, Rühl M. Isolation of bacterial and fungal microbiota associated with *Hermetia illucens* larvae reveals novel insights into entomopathogenicity. *Microorganisms* 2022, 10(2):319.

DOI: https://doi.org/10.3390/microorganisms10020319

Supplementary data available on:
https://www.mdpi.com/2076-2607/10/2/319#supplementary

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Article

Isolation of Bacterial and Fungal Microbiota Associated with *Hermetia illucens* Larvae Reveals Novel Insights into Entomopathogenicity

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Abstract: Larvae of the black soldier fly (BSF) *Hermetia illucens* are polyphagous feeders and show tremendous bioconversion capabilities of organic matter into high-quality insect biomass. However, the digestion of lignocellulose-rich palm oil side streams such as palm kernel meal (PKM) is a particular challenge, as these compounds are exceptionally stable and are mainly degraded by microbes. This study aimed to investigate the suitability of BSF larvae as bioconversion agents of PKM. Since the intestinal microbiota is considered to play a key role in dietary breakdown and in increasing digestibility, the bacterial and fungal communities of BSF larvae were characterized in a culture-dependent approach and screened for their putative entomopathogenicity. The lethality of six putative candidates was investigated using intracoelomal injection. In total, 93 isolates were obtained with a bacterial share of 74% that were assigned to the four phyla *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, and *Proteobacteria*. Members of the genera *Klebsiella*, *Enterococcus*, and *Sphingobacterium* are part of the core microbiome, as they were frequently described in the gut of *Hermetia* larvae regardless of diet, nutritional composition, or rearing conditions. With 75%, a majority of the fungal isolates belonged to the phylum *Ascomycota*. We identified several taxa already published to be able to degrade lignocelluloses, including *Enterococcus*, *Cellulomonas*, *Pichia* yeasts, or filamentous *Fusarium* species. The injection assays revealed pronounced differences in pathogenicity against the larvae. While *Alcaligenes faecalis* caused no, *Diutina rugosa* weak (23.3%), *Microbacterium thalassium* moderate (53.3%), and *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* high (≥80%) lethality, *Fusarium solani* injection resulted in 100% lethality.

Keywords: black soldier fly; palm kernel meal; insect rearing; culturable microbiome; infection; core microbiome; entomopathogens

Citation: Klüber, P.; Müller, S.; Schmidt, J.; Zorn, H.; Rühl, M. Isolation of Bacterial and Fungal Microbiota Associated with *Hermetia illucens* Larvae Reveals Novel Insights into Entomopathogenicity. *Microorganisms* **2022**, *10*, 319. https://doi.org/10.3390/microorganisms10020319

Academic Editors: Michael J. Bidochka and Charles M. Dozois

Received: 17 December 2021
Accepted: 28 January 2022
Published: 29 January 2022

1. Introduction

Hermetia illucens (*Diptera*: *Stratiomyidae*; BSF), commonly known as the black soldier fly, is native to the tropical and subtropical regions of America. Due to anthropogenic influences, it has spread almost worldwide [1]. In addition to a wide tolerance range for environmental factors, their short life cycle of 40–45 d makes the fly an interesting candidate for industrial applications [2]. Based on the polyphagous diet of the larvae, they have an extremely wide substrate spectrum. Various studies report their bioconversion capabilities of different organic matter and waste such as kitchen scraps, pineapple peelings, soybean residue [3], brewer's spent grains [4], fish offal [5], as well as chicken and swine manure [6,7] into high-quality insect biomass. In particular, the high protein and fat content of the larvae are promising for novel uses in the feed sector, as an alternative to fish and soy meal formulations in aquaculture or livestock, respectively [8].

The potential to transform byproducts of agroindustry, livestock, or urban wastes into a sustainable protein source goes hand in hand with possible chemical and microbiological risks that need to be assessed with regard to feed and food safety [9]. Studies demonstrated that the composition of the gut microbial community, including bacteria and fungi, is strongly influenced by the feeding substrate [10,11] and numerous extrinsic parameters [2]. The microbiota harbored by insects are involved in complex processes such as the development of the gastrointestinal tract, modulating the immune system [12], the digestion of plant polymers [13], synthesizing essential amino acids and vitamins [14], pheromone and kairomone synthesis for inter- and intraspecific communication, as well as the defense against pathogen and parasite colonization [15]. In particular, the ability to grow on substrates with a high microbial load, such as feces, implies a highly adaptable immune response. BSF larvae were even able to reduce the concentration of prominent pathogens such as *Salmonella* spp. in human feces or the enterohemorrhagic *Escherichia coli* O157:H7 in chicken manure [16,17]. In contrast, *Salmonella enteritidis* and the food pathogen *Bacillus cereus* could accumulate in the gut of larvae grown on food waste or chicken manure, respectively [16,18]. The mechanistic role of the microbiome in the defense against unwanted bacteria and fungi is still unclear. However, a direct connection between the gut microbial community, the feeding substrate, and the expression of more than 50 putative antimicrobial peptides has already been demonstrated [19]. The inoculation of the substrate with companion bacteria also had positive effects on the growth and developmental parameters of black soldier fly larvae [20].

The intestinal microbiota of the black soldier fly probably contributes heavily to diet breakdown, e.g., through the degradation of plant polymers such as cellulose or lignin, and enables the larvae to utilize a variety of substrates [10]. Concurrently, such a microbial enzyme repertoire offers an exploitation potential for biotechnological applications. Based on this hypothesis, fiber-rich organic byproducts of the palm oil industry could be suitable as a potential feeding regime for the larvae. Palm oil production is one of the fastest-growing industries, mainly due to its comparatively high space efficiency and the steadily increasing demand for vegetable oils worldwide, resulting in the deforestation of large rainforest areas that are being replaced by oil palm (*Elaeis guineensis*) monocultures [21,22]. In 2018, 71.5 million tons of palm oil were produced on a cultivated area totaling 18.9 million ha, of which the main producers, Indonesia and Malaysia, have a share of 84% [23]. With a yield of 20–23% crude palm oil from the fruits, millions of tons of organic byproducts are generated annually, including empty fruit bunches (EFB), palm kernel meal (PKM), or the liquid palm oil mill effluent (POME). Due to its cost efficiency and high fiber content, PKM is mainly used in ruminant diets but is also used successfully as a supplement for monogastric livestock such as poultry and pigs. PKM was also established as feed for BSF larvae [24–26]. The objectives of the present work, therefore, were to isolate and determine the composition of the cultivable intestinal microbiota of BSF larvae and its function in the digestion of PKM. In terms of larval health and yield, putative entomopathogens of BSFs were characterized and identified for the first time.

2. Materials and Methods

2.1. Rearing of Hermetia illucens

BSF larvae were provided by Bio.S Biogas (Grimma, Germany). The larvae were fed *ad libitum* with PKM and maintained in 19.5 × 16.5 × 9.5 cm (l × w × h) polypropylene containers with a density of 150 mg eggs per container at 27 ± 1 °C and 60 ± 10% relative humidity (RH) in darkness [3,27–29]. The PKM was provided by PT Alternative Protein Indonesia (Tebet, Indonesia) and stored in a dry and dark place until it was fed. The developmental stage was determined by the weight (AT261 DeltaRange, Mettler, Giessen, Germany), length, and head capsule width (Keyence VHX-2000 digital microscope, Keyence, Osaka, Japan) as described elsewhere [30].

2.2. Cultivation of Bacterial and Fungal Isolates

In order to cultivate gut-associated microbiota, four agar media were used: LB medium (10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 5 g L^{-1} NaCl, 15 g L^{-1} agar) and TSA medium (17 g L^{-1} casein peptone, 2.5 g L^{-1} K_2HPO_4, 2.5 g L^{-1} glucose, 5 g L^{-1} NaCl, 3 g L^{-1} soy peptone, 15 g L^{-1} agar) for bacterial isolates, and YPD medium (20 g L^{-1} peptone, 10 g L^{-1} yeast extract, 20 g L^{-1} glucose, 20 g L^{-1} agar) and M_2 medium (20 g L^{-1} malt extract, 3 g L^{-1} yeast extract, 15 g L^{-1} agar) for fungal isolates [11]. Fungal growth media were supplemented with 50 mg L^{-1} chloramphenicol.

Sixteen L5 larvae (150 ± 25 mg) were collected from different container positions with a spring steel tweezer and washed with distilled water. After anesthesia at −20 °C, their surface was sterilized twice with 70% ethanol (*v*/*v*). The guts were dissected under a stereomicroscope (S9i, Leica Microsystems, Wetzlar, Germany), weighed, and washed in 0.9% NaCl solution (*w*/*v*). Homogenization was carried out individually with sterile glass pestles in 300 µL LB or fungal enrichment GLY medium (20 g L^{-1} glycerol, 10 g L^{-1} yeast extract), respectively. Ten-fold serial dilutions were produced in 0.9% NaCl (*w*/*v*) solution, of which 100 µL were plated onto the four media mentioned above and incubated aerobically at 27 °C (bacteria) or 24 °C (fungi) in darkness. Colony-forming units (CFU) or conidia were calculated after 2 (bacteria), 4 (yeasts), and 7 d (filamentous ascomycetes) to obtain initial information about the microbiota harbored in a sample. Growing colonies and mycelia were selected based on their morphology.

To obtain pure cultures, isolates were transferred twice to fresh agar plates. For DNA extraction, liquid LB/TSA or YM broth (5 g L^{-1} peptone, 3 g L^{-1} malt extract, 3 g L^{-1} yeast extract, 10 g L^{-1} glucose, 50 mg L^{-1} chloramphenicol) for fungi were inoculated with pure cultures [11] and incubated shaking at 180 rpm and 27 °C for bacteria or 150 rpm and 24 °C for fungi. Bacterial and fungal isolates were stored in 25 or 10% glycerol at −80 °C, respectively.

2.3. DNA Extraction

In total, 1–2 mL of bacterial liquid cultures were centrifuged at 15,000 relative centrifugation force (rcf) for 5 min at room temperature (RT), whereas 15 mL of fungal liquid cultures were centrifuged at 1400 rcf for 10 min at 4 °C. Bacterial DNA was isolated from the resulting pellet using the PureLink® Genomic DNA mini kit (Invitrogen, ThermoFisher Scientific, Waltham, MA, USA) using the protocol specifications for gram-positive cell lysates.

A total of 250 µL of yeast pellets or one micro spatula of filamentous mycelia were ground under liquid nitrogen, transferred into a 1.5 mL centrifugation tube, resuspended in 500 µL lysis buffer (400 mM Tris HCl, 60 mM ethylenediaminetetraacetic acid (EDTA), 150 mM NaCl, 1% sodium dodecyl sulfate) and vortexed. After an incubation of 10 min at RT, 150 µL 3 M potassium acetate solution was added, vortexed, and centrifuged at 17,500 rcf for 10 min at RT. The supernatant and the same volume of isopropanol was then transferred into a new tube and centrifuged under the same conditions to precipitate the DNA. The obtained DNA pellet was washed with 70% ethanol (*v*/*v*) and centrifuged for 1 min under the same conditions.

Purified chromosomal DNA was quantified by NanoDrop 2000 (ThermoFisher Scientific, Waltham, MA, USA) and stored at −20 °C until use.

2.4. 16S rRNA and ITS PCR

For species identification, 16S rRNA and 5.8S-ITS regions were amplified with the following primer pairs: 27F (5′-GGT TAC CTT GTT ACG ACT T-3′), 1492R (5′-AGA GTT TGA TCM TGG CTC AG-3′) [31] and ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′), ITS4 (3′-TCC TCC GCT TAT TGA TAT GC-3′) [32]. PCR was carried out in 25 µL reactions containing 0.5 pM of each primer, 0.2 mM dNTPs, 1 × Phusion GC buffer, Phusion polymerase (ThermoFisher Scientific, Waltham, MA, USA), and 100 ng template DNA. The cycling conditions for 16S rRNA amplification were set to: initial denaturation at 98 °C for 2 min, 35 cycles at 98 °C for 30 s, 58 °C for 30 s, 72 °C for 45 s, and final elongation

at 72 °C for 5 min. 5.8S-ITS region was amplified under the following conditions: initial denaturation at 94 °C for 2 min, 35 cycles at 94 °C for 30 s, 51 °C for 30 s, 72 °C for 15 s, and a final elongation for 5 min at 72 °C.

Approximately 5 µL of the PCR products were then separated electrophoretically in a 1% agarose gel in 1 × TAE buffer (40 mM Tris base, 20 mM acetic acid, 1 mM EDTA) for 30 min at 125 V. Midori Green Advance (Nippon Genetics, Dueren, Germany) served as in-gel DNA stain. Each amplicon was sequenced by Sanger sequencing with the corresponding forward and reverse primers (Microsynth, Balgach, Switzerland). The resulting sequences were analyzed by the Nucleotide BLAST algorithm (http://www.ncbi.nlm.nih.gov/blast, accessed on 16 December 2021) for highly similar sequences in the nucleotide collection (nr/nt) database. A sequence identity of 97% was defined as the threshold. Subsequently, taxonomic classification was performed based on the NCBI Taxonomy Browser (https://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi, accessed on 11 October 2021). In addition to morphological characteristics, isolates of the same species were compared genetically by pairwise alignment (Geneious 9.1.8, Biomatters, Auckland, New Zealand). If differences in the sequences were identified, these were retained as a different genotype.

2.5. Growth Curves, OD_{600}/CFU Relationship, and Antibiotic Susceptibility Tests of Putative Entomopathogens

An extensive literature review was conducted to condense putative candidates from our bacterial and fungal isolates, which were previously reported to have entomopathogenic potential. In order to obtain synchronized bacterial or yeast cultures, single colonies of putative entomopathogens were inoculated in 20 mL liquid LB, TSA, or YPD, depending on the medium on which they were isolated (Supplementary Tables S1 and S2). A representative isolate with the highest sequence identity was selected and used for further infection experiments. Isolates were deposited at the German Collection of Microorganisms and Cell Cultures GmbH (DSMZ, Braunschweig, Germany): *Microbacterium thalassium* (DSM 112768), *Diutina rugosa* (DSM 112794), *Fusarium solani* (DSM 112793), *Alcaligenes faecalis* (DSM 112765), *Klebsiella pneumoniae* (DSM 112766), and *Pseudomonas aeruginosa* (DSM 112767). The cultures were grown overnight at 27 °C and 180 rpm. In total, 500 µL of those cultures were transferred into 100 mL fresh medium and incubated under the same conditions. Growth curves were measured in 30 min intervals until an OD_{600} of 1.0 was reached (Ultrospec 10, Biochrom, Berlin, Germany). At least five samples of 1 mL (OD_{600} = 0.2–1.0) were taken, serially diluted in 0.9% NaCl (*w*/*v*) solution and 100 µL were plated onto the corresponding agar medium. After an incubation of 2 d at 27 °C, $CFU \cdot mL^{-1}$ was calculated. Experiments were carried out as biological triplicates.

Antibiotic susceptibility tests, including the determination of the minimal inhibitory concentration (MIC) for *P. aeruginosa* and *K. pneumoniae* were performed with the VITEK 2 system (BioMérieux, Marcy-l'Étoile, France); susceptibility analysis of *A. faecalis* and *M. thalassium* were performed by quantitative Etest (bestbion dx, Cologne, Germany). For resistance detection, 100 µL of log-phase liquid cultures (OD_{600} = 0.6) were plated on LB agar. The test strip was placed in the center of the plates. After an incubation of 2 d at 27 °C, zones of inhibition were examined. The results were interpreted in accordance with the guidelines of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and the Clinical and Laboratory Standards Institute (CLSI) [33,34].

2.6. Injection Assay

Three single colonies of putative entomopathogenic bacteria or yeasts were inoculated in liquid media and incubated as described above until they reached the log phase. OD_{600} was determined, and 1 mL of the liquid culture was washed twice in sterile 0.9% NaCl (*w*/*v*) solution to avoid interference from the medium. The density was then adjusted to 2×10^8 $CFU \cdot mL^{-1}$ using the linear regression function. In order to achieve a comparable infection dose with the filamentous ascomycete *F. solani*, YPD agar plates were inoculated with a disc (0.6 cm) of an actively growing, one-week-old *F. solani* culture, and incubated at

24 °C for 12 days until sporulation occurred. For infection, *F. solani* conidia were released and harvested by rubbing the surface of plates covered with extraction solution (0.9% NaCl, 0.05% Tween-20) using a sterile Drigalski spatula [35]. Spores were separated completely from larger fragments of the hyphae by glass wool filtration, followed by two washing steps similar to the bacteria procedure and resuspended in 1 mL 0.9% NaCl solution. The number of conidia was determined by a hemocytometer and adjusted to 2×10^8 conidia·mL^{-1} [36].

Ten L5 larvae of similar weight (150 ± 20 mg) from three breeding containers were selected at random. Each experimental group was surface sterilized twice with 70% ethanol (*v*/*v*) before being infected. A total of 5 µL containing 1×10^6 CFU or conidia were ventrally injected with insulin syringes (Micro-Fine + U-100, Becton Dickinson, Franklin Lakes, NJ, USA) in the haemocoel of the third thoracic segment of the larvae [37]. The same number of non-infected and 0.9% NaCl-injected larvae served as negative controls. Post-infection, the larvae were maintained in 19.5 × 16.5 × 9.5 cm (l × w × h) polypropylene containers at 27 ± 1 °C and 60 ± 10% RH in darkness. They were fed with 20 g chicken feed (16% crude protein, 3.5% crude fat, 5% crude fiber, 12.5% crude ash; GoldDott Eierglück, DERBY Spezialfutter, Muenster, Germany) per container. The lethal time (LT_{50}; time until 50% of the individuals were dead) and percent of mortality were monitored in 24 h intervals over 7 d (bacteria, yeast) or 10 d (*F. solani*) post-infection by exposing the larvae to mechanical stimuli. If they did not respond, they were considered dead [38]. Experiments were carried out as biological triplicates.

2.7. Data Processing

Data were processed with Excel 2016 (Microsoft, Redmond, WA, USA) and graphed with OriginPro 2020b (OriginLab Corporation, Northampton, MA, USA). The 16S rRNA and 5.8S-ITS sequences were used to create phylogenetic trees by multiple sequence alignment with the ClustalW software implemented in Geneious [39]. The trees were built using the neighbor-joining method and the JC69 Jukes and Cantor substitution model with 1000 bootstrap replications. β-diversity was calculated using the Jaccard index (I_J). A simple linear regression model of OD_{600} and $\log_{10}$(CFU) was performed to describe the relationship between the photometrically measured density and the viable cell count. Lifetime data post-infection were analyzed by the Kaplan–Meier estimation to generate $S(t)$ survival functions, which were compared pairwise by log-rank test and an error level of $\alpha = 0.05$ for statistical significance.

3. Results

3.1. Taxonomic Composition of the Culturable Gut Microbiota in BSF Larvae Grown on PKM

The cultivable microbial gut community of BSF larvae reared on PKM was predominantly composed of bacteria. With $\geq 7 \times 10^8$ CFU·gut^{-1}, these showed a 100 to 100,000-fold higher abundance of cultivable cells than yeasts or filamentous ascomycetes, respectively (Table 1).

Table 1. Calculation of the bacterial and yeast CFU as well as the ascomycete conidia. Values represent the mean of 16 larval guts (±SD).

	Bacteria		**Yeasts**		**Filamentous Ascomycetes**	
	LB	**TSA**	**YPD**	**M_2**	**YPD**	**M_2**
CFU/conidia gut^{-1} (±SD)	1.06×10^9 ($\pm 9.81 \times 10^8$)	7.04×10^8 ($\pm 4.63 \times 10^8$)	1.10×10^6 ($\pm 3.22 \times 10^5$)	4.67×10^6 ($\pm 2.83 \times 10^6$)	1.40×10^4 ($\pm 7.87 \times 10^3$)	1.31×10^4 ($\pm 9.44 \times 10^3$)
CFU/conidia (mg gut)$^{-1}$ (±SD)	1.35×10^7 ($\pm 1.39 \times 10^7$)	8.52×10^6 ($\pm 5.48 \times 10^6$)	1.47×10^4 ($\pm 3.90 \times 10^3$)	6.56×10^4 ($\pm 4.34 \times 10^4$)	1.90×10^2 ($\pm 1.11 \times 10^2$)	1.63×10^2 ($\pm 1.03 \times 10^2$)

From the total of 16 dissected guts, 138 isolates were obtained (82 bacterial and 56 fungal) on the basis of morphological characteristics. After pairwise comparison, we identified 93 isolates with unique 16S rRNA (Supplementary Table S1) or 5.8S-ITS sequences

(Supplementary Table S2), indicating that the sequences of 45 isolates (32.6%) are redundant. Consequently, these isolates were discarded. All sequences showed 98–100% identity with sequences available in the NCBI nucleotide collection database; isolates with a sequence identity < 100% were integrated into the database. The detailed results of the classification and corresponding accession numbers are shown in Supplementary Tables S1 and S2. Approximately 74% of the isolates were bacteria; the remaining species were fungi. In total, 51% of the bacteria can be assigned to Gram-positive, 38% to Gram-negative, 7% to Gram-variable, and 4% to taxa that cannot be determined by Gram staining (Figure 1). Overall, we were able to clearly identify 15 bacterial and 7 fungal taxa down to the species level, whereby 53 isolates of both domains were identified down to the genus level on the basis of similar sequence identities with at least two or more database entries.

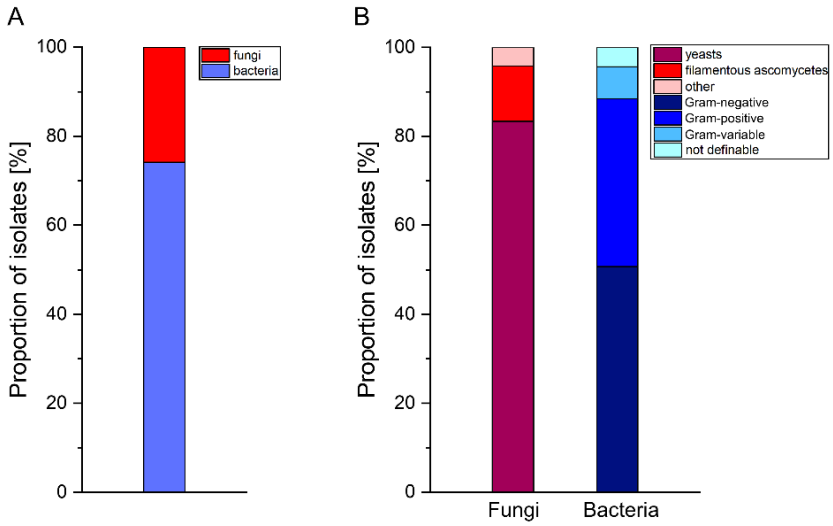

Figure 1. Superordinate categorization of the isolates. (**A**) Ratio of fungal and bacterial isolates. (**B**) left: Distribution of filamentous and yeast-like fungi. 'Other' describes a single member of the *Mucoromycota*. right: Bacterial community classification based on Gram staining characteristics.

The 69 bacterial isolates were assigned to the four phyla *Actinobacteria* (11 isolates), *Bacteroidetes* (nine isolates), *Firmicutes* (21 isolates), and *Proteobacteria* (25 isolates), which are categorized into 7 classes, 9 orders, 16 families, and 23 genera. We found no representatives of other phyla. Accordingly, a new genus was identified with every third isolate. Three prokaryotic isolates could only be assigned to the domain of the bacteria. The α diversity, according to Whittaker describing the diversity within a habitat, showed strong differences between the TSA (46 isolates) and the LB medium (27 isolates). In addition, the two media only had a Jaccard index of $I_J = 0.07$, suggesting a low isolate similarity. More than 66% of the bacteria belonged to the *Proteobacteria* and *Firmicutes*, with a proportion of the isolates of 36% and 30%, respectively (Figure 2).

The phylogenetic relationship within the bacterial community was calculated using the obtained sequences and illustrated in a neighbor-joining tree (Figure 3). Among the most prominent phylum *Proteobacteria*, most isolates belonged to the families *Alcaligenaceae* (10 isolates) and *Enterobacteriaceae* (9 isolates) and were assigned to the genera *Klebsiella* (7 isolates), *Bordetella* (5 isolates), *Alcaligenes* (3 isolates *A. faecalis*), *Achromobacter* (2 isolates), and *Citrobacter* (2 isolates *C. amalonaticus*). Three isolates each belonged to the families *Pseudomonadaceae* (all assigned to the genus *Pseudomonas*) and *Brucellaceae*, which were assigned to the genera *Ochrobactrum*, *Brucella*, and *Bordetella*. The genera *Pseudomonas* (all isolates), *Achromobacter* (both isolates), and *Brucella* were only isolated from TSA agar, whereas *Ochrobactrum* was the only genus that was obtained exclusively from LB agar (Supplementary Table S1). *Bordetella* sp. (five isolates), *Klebsiella pneumoniae* (one to five isolates), and *Pseudomonas aeruginosa* (one isolate) were identified as prominent human pathogens.

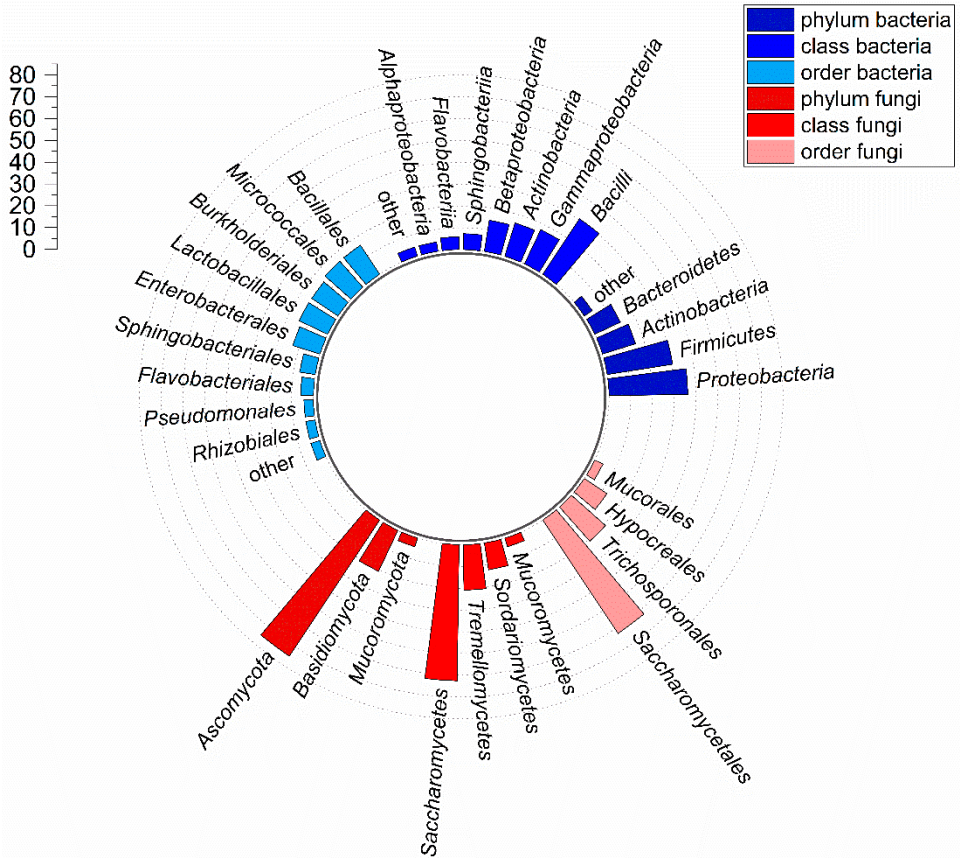

Figure 2. Radial bar plot of the cultivable gut microbiota composition. The proportion of bacterial and fungal phyla, classes and orders were grouped and compared. Dashed lines indicate 10% intervals. Isolates that could not be assigned to a specific taxon were summarized as 'others'.

Within the phylum *Firmicutes*, nine isolates belonged to the family *Enterococcaceae* (all genus *Enterococcus*), eight isolates to the family *Paenibacillaceae* (genus *Paenibacillus* and *Cohnella*), three isolates to the family *Bacillaceae* (all genus *Bacillus*), and one to the family *Lactobacillaceae* (genus *Lactobacillus*). Representatives of the genera *Bacillus*, *Lactobacillus* and *Cohnella* were cultivated exclusively on TSA agar, while all other *Firmicutes* isolates were obtained from cultures grown on TSA and LB agar plates.

The other phyla, with 16% (*Actinobacteria*) and 13% (*Bacteroidetes*), were represented significantly weaker in larval guts. Interestingly, all representatives of the *Actinobacteria* can be assigned to the order *Micrococcales*, whereby four isolates belonged to the family *Micrococcaceae* (genus *Kocuria* and *Micrococcus*), three isolates to the family *Microbacteriaceae* (genus *Microbacterium* and *Leucobacter*), two isolates to the family *Cellulomonadaceae* (both *Cellulomonas flavigena*), one isolate to the family *Dermacoccaceae* (*Dermacoccus nishinomiyaensis*), and one to the family *Promicromonosporaceae* (genus *Cellulosimicrobium*). Furthermore, the isolates of the genera *Dermacoccus*, *Leucobacter*, *Kocuria*, and *Cellulosimicrobium* were exclusively obtained from TSA agar plates.

Within the phylum *Bacteroidetes*, five isolates were assigned to the family *Sphingobacteriaceae* (all genus *Sphingobacterium*) and four to the family *Flavobacteriaceae* (genus *Empedobacter* and *Flavobacterium*) (Figure 3). All *Bacteroidetes* genera were obtained from both LB and TSA agar plates. Despite chloramphenicol supplementation (50 $mg \cdot L^{-1}$), *Sphingobacterium thalpophilum* (isolate 1) could be identified on M_2 medium in addition to TSA.

The 24 fungal isolates were classified into 3 phyla, 4 classes and orders, 7 families, and the same number of genera (1 genus from every 3.4 isolates; Figures 2 and 4). We found no representatives of other phyla. The fungal growth media M_2 (12 isolates) and YPD (10 isolates) reached the highest α diversity, followed by the bacterial growth media TSA (6 isolates) and LB (5 isolates). TSA and LB showed the highest similarity with a

Jaccard index of $I_J = 0.43$, while isolates were grown on M_2 and YPD ($I_J = 0.24$), M_2 and TSA ($I_J = 0.18$), TSA and YPD ($I_J = 0.11$), as well as M_2 and LB ($I_J = 0.10$) had noticeably lower similarities. YPD and LB had no similarity ($I_J = 0$).

Figure 3. Phylogenetic analysis of all bacterial BSF gut isolates based on 16S rRNA sequences. Unrooted neighbor-joining tree using the JC69 Jukes and Cantor substitution model. The numbers indicate support for clade branching (%) of 1000 bootstrap replications.

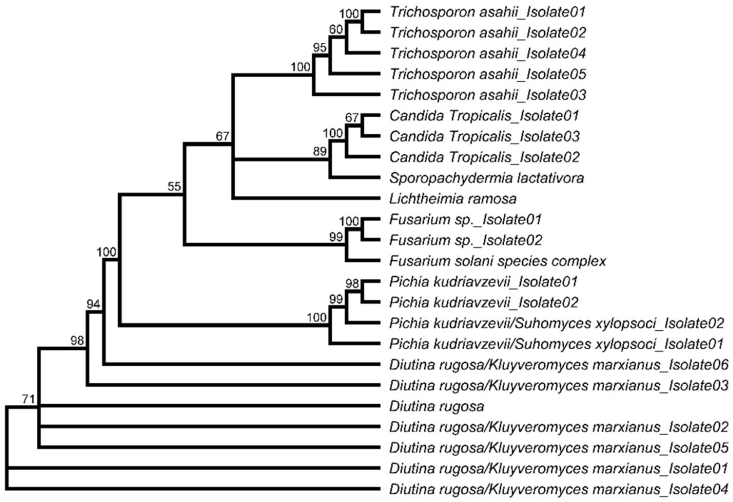

Figure 4. Phylogenetic analysis of all fungal BSF gut isolates based on 5.8S-ITS sequences. Unrooted neighbor-joining tree using the JC69 Jukes and Cantor substitution model. The numbers indicate support for clade branching (%) of 1000 bootstrap replications.

With 75%, a majority of the fungi belonged to the phylum *Ascomycota* (18 isolates) distributed in the orders *Hypocreales* (three isolates) and the dominant *Saccharomycetales* (15 isolates), which contained 63% of the total fungal isolates. Within the phylum *Ascomycota*, seven isolates were assigned to the family *Saccharomycetaceae* (genus *Diutina* and *Kluyveromyces*), four isolates to the family *Pichiaceae* (two isolates *Pichia kudriavzevii* and two isolates *Pichia/Suhomyces*), three isolates to the family *Debaryomycetaceae* (all *Candida tropicalis*), three isolates to the family *Nectriaceae* (all genus *Fusarium*), and one to the family *Dipodascaceae* (*Sporopachydermia lactativora*). Representatives of the families *Saccharomycetaceae* and *Debaryomycetaceae* were isolated from a variety of different media, including YPD, M_2, LB, and TSA agar. In contrast, *S. lactativora* was exclusively obtained from a culture grown on YPD agar (Supplementary Table S2).

The phylum *Basidiomycota* was only represented by five isolates, which were assigned to the family *Trichosporonaceae* (all *Trichosporon asahii*). *T. asahii* was isolated on YPD, M_2, and TSA agar. Except for the *Nectriaceae* family, all members of the *Ascomycota* and *Basidiomycota* were yeasts (83% of total fungal isolates; Figure 1B).

The phylum *Mucoromycota* was exclusively represented by a single isolate, which was assigned to the family *Lichtheimiaceae* (*Lichtheimia ramosa*) and obtained from a culture grown on M_2 agar (Figure 4, Supplementary Table S2).

3.2. Characterization of Putative Entomopathogenic Candidates from BSF Guts

An extensive literature review was conducted to condense putative candidates from our bacterial and fungal isolates, which were previously reported to have entomopathogenic potential. Four bacteria and two fungal species isolated from the BSF larval gut have already been described as putative insect pathogens in the literature: *Microbacterium thalassium* for *Ostrinia nubilalis* (*Lepidoptera*: *Crambidae*) [40], *Alcaligenes faecalis*, *Klebsiella pneumonia*, and *Pseudomonas aeruginosa* for *Galleria mellonella* (*Lepidoptera*: *Pyralidae*) [41–43], as well as *Diutina rugosa* for *Anastrepha ludens* (*Diptera*: *Tephritidae*) [36,44] and *Fusarium solani* for *Dendroctonus frontalis* (*Coleoptera*: *Curculionidae*) [45].

Isolates were deposited at the German Collection of Microorganisms and Cell Cultures GmbH (DSMZ, Braunschweig, Germany): *Microbacterium thalassium* (DSM 112768), *Diutina rugosa* (DSM 112794), *Fusarium solani* (DSM 112793), *Alcaligenes faecalis* (DSM 112765), *Klebsiella pneumoniae* (DSM 112766) and *Pseudomonas aeruginosa* (DSM 112767).

First, the four bacterial candidates were examined for possible antibiotic resistance by the VITEK 2 system and the quantitative Etest. All tested strains were susceptible to meropenem and trimethoprim/sulfamethoxazole. The antibiotic susceptibility test showed

resistance to rifampicin and the β-lactam antibiotics ampicillin and piperacillin, but no evidence on extended-spectrum β-lactamases (ESBL) in *K. pneumoniae*, a member of the *Enterobacteriaceae* (Table 2). Cefuroxime had an intermediate effect on the growth of *K. pneumoniae*. *A. faecalis* had a high level of resistance to commonly used penicillins (ampicillin/sulbactam, piperacillin/tazobactam) and cephalosporins, including cefepime, cefotaxime, and ceftazidime. In addition, *A. faecalis* showed intermediate susceptibility against imipenem and rifampicin. *P. aeruginosa* was resistant to astreonam, piperacillin/tazobactam and rifampicin while being intermediate susceptible to cefepime, ceftazidime, ciprofloxacin, imipenem, and piperacillin. Antibiotics penetrating Gram-positive bacteria were chosen in accordance with CLSI guidelines for infrequently isolated and fastidious bacteria and an *M. paraoxydans* bacteraemia study [33,46]. *M. thalassium* showed resistance to cefepime, clindamycin, and rifampicin, while it was intermediate susceptible to cefotaxime, gentamicin, penicillin, and tetracycline. In total, three of the four isolates were resistant to rifampicin; the antimicrobial effect on *A. faecalis* was intermediate (Table 2).

Table 2. Antibiotic susceptibility test of putative bacterial entomopathogens. MIC ($\mu g \cdot mL^{-1}$) interpretative criteria were taken from the susceptibility breakpoint specifications of CLSI and EUCAST [33,34]. Interpretation abbreviations are defined as follows: susceptible (S), intermediate (I), resistant (R), insufficient evidence (IE), no suitable target (-), not determined (n).

Drug	Gram-Negative						Gram-Positive	
	Alcaligenes faecalis		*Klebsiella pneumoniae*		*Pseudomonas aeruginosa*		*Microbacterium thalassium*	
	MIC	**Interpretation**	**MIC**	**Interpretation**	**MIC**	**Interpretation**	**MIC**	**Interpretation**
Amikacin	n	n	n	n	≤2	S	(-)	(-)
Ampicillin	>256	R	≥32	R	(-)	(-)	>0.75	S
Ampicillin/ Subactam	≥64	R	≤2	S	(-)	(-)	n	n
Aztreonam	n	n	n	n	32	R	(-)	(-)
Cefepime	>48	R	n	n	4	I	>6	R
Cefotaxime	>256	R	≤1	S	(-)	(-)	≥2	I
Cefpodoxime	n	IE	≤0.25	S	(-)	(-)	n	IE
Ceftazidime	>256	R	≤1	S	4	I	n	IE
Cefuroxime	n	n	≤1	I	(-)	(-)	n	IE
Cefuroxime axetil	n	n	≤1	S	(-)	(-)	n	IE
Ciprofloxacin	≥0.19	S	≤0.25	S	0.5	I	≥0.5	S
Clindamycin	(-)	(-)	(-)	(-)	(-)	(-)	16	R
Colistin	n	n	n	n	≤0.5	S	(-)	(-)
Ertapenem	≥0.032	S	≤0.5	S	(-)	(-)	n	IE
Erythromycin	n	IE	n	n	N	n	≤0.5	S
Fosfomycin	n	IE	n	n	(-)	(-)	(-)	(-)
Gentamicin	<1.5	S	≤1	S	≤1	IE	≤12	I
Imipenem	≥2	I	≤0.25	S	2	I	≤0.5	S
Meropenem	≥0.38	S	≤0.25	S	≤0.25	S	≥0.125	S
Moxifloxacin	n	IE	≤0.25	S	(-)	(-)	n	IE
Penicillin	(-)	(-)	(-)	(-)	(-)	(-)	≥0.5	I
Piperacillin	n	n	8	R	16	I	n	IE
Piperacillin/ Tazobactam	>256	R	≤4	S	32	R	n	IE
Rifampicin	≥1.5	I	>32	R	8	R	≥12	R
Tetracyclin	n	IE	n	n	(-)	(-)	≤12	I
Tigecycline	≥0.5	S	n	n	(-)	(-)	n	IE
Tobramycin	n	n	n	n	≤1	S	(-)	(-)
Trimethoprim/ Sulfamethoxazole	≥1.5	S	≤20	S	(-)	(-)	>0.5	S
Vancomycin	(-)	(-)	(-)	(-)	(-)	(-)	>2	S/IE

In order to check their pathogenicity against BSF larvae, growth curves of the bacteria and yeast were first recorded and linear regressions of the CFU, and the corresponding

OD_{600} were determined. *K. pneumoniae* reached an OD_{600} > 1 after 210 min, followed by *A. faecalis* (330 min). *M. thalassium* (450 min) took more than twice the time to reach OD_{600} = 1. Interestingly, *P. aeruginosa* and *D. rugosa* (both 390 min) showed a comparable growth phenotype (Figure 5).

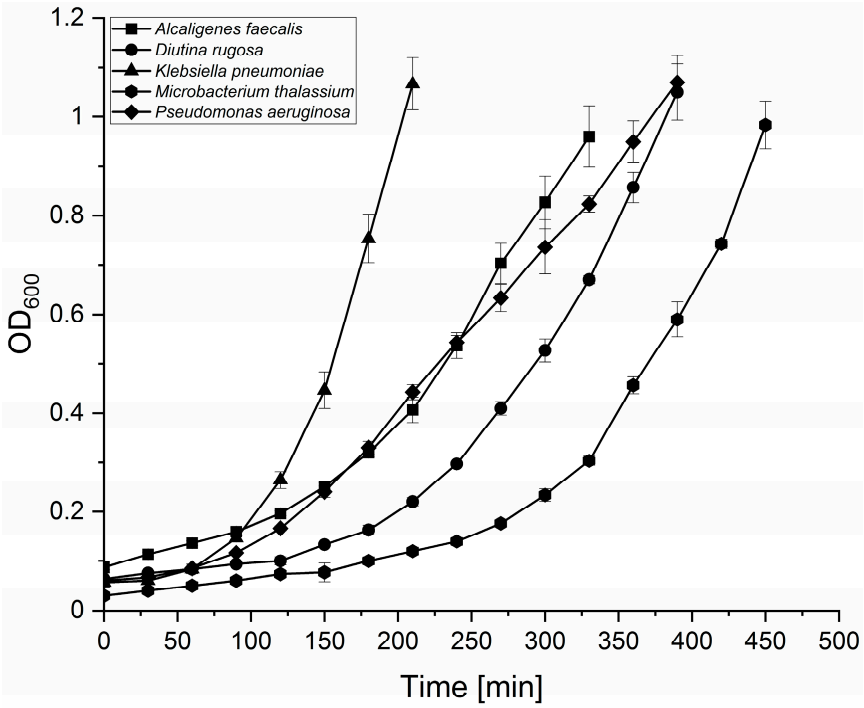

Figure 5. Growth curves of putative entomopathogenic bacterial (*A. faecalis*, *K. pneumoniae*, *M. thalassium*, *P. aeruginosa*) and yeast (*D. rugosa*) candidates. OD_{600} was measured in 30 min intervals; the average optical density OD_{600} (±SD) of three independent liquid cultures is shown.

The regression functions are given in Table 3. All liquid cultures had a comparable high regression coefficient of $R^2 > 0.98$. The number of conidia of the filamentous ascomycete *F. solani* was determined by a hemocytometer.

Table 3. Linear regression model of $\log_{10}(CFU \cdot mL^{-1})$ and OD_{600} from liquid cultures of putative entomopathogenic bacterial (*A. faecalis*, *K. pneumoniae*, *M. thalassium*, *P. aeruginosa*) and yeast (*D. rugosa*) candidates.

Liquid Culture	n	Function	R^2
Alcaligenes faecalis	5	y = 2.18x + 6.84	0.9954
Diutina rugosa	6	y = 1.57x + 6.80	0.9901
Klebsiella pneumoniae	5	y = 3.30x + 5.87	0.9985
Microbacterium thalassium	5	y = 2.79x + 6.25	0.9827
Pseudomonas aeruginosa	7	y = 1.76x + 6.87	0.9964

3.3. In Vivo Evaluation of Putative Entomopathogens in BSF Larvae

To evaluate the pathogenic potential of fungal and bacterial species isolated from the gut of BSF larvae, injection assays were performed. All examined candidates, with the exception of *A. faecalis*, were able to cause an infection in the larvae within seven days post-injection. *A. faecalis* and *D. rugosa* demonstrated no change in survival probability compared to the NaCl-injected control group ($p = 0.10$).

The course of infection and the pathogenicity of *Klebsiella* and *Pseudomonas* species were comparably high (16.7% and 20.0% survival), albeit only *P. aeruginosa* caused the significantly greater killing of the larvae than the moderate *M. thalassium* ($p = 0.02$). Larvae injected with conidia of *F. solani* showed the lowest survival rate ($p < 0.00001$), with 100%

lethality was already reached one day after inoculation (Figure 6A). In agreement, spores had germinated, and outgrowing mycelium indicated rapidly successful host colonization. An increase in the pigmentation of infected tissue due to melanization was observed in BSF larvae, similar to well-established insect infection models such as *G. mellonella* (Figure 6B).

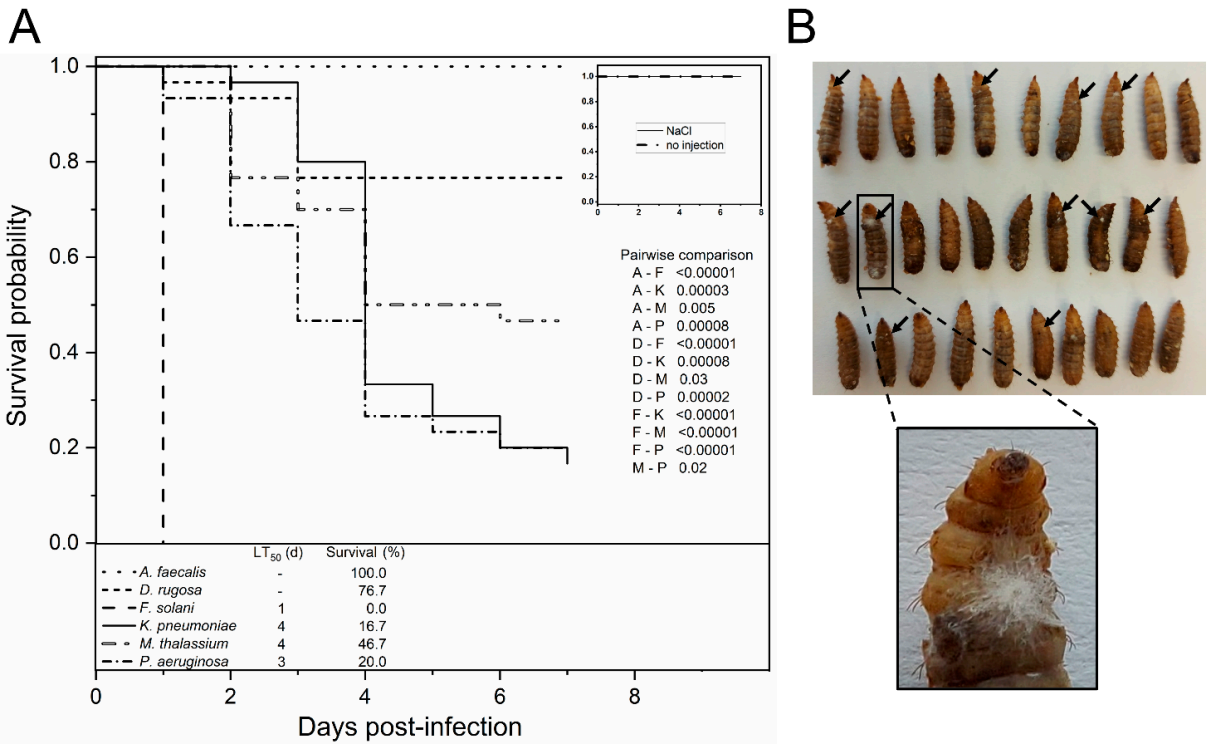

Figure 6. Inoculation of BSF larvae with putative entomopathogens leads to lethality in a species-dependent manner. (**A**) Kaplan-Meier survival functions of 30 larvae injected with 10^6 CFU, conidia, or an equal volume of physiological NaCl (control). Not-injected larvae served as an additional control; larval survival was monitored daily. Lethal time is expressed as LT_{50} values (50% deaths). *p*-Values represent infections compared pairwise by log-rank test, with first letters indicating the genera. (**B**) Images of dead *F. solani* infected larvae one-day post-inoculation. Melanization of infected tissue and mycelium growing out of the injection site (black arrows) can be observed.

4. Discussion

4.1. Analysis of the Cultivable Bacterial and Fungal Gut Microbiota in BSF Larvae Grown on PKM

The BSF has become the focus of science in recent years because its larvae are able to use almost all organic side streams as a feed substrate. The intestinal microbiome seems to play a key role in the dietary breakdown and the increase in digestibility, whereby PKM represents a particular challenge for microorganisms due to the high lignocellulose content (>20% dry matter) [10]. Above all, *Proteobacteria* such as *Rhizobiales*, *Burkholderiales*, *Enterobacteriales*, and members of the *Bacillales* (*Firmicutes*) are known for plant-associated nitrogen fixation [47]. However, studies suggest that *Rhizobiales* could also be endosymbiotically involved in nitrogen uptake in ants [48]. The putative role in the synthesis and provision of amino acids by *Rhizobiales* and *Burkholderiales* bacteria from the gut microbiome of BSF is already discussed elsewhere [49]. Members of the *Enterobacteriaceae*, especially *Enterobacter* or *Klebsiella*, are known for their ability to metabolize complex polysaccharides. The latter genus has already been identified frequently in the gastrointestinal tract (GIT) of BSF larvae reared on food waste, cooked rice, and calf forage and appears to be part of the core microbiota [10,18]. Isolates of *K. pneumoniae* from the GIT of *Bombyx mori* (*Lepidoptera*: *Bombycidae*) showed high β-endoglucanase and α-amylase activity. This points to the participation in the degradation of plant cell walls in the gut of BSF [50]. Typically,

enterococci are also involved in the decomposition of plant polymers; they were detected as main actors in the digestion of lignocellulose in the longhorn beetle *Cyrtotrachelus buqueti* (*Coleoptera*: *Curculionidae*) [51]. Accordingly, the high relative abundance of nine *Enterococcus* isolates suggests a strong lignocellulolytic impact on PKM digestion in the larval GIT of BSF. Besides *Enterococcus*, the genus *Sphingobacterium* constitutes the core microbiome since both have been previously found in BSF larvae, regardless of the diet's nutritional composition or rearing conditions [52–55]. Despite their low abundance, the two phenotypically different *Cellulomonas flavigena* isolates could, due to their cellulase and xylanase activities, make an important contribution to the degradation of cellulose contained in the PKM. In agreement with a study showing that a defined inoculation of companion *Bacillus subtilis* and *B. natto* strains in chicken manure had a positive effect on developmental time and growth performance of BSF, *Bacillus* isolates could consequently promote digestion of PKM [20]. The genera *Microbacterium*, *Micrococcus* and *Cellulomonas* belonging to the *Actinobacteria* were already isolated by subculturing from BSF eggs [56]. It is possible that these genera colonize adult flies and are transmitted vertically during oviposition. In this way, bacteria could be ingested from the egg surface during the early initial colonization of the hatched larvae and establish themselves in the GIT. Interestingly, the exposure of these three genera did not result in any olfactory responses from adult flies, whereas artificial oviposition sites inoculated with *Lactobacillus plantarum* were preferred by *Drosophila melanogaster* (*Diptera*: *Drosophilidae*) [56,57].

Until now, most of the publications on microbes have mainly focused on the bacterial composition in the intestine of BSF, while little is known about the dynamics and functional relationships of mycobiota. Comparison with the literature shows that, apart from *Mucoromycetes*, members of all classes have already been detected in the larval GIT [11]. With *Lichtheimia ramosa*, a member of the *Mucoromycetes* could be identified for the first time. Overall, yeasts represent the majority of fungal isolates with a relative abundance of more than 80%, which indicates an intensive insect-yeast association. Such beneficial interactions are widespread in insects and were frequently found in *Hymenoptera*, *Coleoptera*, and *Diptera* (especially fruitflies), which BSFs also belong to (reviewed in [58]). Several isolated yeasts, including *Candida*, *Pichia*, and *Trichosporon*, have already been detected in a culture-independent study in the gut of BSF larvae, which were grown on chicken feed or vegetable waste, respectively [11]. In particular, members of the *Saccharomycetales* such as *Candida* sp. and *Pichia* sp. are known to express antimicrobial peptides (AMPs), which are often directed against closely related yeasts and, thus, possibly protect the larvae against pathogen colonization [59]. Species of both genera (*C. tropicalis*, *P. kudriavzevii*) have also been described in palm wine from the oil palm therefore it is a reasonable assumption that they originate from the plant [60]. Further, *P. kudriavzevii* is described as a candidate for bioconversion of hemicellulosic materials into ethanol [61]. It is likely that *D. rugosa* (syn. *Candida rugosa*) is involved in the lipid degradation of palm (kernel) oil from PKM that has not been completely extracted during the milling process, as most frequently commercially used yeast lipases come from *Diutina* [62]. In agreement with PKM-fed larvae, *D. rugosa* was identified in substrate and frass samples of chicken feed and cottonseed press cake, suggesting a beneficial role in a functional or pathogen protective manner [63]. In addition to bacteria and yeasts, filamentous fungi of the phyla *Ascomycota* and *Basidiomycota* are known to produce various lignocellulolytic enzymes. These enzymes are secreted into their environment and, thus, making a significant contribution to the natural decay of plant biomass. Most ascomycetes are able to hydrolyze (hemi-) cellulose but are deficient in lignin degradation. The plant pathogen *Fusarium solani* f. sp. *glycines* (*Nectriaceae*), on the other hand, secretes laccases and lignin peroxidases, which enable lignin degradation and imply a similar function of *Fusarium* isolates in the GIT of BSF [64]. Slow degradation rates of lignin were also indicated for *F. solani* strains and other *Fusarium* sp. varieties [65]. This ability could also be related to the suspicion that *F. solani* is the causative agent of fatal yellowing disease in palms. Members of this genus could therefore be substrate endogenous and, consequently, have established themselves in the intestine of the larvae [66]. For

Monochamus marmorator (*Coleoptera*: *Cerambycidae*), it could be shown that the ascomycete *Trichoderma harzianum* enables the beetle to degrade cellulose. Larvae of *M. marmorator* were only able to metabolize cellulose if *T. harzianum* was present in the feed. The fungus was not an integral part of the mycobiome but was only ingested through feed consumption [67]. Similar functions of mycobiota are also conceivable in larvae of BSF, which also ingest various fungi depending on the substrate [11].

Many studies reveal pronounced differences in the composition of the microbiome of BSF larvae, significantly affected by the diet. At the same time, Klammsteiner et al. (2020) and a comparison of our data with previously published community structures show that ingested substrates strongly influence the gut microbiome, but a non-impacted core community seems to be omnipresent [10,11,52–55].

4.2. Investigation of Putative Entomopathogenic Isolates

Since the BSF has only played an increasing role commercially for a few years, research on entomopathogens and their infection mechanisms have received little attention. In addition, many studies postulate an extraordinary colonization resistance against pathogens, which is mainly based on speculation and has not been adequately investigated. These hypotheses are mostly based on larval ability to live in environments such as feces, compost, or even carrion, representing particularly microbially contaminated regimes. Although there has not yet been a widespread outbreak in commercial breeding, the economic risk of diseases caused by entomopathogens has increased due to further upscaling of production [16,17,20,68,69]. For this reason, all bacterial and fungal isolates from the gut of PKM-fed larvae were examined for the mention of entomopathogenicity as part of an extensive literature search, whereby six putative candidates were identified [36,40–45]. The intracoelomal injection of putative entomopathogenic bacteria and fungi demonstrated pronounced differences in the survival probability. *A. faecalis* was not able to cause an infection in the larvae, suggesting an inefficient infection strategy against dipteran species. However, there is limited knowledge on the host spectrum of *A. faecalis*, as it was described only once in a study with *G. mellonella* [43]. The AflP-1A/1B binary toxin of *Alcaligenes* sp. was shown to be functionally homologous to the insecticidal Cry34Ab1/Cry35Ab1 proteins from *Bacillus thuringiensis*, inducing the perforation of the intestinal epithelium of coleopteran larvae such as *Diabrotica virgifera* (*Coleoptera*: *Chrysomelidae*) [70]. Even though *D. rugosa* caused an infection in a few BSF larvae, survival did not differ significantly from the NaCl-injected control. In accordance with our data, Salas et al. (2018) also categorized *D. rugosa* as a weakly pathogenic species in the Mexican fruit fly, *Anastrepha ludens*, due to a high yield of larvae and pupae grown in a yeast-inoculated diet [44]. *M. thalassium* caused moderate larval killing, which suggests the presence of virulence factors that allow the bacterium to bypass the larval immune response, but it does not appear to be a highly adapted system for *Diptera*. To date, no information on the infection mechanism is available in the literature, and merely one study demonstrated a moderate lethality in lepidopteran *Ostrinia nubilalis* larvae [40].

In contrast, *P. aeruginosa* and *K. pneumoniae* are well-studied in vertebrates, which is mainly due to the fact that they are prevalent nosocomial germs. Both species could, however, also be linked to the infection of *Diptera* such as *D. melanogaster* or *Aedes aegypti* (*Diptera*: *Culicidae*) and showed a high lethality of about 80% in BSF larvae [71,72]. In accordance, *Pseudomonas* was found frequently during early developmental stages on chicken feed, perceptible from the remarkable green substrate discoloration and the typical odor of linden blossoms (data not shown). Substrate colonization by *Pseudomonas* species may therefore contribute to lower larval survival rates at an early stage. In addition to fimbriae or pili, which enable adhesion to surfaces, *Klebsiella* and *Pseudomonas* are characterized by binding antimicrobial substances of the host immune system and protecting themselves lipopolysaccharide *O*-antigen-mediated from the opsonization by complement factor C3b. Moreover, exposed cell structures of *K. pneumoniae* are masked by a polysaccharide capsule, which protects it from phagocytosis [73,74]. *Pseudomonas* also has a broad repertoire of

factors that allow it to damage the host. These lead to pore induction and perforation of cell membranes, as well as enzymatic modifications catalyzing depolymerization of actin filaments of the cytoskeleton or the ADP-ribosylation of elongation factor 2 by exotoxin A [75,76]. It is, therefore, a reasonable assumption that the high lethality resulted from the various virulence factors of both *Gammaproteobacteria*, which enable them to be highly adapted during different phases of infection (adhesion, invasion, establishment, reproduction, damage). Nevertheless, a recent study, which focused on the antibacterial effect of the HP/F9 peptide from the hemolymph of BSF larvae, revealed an effective *K. pneumoniae* growth inhibition in vitro and an in vivo reduction of inflammation in infected mice lungs [77]. BSF larval extracts also showed antibacterial activity against *P. aeruginosa* in substituted liquid cultures [68]. These findings indicate differences in pathogenicity and sensitivity to the immune response in a strain-dependent manner.

The inoculation of *F. solani* spores led to a tremendously high killing rate within a very short incubation time. Pathogenicity against BSF larvae is described here for the first time, although several members of the *Nematocera* such as *Culex pipiens* (*Diptera*: *Culicidae*), *Aedes cantans*, *A. detritus* (*Diptera*: *Culicidae*), and *Anopheles stephensi* (*Diptera*: *Culicidae*), as well as the brachyceran *Tetanops myopaeformis* (*Diptera*: *Ulidiidae*) were already killed by *Fusarium* sp. [78,79]. *Fusarium* species synthesize a broad portfolio of mycotoxins, including beauvericin, enniatins A, A1, B and B1, moniliformin, and fusaproliferin. Cyclodepsipeptides such as beauvericin and enniatin are able to integrate into the cell membrane. Due to their ring-shaped structure, they form cation-selective channels. In addition, beauvericin penetrates the cell nucleus and interacts with the host DNA, whereby beauvericin-DNA adducts can form [79,80]. Moniliformin, on the other hand, modifies various enzymatic processes in the host, which results in cell damage. The inhibition of glutathione-peroxidase and -reductase leads to an increase in oxidative stress; at the same time, transketolase and aldose reductase are also inhibited, which disrupts carbohydrate metabolism [80]. This remarkable variety of damaging secondary metabolites probably contribute significantly to the high lethality of infected larvae. Which mycotoxins are fundamentally essential for pathogenicity and how fungicidal substances affect survival rates of this highly virulent ascomycete should be clarified in further experiments.

The treatment of pathogens is of particular interest and might be difficult due to associated antibiotic resistance. Antibiotic resistance profiles of putative bacterial entomopathogens were determined by susceptibility tests, revealing several intermediate and resistant acting isolates. There is the possibility, similar to conventional livestock farming, to administer prophylactic antibiotics in insect breeding in order to prevent the spread of pathogens. However, this would also influence the beneficial microbiota of the BSF larvae, which may have an impact on growth performance and developmental rates. For example, the long-term medication of *Parasemia plantaginis* (*Lepidoptera*: *Erebidae*) larvae with fumagillin affected life-history traits, as well as their reproduction, negatively [81]. In addition, the widespread use of antibiotics has to be refused as it has been proven that it significantly contributes to the development of novel resistance mechanisms and promotes their spread. A more rational approach would be a targeted medication with specific antimicrobial substances as soon as a dominant pathogen has been detected in a breed, e.g., greening pseudomonads. The most efficient and gentle way would be the use of probiotics, which compete with pathogens for resources and habitat. However, further research is required to identify such probiotic microorganisms for BSF. Members of the genus *Bacillus* could be suitable probiotics since strains of *B. subtilis* and *B. natto* have already been positively associated with the development of BSF larvae [20]. It is conceivable that some yeasts are able to eliminate entomopathogenic closely related species by secreting AMPs [59]. The inoculation of core microbiota could also contribute to restoring the healthy balance of the microbiome by displacing pathogens. Furthermore, there is a possibility that antibiotic-resistant bacteria or their resistance determinants will be spread along the feed chain. Since BSF larvae are a promising alternative to fish meal and soy protein in livestock feeding, corresponding bacteria could colonize food and animals; thus

reaching humans directly by interaction with animals and their excretions such as feces, urine, and saliva (especially farmers) or indirectly through consumption of contaminated food products [82,83].

4.3. Conclusions

In conclusion, our study shows that BSF larvae are suitable as a bioconversion agent for PKM, whereby the gut microbiota seems to play an important role. We identified several taxa that are able to degrade lignocelluloses, including *Enterococcus*, *Cellulomonas*, *Pichia* yeasts, or filamentous *Fusarium* species, which could increase the digestibility for the larvae. Moreover, the isolates generated could have the potential for industrial applications. As postulated by several studies, BSF larvae seem to have an adaptive immune system that enables them to survive even on substrates with high microbial contamination. On the other hand, our results show for the first time that both fungal and bacterial isolates from the gut of the larvae occasionally have a strong entomopathogenic potential when injected. Further research on the identification of dipteran entomopathogens and their infection mechanisms should be carried out. In the future, this could combat the spread of diseases caused by entomopathogens and prevent large monetary losses through the timely use of appropriate diagnostic protocols [69].

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/microorganisms10020319/s1, Table S1: Taxonomic classification of bacterial isolates from the gut of BSF larvae grown on PKM. For isolates whose sequences matched two database entries, both suggestions were given. Prokaryotic isolates that could only be assigned to the bacteria were listed as *Bacteria* sp., Table S2: Taxonomy of fungal isolates from the gut of BSF larvae grown on PKM. For isolates whose sequences matched two database entries, both suggestions were given.

Author Contributions: Conceptualization, P.K. and S.M.; methodology, P.K., S.M. and J.S.; validation, P.K., M.R. and H.Z.; formal analysis, P.K. and S.M.; investigation, P.K., S.M. and J.S.; resources, H.Z. and M.R.; data curation, P.K., S.M. and M.R.; writing—original draft preparation, P.K.; writing—review and editing, P.K., M.R. and H.Z.; visualization, P.K.; supervision, P.K.; project administration, P.K.; funding acquisition, H.Z. and M.R. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Hessian Ministry of Science and Art in the course of the LOEWE center for Insect Biotechnology. Part of the work was funded by PT Alternative Protein Indonesia.

Institutional Review Board Statement: Not applicable.

Data Availability Statement: All relevant data is contained within the article.

Acknowledgments: The authors thank Bio S. Biogas for providing BSF larvae. We are particularly thankful for the methodological expertise and useful comments on antimicrobial susceptibility by Eugen Domann (Justus Liebig University Giessen, Germany) and Silke Zechel-Gran for assistance with infection assays.

Conflicts of Interest: The authors declare no conflict of interest.

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Research Article 3 – Diet fermentation leads to microbial adaptation in black soldier fly (*Hermetia illucens*; Linnaeus, 1758) larvae reared on palm oil side streams.

Klüber P, Tegtmeier D, Hurka S, Pfeiffer J, Vilcinskas A, Rühl M, Zorn H. Diet fermentation leads to microbial adaptation in black soldier fly (*Hermetia illucens*; Linnaeus, 1758) larvae reared on palm oil side streams. *Sustainability* 2022; 14(9):5626.

DOI: https://doi.org/10.3390/su14095626

Supplementary data available on:
https://www.mdpi.com/2071-1050/14/9/5626#supplementary

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Diet Fermentation Leads to Microbial Adaptation in Black Soldier Fly (*Hermetia illucens*; Linnaeus, 1758) Larvae Reared on Palm Oil Side Streams

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Abstract: Insects offer a promising alternative source of protein to mitigate the environmental consequences of conventional livestock farming. Larvae of the black soldier fly (*Hermetia illucens*; Linnaeus, 1758) efficiently convert a variety of organic side streams and residues into valuable proteins, lipids, and chitin. Here, we evaluated the suitability of two palm oil industry side streams—empty fruit bunches (EFB) and palm kernel meal (PKM)—as larval feed, and their impact on the larval gut microbiome. Among 69 fungal species we screened, *Marasmius palmivorus*, *Irpex consors*, and *Bjerkandera adusta* achieved the fastest growth and lignin degradation, so these fungi were used for the pretreatment of 7:3 mixtures of EFB and PKM. Larvae reared on the mixture pretreated with *B. adusta* (BAD) developed significantly more quickly and reached a higher final weight than those reared on the other pretreatments or the non-fermented reference (NFR). Amplicon sequencing of the BAD and NFR groups revealed major differences in the larval gut microbiome. The NFR group was dominated by facultatively anaerobic *Enterobacteriaceae* (typical of *H. illucens* larvae) whereas the BAD group favored obligately anaerobic, cellulolytic bacteria (*Ruminococcaceae* and *Lachnospiraceae*). We hypothesize that fungal lignin degradation led to an accumulation of mycelia and subsequent cellulolytic breakdown of fiber residues, thus improving substrate digestibility.

Keywords: black soldier fly; palm oil; empty fruit bunches; palm kernel meal; sustainable insect feed; amplicon sequencing; microbiome; fermentation; insect rearing; *Bjerkandera adusta*

Citation: Klüber, P.; Tegtmeier, D.; Hurka, S.; Pfeiffer, J.; Vilcinskas, A.; Rühl, M.; Zorn, H. Diet Fermentation Leads to Microbial Adaptation in Black Soldier Fly (*Hermetia illucens*; Linnaeus, 1758) Larvae Reared on Palm Oil Side Streams. *Sustainability* **2022**, *14*, 5626. https://doi.org/10.3390/su14095626

Academic Editors: Christos G. Athanassiou, Christos I. Rumbos, David Deruytter and János-István Petrusán

Received: 20 March 2022
Accepted: 3 May 2022
Published: 6 May 2022

1. Introduction

The larvae of the black soldier fly, *Hermetia illucens* (BSF; Linnaeus, 1758; Diptera: Stratiomyidae), are polyphagous with a remarkably efficient feed conversion ratio, making them suitable for the bioconversion of various animal and vegetable organic substrates into valuable insect proteins and lipids [1–3]. BSF larvae can even utilize substrates with a high microbial load, such as livestock manure, reducing the total dry matter and nitrogen content by more than 50% [2,4]. BSF has a short lifecycle of 40–45 d, and can be farmed in space-saving vertically stacked facilities with low technological requirements. BSF larvae therefore offer a promising and sustainable alternative for soy protein in the feed sector, helping to counter the slash and burn clearance of tropical forests. Since 2017, the European Union has also authorized the use of seven insect species including BSF for aquaculture feeding [5].

The versatility of BSF larvae is dependent on the gut microbiome [6,7]. For example, the degradation of complex plant polymers, such as cellulose and lignin, would not be

possible without associated microbes. The structure of bacterial and fungal communities in the BSF larval gut is strongly affected by the feed substrate and its properties, but also by extrinsic factors, such as rearing location, scale, or temperature [8–10]. In this context, the diet-dependent expression of antimicrobial peptides—defense molecules of the insect innate immune system—plays a key role in the regulation of gut microbial composition [11] although there is also an omnipresent core community [7,8,12,13]. The feeding status (especially periods of starvation) also influence the microbiome and its associated metabolic functions, thus potentially disrupting larval growth and feed conversion efficiency [14]. However, the inoculation of poultry manure with companion bacteria was able to promote larval development and is a promising biotechnological strategy to optimize growth periods [15]. In contrast to the bacterial community, little is known about the mycobiome of BSF larvae. Fungi are important in the digestive capabilities of many insects, including cerambycid beetle larvae, fungus-growing termites, and siricid woodwasps [16]. By secreting a broad enzyme repertoire, fungi mediate many further processes such as the degradation of toxic compounds and the production of bioactive substances, as previously reported in BSF [17]. It is necessary to determine the fungal composition of the larval gut to evaluate their hazard potential as an animal feedstock, given the ability of many fungi to synthesize mycotoxins [18].

The growing worldwide demand for proteins and lipids contrasts with the inefficient use of agro-industrial raw materials and the modern throwaway society. Large amounts of underutilized organic material accumulate as side streams in many parts of the agri-food industry, including palm oil production to meet the increasing demand for vegetable oils [19]. In 2018, 71.5 million tons of crude palm oil was produced on an area of 18.9 million ha [20]. The milling process and subsequent refining and fractionation steps produce side streams and residues such as empty fruit bunches (EFB), palm kernel meal (PKM), as well as sludge and mill effluents (POME), amounting to many millions of tons per year [21]. Although PKM is already used as an inexpensive dietary supplement for ruminants and has been proposed as a sustainable option for BSF rearing [13,22], the utilization of EFB is more challenging due to its high content of lignocellulose. For this reason, it has not been used as a feed, and it is mostly burned so far. The upcycling of palm oil side streams by fermentation and feeding them to insects has not been investigated so far.

The compact structure of lignocelluloses requires special enzyme systems that are exclusively found in wood-degrading fungi. White-rot fungi representing the Basidiomycota are particularly suitable for the depolymerization of lignocelluloses because they produce enzyme cocktails that break down cellulose, hemicellulose, and lignin [23]. This makes them excellent candidates for the pretreatment of lignocellulose-rich EFB by fermentation, although feeding studies using EFB or mixtures of EFB and PKM have not been reported thus far.

Here we investigated the suitability of fermentation as a pretreatment for mixtures of EFB and PKM to improve digestibility, and the corresponding changes in BSF life-history traits as well as diet-specific adaptations in the larval gut microbiome. Extensive screening followed by feeding trials was carried out to find a suitable fungus for the fermentation step. The bacterial and fungal communities in the feed, BSF larval guts, and frass were then characterized by Illumina high-throughput sequencing of the 16S rRNA gene and the fungal internal transcribed spacer (ITS) regions.

2. Materials and Methods

2.1. Fungal Screening and Substrate Preparation

The palm oil side streams were provided by PT Alternative Protein Indonesia (Tebet, Indonesia). EFB was ground to powder (~1 mm particle size) in an SM 2000 cutting mill, autoclaved and set to a moisture content of 60% (Retsch, Haan, Germany). For fungal screening, 50 g of the powder was placed in 250 mL Erlenmeyer flasks before adding 3 mL of homogenized liquid culture representing a particular fungus from the strain collection of the Institute of Food Chemistry and Food Biotechnology (University of Giessen, Germany).

Technical duplicates of 69 strains were incubated for 10 d at 30 °C in the dark. Fungal growth and lignocellulose degradation were evaluated visually, with degradation revealed by EFB bleaching [24].

For solid-state fermentation of the feed substrate, submerged cultures of the basidiomycetes *Bjerkandera adusta*, *Irpex consors*, and *Marasmius palmivorus* were processed in a T25 digital Ultra-Turrax homogenizer (IKA, Staufen im Breisgau, Germany) at 10,000 rpm for 30 s. An EFB + PKM mixture in the ratio of 7:3 was chosen because the side streams arise in this ratio. We then inoculated 250 g of the EFB + PKM mixture (7:3 ratio) with 15 mL of the corresponding fungal suspension in 2 L Erlenmeyer flasks (5% *w*/*v*) and incubated the cultures for 28 d at 28 °C and 60% relative humidity in the dark. The corresponding fermented feed was described as BAD (*B. adusta* fermented), ICO (*I. consors* fermented), or MPA (*M. palmivorus* fermented), respectively. The unfermented EFB + PKM mixture was used as the control (NFR). The feed was stored at −20 °C and thawed immediately before use. Chicken feed (CF) was used as a positive control, as in previous studies [1,7,10,25,26]. We used GoldDott Eierglück chicken feed (Derby Spezialfutter, Muenster, Germany), which was prepared by grinding in a Mockmill 200 grain mill (Wolfgang Mock, Otzberg, Germany) to a particle size of 0.1–1.5 mm.

2.2. Insect Rearing

BSF were originally obtained from Bio.S Biogas (Grimma, Germany). The larvae were maintained in 19.5 × 16.5 × 9.5 cm (l × w × h) plastic containers within a climate chamber at 27 ± 1 °C, 65 ± 5% relative humidity, in constant darkness [7,27]. Adult flies were kept in 60 × 60 × 90 cm (l × w × h) mesh cages (Bioform, Nuremberg, Germany) in a greenhouse at 25 ± 1 °C, 40 ± 10% relative humidity, and a 12 h photoperiod. Water-soaked paper towels provided drinking water *ad libitum*. Wooden board stacks held with rubber bands served as an artificial oviposition system, as previously described [27]. We harvested 200 mg eggs per plastic container using a plastic spatula. The containers were sprayed daily with water. Feed was added at 48 h intervals as soon as ≥50% of the eggs had hatched. To prevent contamination, disposable nitrile gloves were worn to handle and administer the feed.

2.3. Developmental Parameters

All developmental parameters were recorded as biological triplicates for each diet group. Data from the CF diet controls were recorded in collaboration with Tegtmeier and colleagues, except for fertility and longevity-related data [7]. We placed 150 mg eggs in three replicate containers per substrate to record the hatching time. Depending on the feed, the larvae reached a manageable size (3–4 mm; instar L3) after 5–10 d, allowing the analysis of growth curves. The mean larval weight was determined (at 48 h intervals until ≥50% of the individuals reached the prepupal stage) by placing random specimens (n = 25) on an AT261 DeltaRange analytical balance (Mettler, Giessen, Germany). The distinction between larval instars was standardized based on the head capsule width and the weight [28]. The survival rates of the larva-prepupa, prepupa-pupa, and pupa-imago stages were determined in 19.5 × 16.5 × 9.5 cm (l × w × h) plastic containers with a stocking density of 100 individuals. The transition between two developmental stages was documented as soon as ≥50% of the population reached the next developmental stage. We recorded the final weight (as above) and final length (using a VHX-2000 digital microscope; Keyence, Osaka, Japan) of 50 L5 larvae, prepupae, pupae and 60 imagines). Adult flies were collected within 24 h of emergence, inactivated by placing on ice and stored at −20 °C before measurement. The sex of adult flies was determined according to external dimorphisms, including antennal and genital structures [29].

Reproductive parameters were evaluated in 20 temporally synchronized pairs of adult flies (within 24 h of emergence) in conical 8.4 × 8.4 × 11.4 cm (l × w × h) polypropylene boxes in the greenhouse. Each pair was placed in its own box, which contained an artificial oviposition system consisting of two stacked 4 × 4 cm wooden boards spaced and held in

place by magnetic tape strips. A circular 4.5 cm mesh insert in the lid enabled gas exchange and the daily provision of water by spraying. The adult lifespan and the deposition of eggs were checked and documented daily. Fresh egg clutches were collected, weighed, unclustered carefully using a metal spatula, and counted under an S9i stereomicroscope (Leica Microsystems, Wetzlar, Germany).

2.4. 16S rRNA Gene and ITS Amplicon Sequencing

DNA samples and amplicon sequencing were prepared according to Tegtmeier et al. [7] in order to compare the results to the amplicon data of BSF larvae reared on chicken feed (standard diet for BSF rearing). Briefly, L5 larvae weighing 100–150 mg were collected with spring steel tweezers. Frass samples were taken from three different vertical sites and horizontal levels and pooled to cover a large proportion of the different microbes colonizing the frass. Samples of the NFR and BAD feed were taken at three time points during the feeding period and were also pooled. All samples were stored at −20 °C before processing. The larvae were cleaned with sterile water and then surface sterilized twice with 70% ethanol. To dissect the larval gut, the haemocoel was opened laterally with microscissors and the gut was removed under a stereomicroscope using Dumont (Montignez, Switzerland) No. 5 tweezers. Six single guts per diet as well as three replicates of 100 mg of the corresponding feed and frass samples were disrupted by bead beating in a FastPrep-24 (MP Biomedicals, Solon, OH, USA) for 90 s at 6.5 $m \cdot s^{-1}$. DNA was isolated using the NucleoSpin soil kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's specifications. The yield and purity of the DNA were determined using a Take3 spectrophotometer (BioTek Instruments, Winooski, VT, USA).

Two-step PCR library preparation and amplicon sequencing were carried out by LGC Genomics (Berlin, Germany) on the MiSeq V3 platform (Illumina, San Diego, CA, USA). Primers U341F (5′-CCT AYG GGR BGC ASC AG-3′) and U806R (5′-GGA CTA CNN GGG TAT CTA AT-3′) were used to amplify the 16S rRNA hypervariable V3–V4 region of bacteria and archaea [30], whereas primers fITS7 (5′-GTG ART CAT CGA ATC TTT G-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) were used to amplify the fungal ITS2 region [31,32]. We aimed for ~20,000 paired-end reads per sample with a read length of 300 bp. Samples were multiplexed and pooled for sequencing. Demultiplexing and the clipping of adapters and primers was carried out using bcl2fastq 2.17.1.14, followed by analysis with QIIME 2020.6 [33]. Only forward ITS sequencing reads were used, and the read-through adapters and primers were removed using the cutadapt plugin [34]. The DADA2 plugin [35] was used for combined error correction, quality control, filtering chimeric sequences, and the creation of an ASV table showing the number of sequences for each ASV per sample.

Taxonomic classification was achieved using self-trained naïve Bayes classifiers based on SILVA 132 [36] and UNITE 8.2 [37] QIIME-compatible releases with 99% sequence identity for 16S and ITS, respectively. Reference data from SILVA were trimmed before training to include only the 16S rRNA gene region amplified by the primers [38]. The confidence for classification was 0.7 (16S) or 0.94 (ITS) as recommended [39]. Mitochondrial and chloroplast sequences were discarded. Alpha diversity was calculated based on Faith's phylogenetic diversity [40] and observed ASVs. Rarefied data to equal sequencing depths of 8819 (16S) and 12,846 (ITS) were used to evaluate beta diversity with UniFrac [41] distance metrics.

We used discrete false-discovery rate (DS-FDR) to test for diet-dependent differential abundance in gut samples [42]. We collapsed the classified ASVs at the species level to run the test on log2-transformed data, testing for differences in mean values ($p = 0.05$, permutations = 1000) with the corresponding QIIME2 plugin. We screened for representatives of the positively and negatively correlated taxa (based on DS-FDR) using the Integrated Microbial Genomes Expert Review (IMG/ER) platform (https://img.jgi.doe.gov/cgi-bin/er/main.cgi, accessed on 28 July 2021) [43], and selected 140 genomes of the 28 most positively correlated taxa (highest *p*-value) as well as 140 genomes of the 28 most negatively correlated taxa (lowest *p*-value). Only taxa that were classified to the family level were included.

For taxa that were not classified to the genus level, only genomes of representatives with ≥91% sequence identity to the target sequence were included. The genomes were screened for genes involved in cellulose degradation (endoglucanases EC 3.2.1.4, exoglucanases EC 3.2.1.91 and β-glucosidases EC 3.2.1.21) using the KEGG Orthology (KO) database implemented in IMG/ER [44].

2.5. Statistics

Statistical analysis and visualization were carried out using Excel 2016 (Microsoft, Redmond, WA, USA) and R 4.1 [45] with the packages ggpubr, plyr, qiime2R, scales, and tidyverse. Diet-dependent differences in the relative abundance of bacterial or fungal taxa were determined using Student's (homogeneous variance) or Welch's (inhomogeneous variance) *t*-test and an error level of $\alpha = 0.05$ for statistical significance. The homogeneity of variance was calculated with Levene's test. Developmental parameters were compared by one-way ANOVA and means were separated using the Bonferroni–Holm test [46]. Sex-specific and total adult longevity were analyzed using the Kaplan–Meier estimation to generate $S(t)$ survival functions, which were compared pairwise by log rank tests at an error level of $\alpha = 0.05$ for statistical significance in OriginPro 2020b (OriginLab, Northampton, MA, USA). For PERMANOVA [47], we grouped all samples combing feed (NFR, BAD) and source (feed, gut, and frass) separately for 16S and ITS. We used the corresponding unweighted UniFrac distance metric and tested pairwise on the six groups with 999 permutations, with *p*-value correction for multiple tests based on the Benjamini–Hochberg procedure [48].

3. Results

3.1. Fungal Screening and BSF Growth Performance

In order to identify a suitable fungus for the pretreatment of palm oil side streams, we screened 69 strains, including 61 Basidiomycota and 8 Ascomycota. We found that 29 strains were unable to grow on EFB, and that *Mycena pseudocorticola* and *Omphalotus illudens* achieved only weak growth. Members of the family *Pleurotaceae* showed comparatively fast growth, but the fastest-growing species were the white-rot fungi *Marasmius palmivorus*, *Irpex consors*, and *Bjerkandera adusta*. Bleaching of the dark lignin also indicated these three species and eight others (with *Xylaria longipes* representing the only ascomycete) as putative candidates for the fermentation of palm oil side streams (Table 1).

Table 1. Fungal screening for growth [1] and lignin degradation [2] during the solid-state fermentation of empty fruit bunches (EFB).

Phylum	Class	Order	Family	Species	Growth	Optical Lignin Degradation
Ascomycota	Leotiomycetes	Helotiales	*Mollisiaceae*	*Mollisia lividofusca*	-	-
				Mollisia pilosa	-	-
	Sordariomycetes	Glomerellales	*Plectosphaerellaceae*	*Plectosphaerella* sp.	-	-
		Hypocreales	*Cordycipitaceae*	*Isaria farinosa*	+	-
			Hypocreaceae	*Trichoderma longipile*	+	-
				Trichoderma minutisporum	+	-
				Trichoderma polysporum	+	-
		Xylariales	*Xylariaceae*	*Xylaria longipes*	++	+
Basidiomycota	Agaricomycetes	Agaricales	*Agaricaceae*	*Coprinus comatus*	+	-
				Coprinus xanthothrix	++	+
			Crepidotaceae	*Crepidotus* sp.	-	-
			Marasmiaceae	*Marasmius palmivorus*	+++	+
			Mycenaceae	*Mycena pseudocorticola*	(−)	-
			Omphalotaceae	*Omphalotus illudens*	(−)	-
			Physalacriaceae	*Armillaria bulbosa*	+	-

Table 1. *Cont.*

Phylum	Class	Order	Family	Species	Growth	Optical Lignin Degradation
				Armillaria gallica	-	-
				Armillaria mellea	-	-
				Armillaria tabescens	-	-
			Pleurotaceae	*Pleurotus calyptratus*	+	-
				Pleurotus cornucopiae	-	-
				Pleurotus eryngii	+	-
				Pleurotus flabellatus	++	-
				Pleurotus floridanus	++	+
				Pleurotus ostreatus (POS1)	++	+
				Pleurotus ostreatus (POS2)	++	+
				Pleurotus ostreatus (POS3)	+	+
				Pleurotus ostreatus (POS4)	-	-
				Pleurotus ostreatus (POS5)	+	-
				Pleurotus pulmonarius (PPU1)	+	-
				Pleurotus pulmonarius (PPU2)	+	-
				Pleurotus salmoneo-stramineus	+	-
				Pleurotus sapidus	++	-
			Psathyrellaceae	*Coprinellus flocculosus*	-	-
			Strophariaceae	*Agrocybe aegerita*	+	-
				Agrocybe perfecta	-	-
				Hypholoma fasciculare	-	-
				Pholiota lignicola	-	-
		Auriculariales	*Auriculariaceae*	*Auricularia mesenterica*	-	-
		Gloeophyllales	*Gloeophyllaceae*	*Gloeophyllum trabeum*	+	-
		Hymenochaetales	*Hymenochaetaceae*	*Inonotus dryadeus*	+	-
		Polyporales	*Fomitopsidaceae*	*Fomitopsis pinicola*	-	-
				Laetiporus sulphureus	-	-
				Piptoporus betulinus	-	-
			Irpicaceae	*Irpex consors*	+++	+
				Irpex vellereus	++	-
			Meruliaceae	*Bjerkandera adusta*	+++	+
				Ceriporiopsis rivulosa	-	-
				Emmia lacerata	+	-
				Phlebia radiata	+	-
			Phanerochaetaceae	*Byssomerulius corium*	+	-
				Phanerochaete chrysosporium	++	+
			Polyporaceae	*Dichomitus albidofuscus*	+	-
				Dichomitus campestris	++	-
				Dichomitus squalens	+	-
				Fomes fomentarius	-	-
				Ganoderma lucidum	-	-
				Ganoderma sp.	-	-
				Lentinus crinitus	-	-
				Lenzites betulinus	+	-
				Microporus affinis	+	-
				Pycnoporus cinnabarinus	-	-
				Pycnoporus coccineus	+	-
				Pycnoporus sanguineus	-	-
				Trametes versicolor (TVE1)	++	+
				Trametes versicolor (TVE2)	-	-
			Sparassidaceae	*Sparassis crispa*	-	-
		Russulales	*Bondarzewiaceae*	*Heterobasidion annosum*	-	-
			Peniophoraceae	*Peniophora lycii*	+	-
			Stereaceae	*Stereum* sp.	++	-

[1] Growth was categorized as follows: - none, (−) weak, + moderate, ++ good, and +++ excellent growth. [2] A binary system was used to evaluate lignin degradation (- no, + yes).

Based on the initial screen, we used *M. palmivorus*, *I. consors*, and *B. adusta* to ferment the EFB + PKM mixture (7:3 ratio). The corresponding fermented feed was described as MPA, ICO, or BAD, respectively. We carried out comparative feeding studies in BSF larvae using CF as a high-quality control and the untreated EFB + PKM mixture as a low-quality standard diet, described as the non-fermented reference (NFR). The final larval weight differed significantly between the diets, and larvae reared on CF were the heaviest ($F_{4,10} = 58.04$; $p < 0.002$) (Figure 1). Neither MPA nor ICO led to an increase in larval weight

compared to NFR, whereas larvae reared on the BAD diet were 25% heavier than their NFR counterparts ($F_{2,6}$ = 74.84; p = 0.042). All the diets influenced larval development. CF resulted in the shortest developmental period, followed by BAD and ICO. However, MPA extended the larval development phase to 79.0 d, approximately twice the duration of the NFR larvae ($F_{4,10}$ = 994.61; $p < 0.0001$). The larvae reared on the BAD diet developed significantly faster than those in the NFR, MPA, and ICO groups ($F_{3,8}$ = 848.13; p = 0.034).

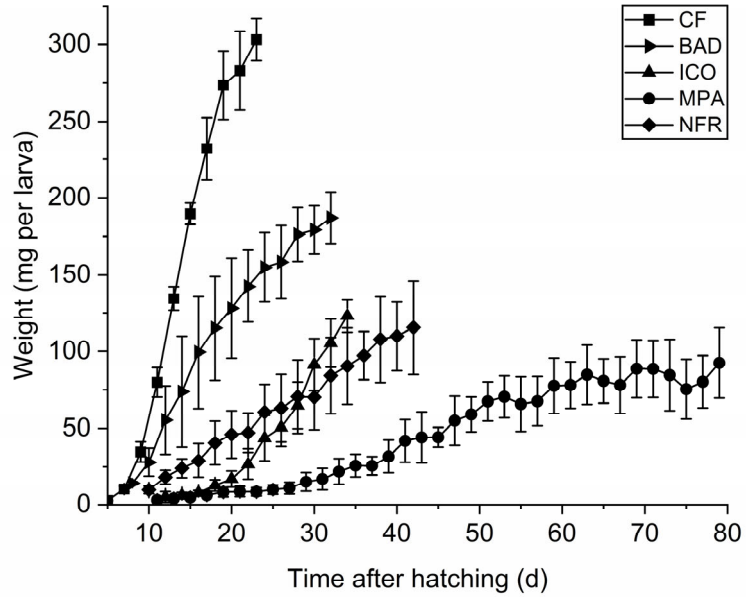

Figure 1. Growth curves of BSF larvae reared on chicken feed (CF), *B. adusta* (BAD), *I. consors* (ICO), or *M. palmivorus* (MPA) fermented EFB + PKM (7:3) mixtures, as well as a corresponding non-fermented reference (NFR). Data are mean larval weights (±SD) of three replicate boxes per diet (n = 25).

3.2. Life-History Traits

We also compared additional life-history traits between larvae raised on the standard diets (CF, NFR) and the BAD diet. The time until ≥50% of the eggs hatched did not differ between the diets. Larval development differed significantly between the diets, with CF larvae reaching the prepupal stage first (Table 2). Larvae reared on the BAD diet developed 27% faster than their NFR counterparts ($F_{2,6}$ = 136.50; p = 0.001). Prepupal and pupal development (including metamorphosis) did not differ between the diet groups. However, the total duration of development differed between the groups, being 19% quicker on the BAD diet and 24% quicker on the CF diet compared to the NFR diet ($F_{2,6}$ = 36.58; $p < 0.004$). The same pattern was also observed for the total preoviposition period ($F_{2,6}$ = 39.88; $p < 0.002$), whereas no significant diet effect was found in the adult preoviposition period. Larvae reared on the CF diet showed the highest larva-to-prepupa developmental success (100%), differing significantly from the BAD diet ($F_{2,6}$ = 8.10; p = 0.0001). In contrast, the prepupa-to-pupa developmental success was 9% higher for larvae reared on the BAD diet compared to those on the CF diet ($F_{2,6}$ = 132.58; p = 0.0001). The proportion of adult flies emerging from pupae was 97–100% in all feeding trials. Oviposition lasted 2–10 d. The longevity of adult males (χ^2 = 51.67; $p < 0.0001$), adult females (χ^2 = 65.98; $p < 0.0001$), and all adults (χ^2 = 91.31; $p < 0.0001$) differed significantly between CF and both other diets. In general, male adults lived longer than females in the same diet group (Table 2).

Table 2. Diet-dependent temporal and physiological parameters of BSF throughout development. BSF larvae were reared on chicken feed (CF), a non-fermented reference (NFR), or a *B. adusta* fermented diet (BAD). Data are means ± SD. The transition between two developmental stages was defined as when ≥50% of a population reached the next stage. Different letters (a–c) within a row indicate statistically significant differences between diets ($p < 0.05$; one-way ANOVA; Kaplan–Meier estimator for adult longevity).

Parameters		Sampling Size	CF [8]	NFR	BAD
Hatching time (d)		≥50%	3.0 ± 0.8 [a]	3.3 ± 0.5 [a]	3.0 ± 0.0 [a]
Larval development (d) [1]		≥50%	22.3 ± 0.5 [a]	41.3 ± 0.9 [b]	30.3 ± 1.7 [c]
Prepupa-pupa (d)		*n* = 300	11.3 ± 1.7 [a]	8.0 ± 0.0 [a]	7.7 ± 1.3 [a]
Intrapuparial metamorphosis (d) [2]		*n* = 300	10.7 ± 0.5 [a]	10.0 ± 0.8 [a]	9.7 ± 0.9 [a]
Adult preoviposition period (d) [3]		*n* = 60	7.7 ± 0.8 [a]	9.6 ± 1.0 [a]	6.1 ± 1.0 [a]
Total preoviposition period (d) [4]		*n* = 60	52.0 ± 2.0 [a]	69.0 ± 2.6 [b]	53.7 ± 1.6 [a]
Total development (d) [5]		*n* = 300	47.3 ± 1.7 [a]	62.7 ± 1.9 [b]	50.7 ± 2.1 [a]
Oviposition period (d)		*n* = 60	7.3 ± 2.1 [a]	4.0 ± 2.2 [a]	4.00 ± 0.8 [a]
Oviposition span (min-max d)		*n* = 60	5–10	2–7	3–9
Successful development larva-prepupa (%)		*n* = 300	100.0 ± 0.0 [a]	61.0 ± 16.8 [ab]	81.3 ± 1.0 [b]
Successful development prepupa-pupa (%)		*n* = 300	90.7 ± 0.5 [a]	98.2 ± 0.5 [b]	99.5 ± 0.7 [b]
Successful development pupa-adult (%)		*n* = 300	97.0 ± 2.4 [a]	98.7 ± 1.0 [a]	100.0 ± 0.0 [a]
Adult longevity (d)	♂	*n* = 60	17.7 ± 0.5 [a]	13.3 ± 0.5 [b]	13.0 ± 0.8 [b]
	♀	*n* = 60	14.7 ± 0.2 [a]	11.7 ± 0.5 [b]	11.3 ± 0.5 [b]
	total	*n* = 120	16.2 ± 0.2 [a]	12.7 ± 0.5 [b]	12.0 ± 0.00 [b]
Adult longevity (min-max d)		*n* = 120	4–25	7–24	5–19
Final larval weight (mg)		*n* = 150	303.0 ± 13.6 [a]	149.3 ± 7.2 [b]	187.0 ± 16.7 [c]
Final larval length (mm)		*n* = 150	26.0 ± 0.1 [a]	20.4 ± 0.1 [b]	22.6 ± 0.1 [c]
Weight prepupa (mg)		*n* = 150	219.6 ± 18.7 [a]	130.3 ± 2.3 [b]	179.7 ± 2.7 [c]
Length prepupa (mm)		*n* = 150	23.4 ± 0.1 [a]	19.0 ± 0.7 [b]	20.8 ± 0.1 [c]
Weight pupa (mg)		*n* = 150	169.0 ± 10.7 [a]	121.0 ± 7.3 [b]	151.6 ± 4.9 [ab]
Length pupa (mm)		*n* = 150	22.7 ± 0.1 [a]	20.1 ± 0.5 [b]	21.1 ± 0.3 [ab]
Weight adult (mg)	♂	*n* = 90	89.4 ± 8.5 [a]	67.6 ± 6.6 [a]	77.7 ± 2.9 [a]
	♀	*n* = 90	103.6 ± 8.3 [a]	83.5 ± 10.4 [a]	94.9 ± 4.7 [a]
	total	*n* = 180	98.1 ± 8.0 [a]	75.5 ± 8.0 [a]	87.2 ± 2.3 [a]
Length adult (mm) [6]	♂	*n* = 90	16.8 ± 0.3 [a]	15.2 ± 0.3 [b]	16.1 ± 0.1 [a]
	♀	*n* = 90	17.5 ± 0.3 [a]	16.4 ± 0.7 [b]	17.3 ± 0.2 [a]
	total	*n* = 180	17.2 ± 0.2 [a]	15.8 ± 0.5 [b]	16.8 ± 0.1 [a]
Sex ratio (♀/♂)		*n* = 180	1.5 ± 0.2 [a]	1.0 ± 0.2 [a]	1.3 ± 0.4 [a]
Fertility (egg clutches/10 females)		*n* = 60	7.8 ± 0.9 [a]	1.4 ± 0.4 [b]	8.2 ± 0.9 [a]
Egg clutch size (eggs/clutch)		*n* = 10	676.0 ± 59.6 [a]	259.9 ± 59.7 [b]	541.3 ± 59.1 [c]
Span of egg clutch size (min-max eggs)		*n* = 10	548–763	172–334	375–589
Egg clutch weight (mg)		*n* = 10	16.5 ± 3.8 [a]	4.6 ± 1.3 [b]	11.1 ± 1.3 [c]
Egg weight (mg/egg) [7]		*n* = 10	0.024 ± 0.005 [a]	0.018 ± 0.002 [a]	0.021 ± 0.001 [a]

[1] Period from hatching to ≥50% prepupae. [2] Period until ≥50% of adults emerged from pupae. [3] Period from adult emerging to oviposition. [4] Period from hatching to oviposition. [5] Period from oviposition to adults emerging. [6] Determined as distance between cranial antennal attachments and abdominal genital structures. [7] Calculated by dividing the clutch weight by the egg count. [8] Data for CF (except for fertility and longevity-related data) have been published before [7] and are included here for comparison.

The final larval weight ($F_{2,6} = 74.84$; $p < 0.04$) and length ($F_{2,400} = 672.70$; $p < 0.0001$) differed significantly between diets, with the largest and heaviest larvae reared on the CF diet, followed by those reared on the BAD diet. The same profile for weight ($F_{2,6} = 22.22$; $p < 0.04$) and length ($F_{2,400} = 348.58$; $p < 0.0001$) was observed at the prepupal stage. Larvae reared on the CF diet produced pupae that were heavier ($F_{2,6} = 13.26$; $p = 0.022$) and longer ($F_{2,400} = 134.33$; $p < 0.0001$) than NFR counterparts, but there was no statistically significant difference between the CF and BAD diets. Neither the total adult weight nor the sex-specific weight showed significant differences between the diets. The adult males ($F_{2,235} = 96.18$; $p < 0.0001$), adult females ($F_{2,310} = 53.97$; $p < 0.0001$), and all adults ($F_{2,545} = 117.20$; $p < 0.0001$) were shortest in the NFR group, but up to 11% longer in the CF

group and up to 6% longer in the BAD group. In general, females were 16–24% heavier and 4–8% longer than the corresponding males, regardless of the diet. All dietary groups had similar sex ratios, with a slight excess of females. The number of egg clutches deposited per female was 5.9-fold higher in the BAD group compared to the NFR group, and even exceeded the performance of the CF group ($F_{2,6}$ = 55.86; p = 0.0005). Furthermore, the egg clutch size in the BAD group was 2.1-fold higher than the NFR group ($F_{2,27}$ = 114.63; $p < 0.0001$), and the egg clutch weight was 2.4-fold higher than the NFR group ($F_{2,27}$ = 74.36; $p < 0.0005$), but in neither parameter did the BAD group outperform the CF group. We observed no significant dietary impact on the weight of individual eggs.

3.3. Taxonomic Composition of the Bacterial Gut Microbiome

To identify differences in the bacterial and archaeal gut microbiome between larvae fed on the BAD and NFR diets, we performed 16S rRNA gene sequencing on 24 samples (six guts, three feed samples, and three frass samples per diet). We obtained 527,684 raw read pairs, 406,803 of which remained after quality control and the removal of chimeric sequences (8819–46,354 reads per sample). The mean read length was 421 bp after merging forward and reverse reads. Rarefaction curves suggested a sufficient sequencing depth (Figure 2).

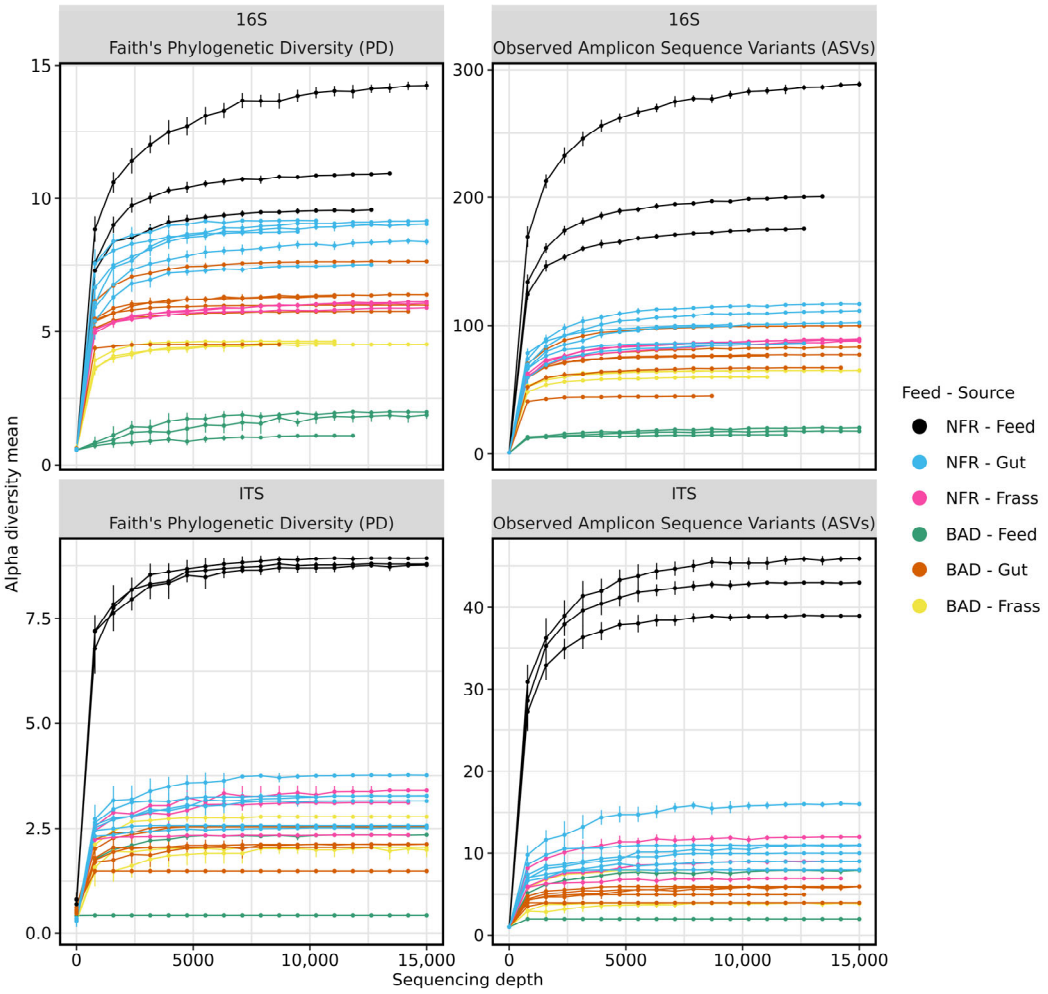

Figure 2. Rarefaction curves of Faith's phylogenetic diversity (**left**) and amplicon sequence variants (**right**) showing the alpha diversity of 16S rRNA gene and ITS amplicon sequencing. Almost all samples of the non-fermented reference (NFR) and *B. adusta* fermented diet (BAD) reach a plateau at ~9000 reads. Samples with a lower number of reads also reach the plateau. Greater sequencing depth would therefore not lead to an increase in taxonomic richness. Standard deviations are shown as bars.

Data processing using DADA2, followed by classification using our self-trained naïve Bayes classifier on SILVA 132 and the removal of mitochondrial and chloroplast sequences resolved the dataset to 693 unique amplicon sequence variants (ASVs) across all 24 samples. All the ASVs were assigned to domain Bacteria (i.e., no Archaea were found). We classified 621 of the 693 ASVs down to the genus level. Principal coordinates analysis (PCoA) of weighted UniFrac distance metrics indicated that all frass samples from both diets clustered together with four gut samples from larvae reared on the NFR diet (Figure 3A). In contrast, unweighted distances indicated differences between all sample groups. However, the unweighted distances revealed a higher similarity between gut samples from larvae reared on the BAD diet and the corresponding frass samples (Figure 3B). The BAD and NFR feed samples showed greater differences when comparing unweighted distances and smaller differences when comparing weighted distances. The feed and frass samples of both diets showed little similarity, although the differences in the BAD group were greater (Figure 3A,B).

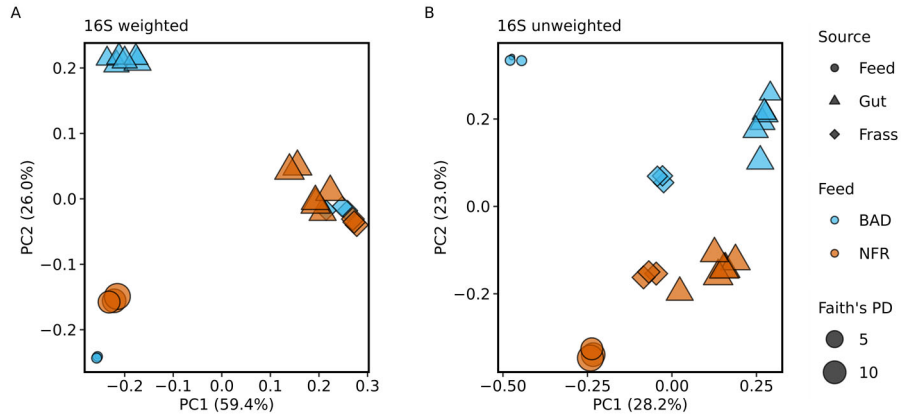

Figure 3. Principal coordinates analysis (PCoA) when comparing the microbiome between the non-fermented reference (NFR) and *B. adusta* fermented diet (BAD) groups. (**A**,**B**) PCoA comparing the bacterial communities based on weighted and unweighted UniFrac distance metrics. The sample type is represented by symbols (feed, gut, and frass) and the dietary group affiliation by colors. The symbol size represents Faith's phylogenetic diversity (PD).

Bacteria were detected in all samples. The BAD feed almost exclusively contained *Bacillaceae* (mostly *Bacillus coagulans*), with a relative abundance of 99.9%. The NFR feed was more diverse, but was still dominated by *Bacillaceae* (48–54.3%), followed by *Paenibacillaceae* (8.8–10.8%), *Nocardiopsaceae* (4–10.1%), *Planococcaceae* (5.3–6.1%), and *Xanthomonadaceae* (3.9–5.7%) (Figure 4). In addition, all NFR feed samples contained seven low-abundance families (0.4–2.3%): *Pseudonocardiaceae*, *Streptomycetaceae*, *Leuconostocaceae*, *Staphylococcaceae*, *Streptosporangiaceae*, *Micromonosporaceae*, and *Thermomonosporaceae* (Supplementary Table S1). Whereas *Bacillaceae* were abundant in all diet samples, they were minor components in the gut samples (2.7–4.7% in NFR and just ~0.07% in BAD gut samples). *Ruminococcaceae* was the predominant family (40.8–50.3%) in all BAD gut samples, although it was not present in the BAD feed. Some members of the *Ruminococcaceae* were also present at a lower relative abundance in all NFR feed (0.04–0.16%) and NFR gut samples (0.6–8.4%), thus showing remarkable enrichment in the guts of larvae reared on the BAD diet ($p < 0.0001$). *Lachnospiraceae*, *Enterococcaceae*, *Rhizobiaceae*, and *Enterobacteriaceae* were core families in all gut samples, regardless of the diet. However, members of the first two families were significantly enriched in BAD gut samples ($p < 0.0002$ and $p = 0.001$), whereas family *Rhizobiaceae* was less abundant in BAD compared to NFR gut samples ($p < 0.0002$). *Enterobacteriaceae* was the predominant family (39.7–68.4%) in all replicate NFR gut samples (Figure 4). Most of the taxa identified in the BAD gut samples were not found in the feed or their abundance was low. The family *Chitinophagaceae* (0.4–1.7%) was exclusively detected in the NFR gut samples, whereas *Beutenbergiaceae* (2.1–3.9%) and *Christensenellaceae*

(1.3–2.7%) were only found in BAD gut samples (Supplementary Table S1). Interestingly, the family *Actinomycetaceae* was identified in all replicate gut samples of both diets, but was completely absent in the corresponding feed and frass samples. However, the relative abundance was significantly higher in the BAD gut samples ($p < 0.0002$).

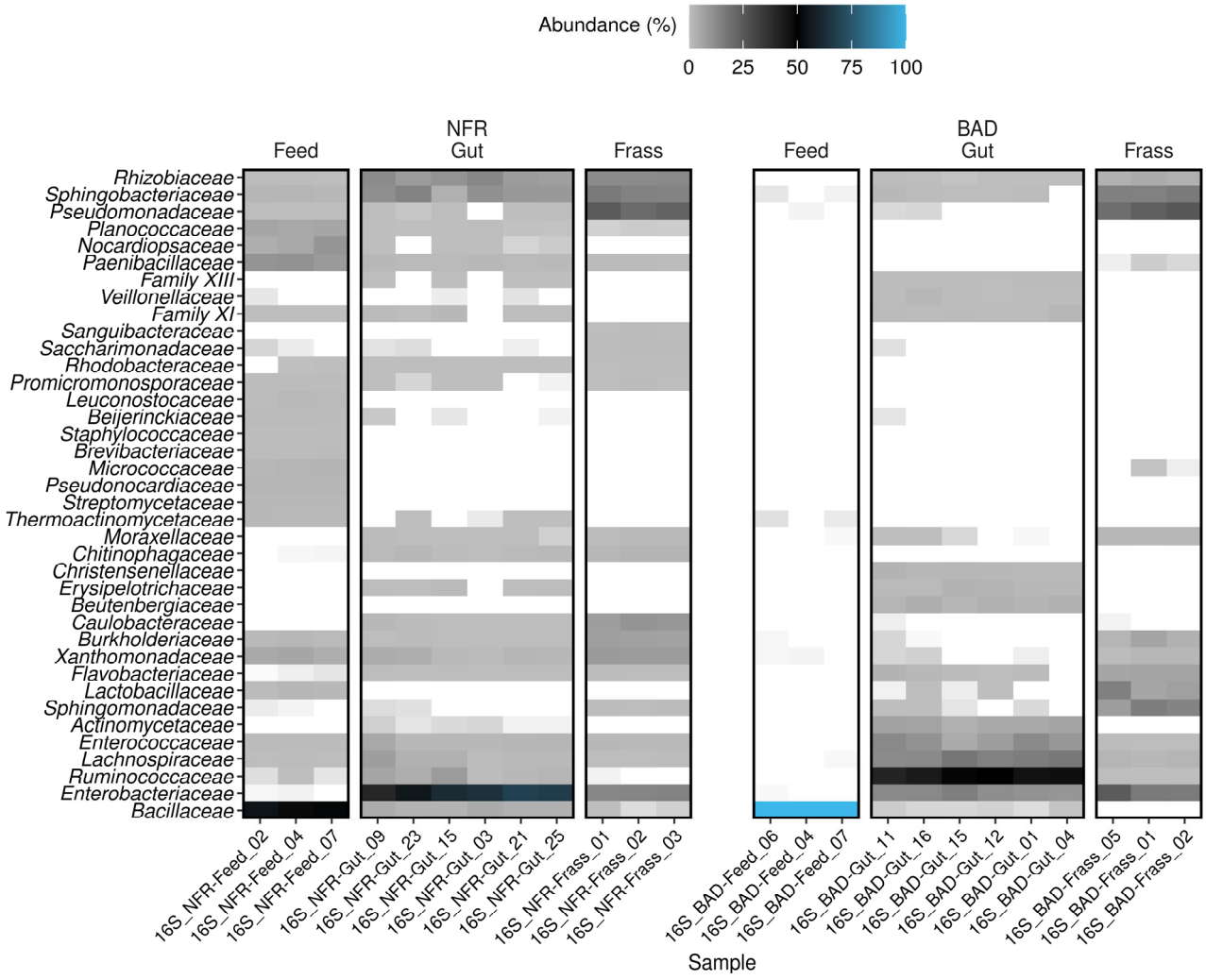

Figure 4. Heat map showing the family-level composition of the bacterial community characterized by amplicon sequencing of the 16S rRNA gene. Samples (feed, gut, and frass) of the non-fermented reference (NFR) and *B. adusta* fermented diet (BAD) groups are shown. Only the 38 most abundant classified families are listed. Families without a suitable classification (uncultivated, undefined, and not applicable) are excluded. The relative abundance (%) is shown as a color gradient that runs from light gray through black to blue, the latter representing the highest abundance. White areas indicate that the family was not detected in the sample.

We also found striking differences between the two diet groups at the genus level. *Klebsiella* was the most prominent genus in all NFR gut samples (31.7–64.8%), but the relative abundance fell to <3% in five of the six replicate BAD gut samples. Instead, *Ruminococcaceae* UCG-014, *Ruminiclostridium* 5, and *Enterococcus* were the dominant genera in all six guts of larvae reared on the BAD diet, with a cumulative abundance of ≥50% (Supplementary Figure S1, Supplementary Table S1). The majority of bacterial families in BSF larval guts could also be found in frass samples, albeit with marked differences in abundance. However, *Sanguibacteraceae* was only detected in the NFR frass and was completely absent from gut samples. The bacterial community in all BAD frass samples was dominated by the *Pseudomonadaceae* (20–25.8%), *Enterobacteriaceae* (16.6–24.9%),

Sphingobacteriaceae (14.9–16.8%), *Sphingomonadaceae* (8.1–16%), *Lactobacillaceae* (5.8–15.1%), *Flavobacteriaceae* (5.7–6.4%), *Burkholderiaceae* (2.2–6.2%), and *Rhizobiaceae* (almost all *Ochrobactrum*; 3.3–4.6%), whereas NFR frass was dominated by *Pseudomonadaceae* (20.1–24.4%), *Sphingobacteriaceae* (14.1–16.6%), *Enterobacteriaceae* (13.3–14.1%), *Rhizobiaceae* (12–12.8%), *Caulobacteriaceae* (7.8–10%), *Xanthomonadaceae* (8.1–8.7%), and *Burkholderiaceae* (6.6–7.5%). The frass samples of both diets showed a common intersection of predominantly represented families, albeit with pronounced differences in relative abundance. The most prominent families in the BAD frass were either absent from the corresponding feed or present at very low abundance in one or two replicate feed samples (0.009–0.038%). In contrast, the most prominent families in the NFR frass were also detected in the feed, with *Caulobacteriaceae* as the major exception (Supplementary Figure S1, Supplementary Table S1). All NFR feed samples and most gut samples showed higher bacterial alpha diversity than corresponding samples from the BAD group. BAD feed showed the lowest alpha diversity as determined by Faith's phylogenetic diversity and the observed ASVs (Figure 2 top row, Figure 3A,B). A PERMANOVA revealed significant differences between the diet groups in terms of the beta diversity of the bacterial gut community (F_{pseudo} = 18.00; p_{adjust} = 0.002).

3.4. Taxonomic Composition of the Fungal Gut Microbiome

The same samples discussed above were also used for ITS sequencing to compare the composition of the fungal community in the BAD and NFR groups. The 24 samples yielded 570,372 raw reads, which we reduced to 530,816 after quality control and trimming (12,846–43,666 per sample). The read length was 186–273 bp. Rarefaction curves are shown in Figure 2. We identified 167 unique ASVs across all 24 samples using DADA2. With a minimum confidence of 0.94 for our self-trained naïve Bayes classifier based on UNITE 8.2, we were able to classify 142 of those 167 unique ASVs to the genus level.

As discussed above for the bacterial community, PCoA indicated pronounced differences in the mycobiome between the BAD and NFR feed samples (Figure 5). Weighted UniFrac distances suggested high similarity between gut and frass samples from the NFR group and frass samples from the BAD group, whereas the unweighted PCoA showed greater differences between these groups (Figure 5A,B). Individual gut samples from both diet groups clustered closer together in the weighted UniFrac model than in the unweighted model (Figure 5A). Interestingly, PCoA of unweighted UniFrac distances revealed substantial interindividual differences between BAD feed samples (Figure 5B).

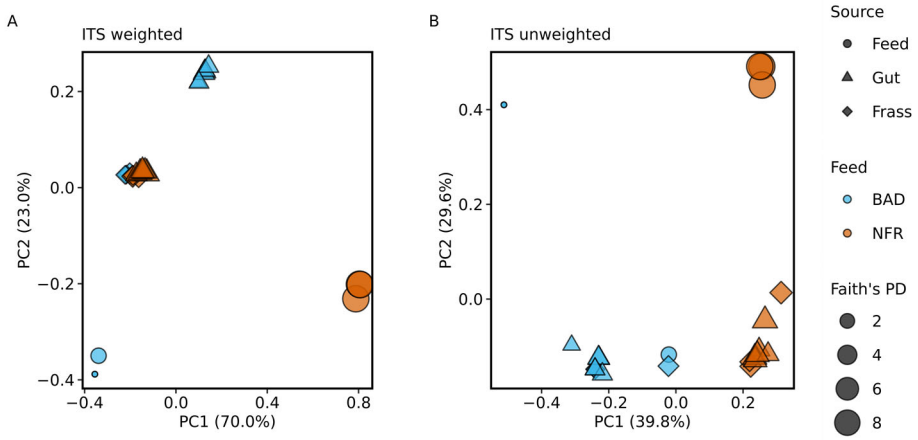

Figure 5. Principal coordinates analysis (PCoA) when comparing the mycobiome between the non-fermented reference (NFR) and *B. adusta* fermented diet (BAD) groups. (**A**,**B**) PCoA comparing the fungal communities based on weighted and unweighted UniFrac distance metrics. The sample type is represented by symbols (feed, gut, and frass) and the dietary group affiliation by colors. The symbol size represents Faith's phylogenetic diversity (PD).

Fungi were detected in all samples of both diet groups. The BAD feed almost exclusively featured *Phanerochaetaceae* (all *B. adusta*) with a relative abundance of 93.1–100% (100% in two of three replicates). In one BAD feed replicate *Trichosporonaceae* were detected with a relative abundance of 5.6%. As observed for the bacterial community, the diversity of the fungal community was higher in NFR feed samples. This was anticipated because the feed was inoculated solely with *B. adusta* after autoclaving. The NFR feed was dominated by ascomycete families: *Saccharomycetales* family *incertae sedis* (mostly *Candida tropicalis*; 53.9–60.8%), *Aspergillaceae* (mostly *Xeromyces*; 14.5–21.8%), and *Trichocomaceae* (mostly *Thermomyces*; 9.3–11.2%) (Figure 6, Supplementary Figure S2). All NFR feed samples also contained six low-abundance (0.04–1.2%) families: *Debaryomycetaceae*, *Wallemiaceae*, *Agaricaceae*, *Saccharomycopsidaceae*, *Rhizopodaceae*, and *Cladosporiaceae*. The families *Chaetomiaceae*, *Sordariaceae*, and *Saccharomycetaceae* were found in all NFR feed samples (0.1–1.1%) and in at least one frass sample, but were completely absent from the guts of larvae reared on the NFR diet. The members of all predominant families in samples of both feeds were only weakly detected in the corresponding gut samples (0.3–3.1%) and frass samples (0.1–3.1%; *Aspergillaceae* was identified in two of three replicates) (Supplementary Table S2).

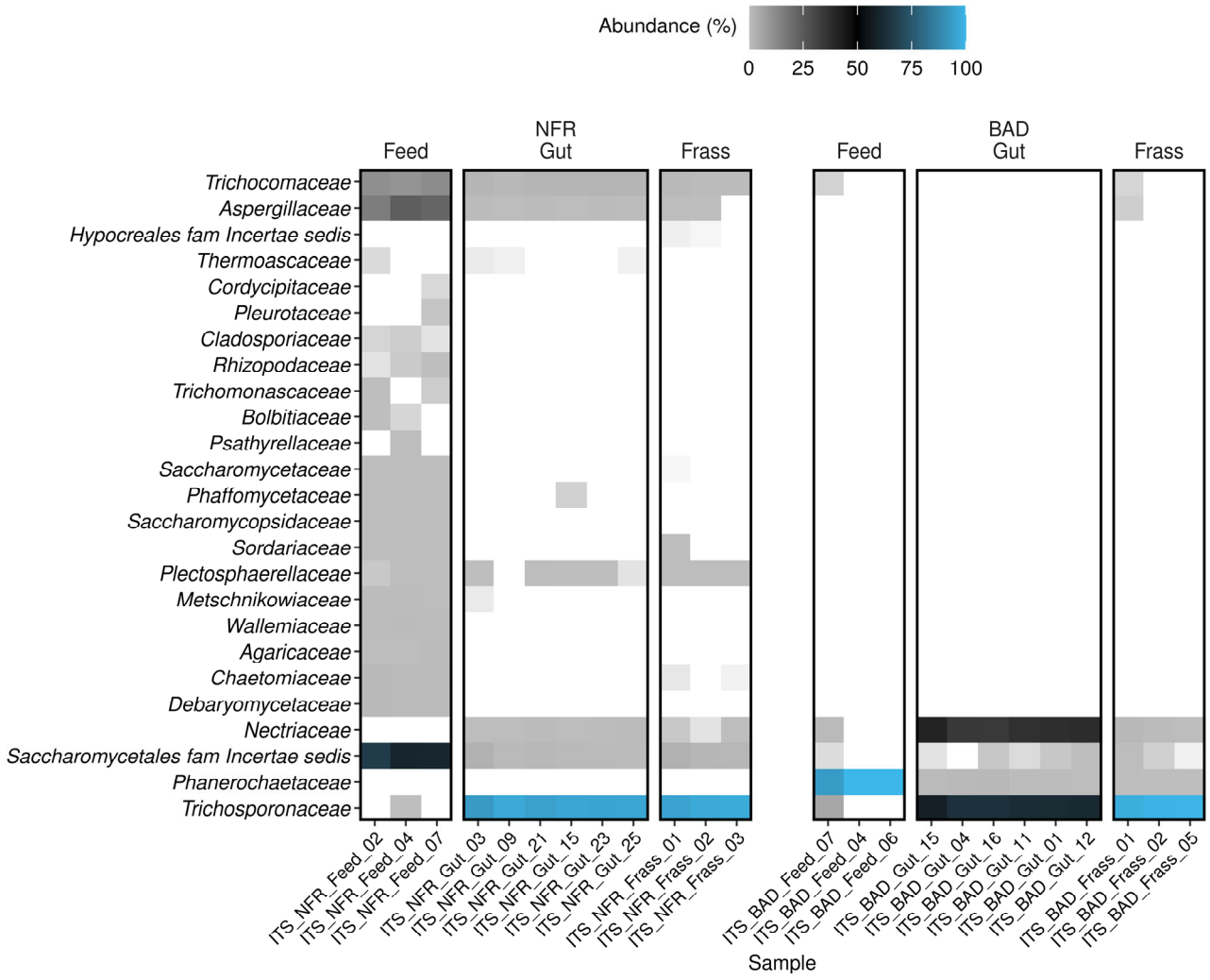

Figure 6. Heat map showing the family-level composition of the fungal community characterized by ITS amplicon sequencing. Samples (feed, gut, and frass) of the non-fermented reference (NFR) and *B. adusta* fermented diet (BAD) groups are shown. All 25 classified families are listed. Families without a suitable classification (uncultivated, undefined, and not applicable) are excluded. The

relative abundance (%) is shown as a color gradient that runs from light gray through black to blue, the latter representing the highest abundance. White areas indicate that the family was not detected in the sample.

Trichosporonaceae and *Nectriaceae* were identified in all gut samples, regardless of the diet. A striking enrichment of the first family (entirely *Trichosporon asahii*) in all guts of larvae reared on the NFR (91.6–95.6%) and BAD (57.9–63.8%) diets was observed, albeit with diet-dependent significant differences in the relative abundance ($p < 0.0001$). Interestingly, *Trichosporonaceae* was only weakly detected in a single replicate of NFR feed (0.16%). In contrast to the NFR diet, the *Nectriaceae* (almost exclusively *Fusarium*) represented the second most prominent family (34.6–41.2%; $p < 0.0001$) in the BAD gut samples, although they were only found in one of three feed replicates (~1.1%). However, *Nectriaceae* was also detected with low abundance in all NFR frass samples but was completely absent in the corresponding feed. The majority of taxa detected in larval guts from the BAD and NFR groups were present in at least one of the feed samples in an inverse relative abundance (Figure 6, Supplementary Table S2). *Trichocomaceae* (1.5–2.3%), *Aspergillaceae* (0.4–0.8%), and *Plectosphaerellaceae* (0.04–0.3% in five replicates) were exclusively detected in the guts of larvae reared on the NFR diet, whereas the family *Phanerochaetaceae* (0.3–1.6%) was only found in guts of larvae reared on the BAD diet. Most fungal families present in the larval guts were also found in frass samples, albeit with marked differences in abundance. The fungal community in all NFR frass samples was dominated by members of the *Trichosporonaceae* (94.4–96.6%), followed by *Saccharomycetales* family *incertae sedis* (mostly *Candida*; 1.6–3.1%), *Trichocomaceae* (all *Thermomyces*; 0.7–1.2%), *Plectosphaerellaceae* (all *Chordomyces*; 0.2–0.3%), and *Aspergillaceae* (~0.2% in two replicates), whereas BAD frass samples contained *Trichosporonaceae* (97.8–99.5%), *Nectriaceae* (0.3–1.5%), *Phanerochaetaceae* (0.1–0.3%), and *Saccharomycetales* family *incertae sedis* (*Diutina* and *Candida*; 0.02–0.3%). The frass samples of both diets showed a common intersection of predominantly represented families, albeit with pronounced differences in their relative abundance ($p = 0.032$). The larval consumption of BAD feed strongly reduced the relative abundance of the fermentation strain *B. adusta*. The most prominent families in the BAD frass were identified with comparable abundance in one replicate feed sample (except for *Phanerochaetaceae* and *Trichosporonaceae*), whereas the most prominent families in the NFR frass, apart from *Trichosporonaceae*, were detected with a higher abundance in all corresponding feed replicates (Supplementary Figure S2, Supplementary Table S2). When larvae were reared on the NFR diet, almost all gut and frass samples showed higher fungal alpha diversity compared to the BAD diet. Faith's phylogenetic diversity and the observed ASVs suggested the highest alpha diversity for NFR feed (Figure 2 bottom row, Figure 5A,B). A PERMANOVA revealed significant differences between the diet groups in the beta diversity of the fungal gut community ($F_{pseudo} = 35.17$; $p_{adjust} = 0.001$).

4. Discussion

4.1. Rationale for the Pretreatment of Palm Oil Side Streams

BSF larvae do not naturally specialize on lignocellulose degradation in the same manner as termites and other xylophagous species. Lignin in the BSF diet is predominantly metabolized by fungi, which secrete extracellular laccases and peroxidases for this purpose, although there is evidence that some bacteria also facilitate lignocellulose utilization. Nevertheless, the efficiency and capacity of fungi appears higher, as indicated by the symbiosis of several fungus-growing termite (subfamily Macrotermitinae) and ant (tribe Attini) species [49]. Termites that do not cultivate fungi promote the digestion of lignocellulose by deploying mechanical processes, such as initial grinding of plant material with their mandibles and subsequent ball-milling in the gizzard to facilitate chemical and enzymatic digestion [50,51]. Endogenous enzymes such as phenol-oxidizing laccases from the salivary glands, as described for *Reticulitermes flavipes* (Kollar, 1837; Blattodea: Rhinotermitidae), can modify lignocelluloses and increase accessibility for glycoside hydrolases [52]. The chem-

ical oxidation of lignin by hydroxyl radicals originating from the Fenton reaction is also possible in the foregut. In addition, the alkaline pH of the anterior hindgut compartment promotes autoxidation, which cleaves lignin-carbohydrate complexes [51]. Comparable physiological adaptations are not present in BSF larvae. Therefore, the lignocellulose-rich diet was pretreated by fermentation with a lignin-degrading fungus and we investigated its effects on BSF development and the microbiome of the feed, gut and frass.

4.2. Pretreatment by Fermentation Influences BSF Life-History Traits

This is the first study exploring the growth and development of insects reared on fermented side streams from the palm oil industry. We were able to rear BSF larvae on non-fermented and fermented diets consisting of the palm oil side streams EFB and PKM. Both diets seemed to be sufficient for the full development of flies at the laboratory scale, albeit with significant temporal and physiological differences. Fermentation with the white-rot fungus *B. adusta*, resulting in the BAD diet, promoted faster larval development and thus achieved a significantly shorter life cycle, which would allow earlier harvesting in an industrial process. In addition, L5 larvae fed on the BAD diet reached a 25% higher final larval weight. Similar weight gain (~30%) and developmental acceleration (up to 10%) were reported when inoculating poultry manure with companion *Bacillus subtilis* [15]. In addition, feed supplemented with BSF-associated *Bacillus licheniformis* HI169 improved the growth rate and final larval weight compared to untreated controls [53]. Our observations may reflect both the direct and indirect effects of pretreatment. White-rot fungi are suitable for the degradation of agro-industrial side streams because they are naturally adapted to degrade plant material rich in lignocelluloses by secreting exoenzymes, enabling their saprophytic lifestyle [54,55]. The enrichment of fungal biomass and depolymerized lignocellulose components may have increased the digestibility of the feed and hence nutrient accessibility, explaining the positive effect on the development and reproduction of adult flies. On the other hand, we were able to identify a clear shift in the bacterial and fungal gut community associated with fermentation. Although the relative abundance of *B. adusta* was almost 100% in the BAD feed, this species was clearly unable to colonize the gut of the BSF larvae. Instead, it was outcompeted by other taxa, as discussed later. However, *B. adusta* may have broken up rough structural motifs of the lignocellulose in the feed and created a suitable medium for colonization by cellulose-degrading microbes forming the gut microbiome, supporting mutualistic interactions [56–58]. In addition to the provision of metabolites or protection against pathogens, symbionts intervene in the feed repertoire of insects to facilitate the utilization of nutritionally unfavorable substrates [59]. Cooperation with bacteria, filamentous fungi, yeast and protozoa contributes to the digestion of plant biomass in phytophagous insects, strongly influencing their development [60,61]. Such cooperation takes many forms, including obligatory intracellular relationships in aphids and other sap-feeding insects, gut-associated communities in beetles and wood bees, and specialized dependent relationships in fungus-cultivating termites, such as *Macrotermes annandalei* (Silvestri, 1914; Blattodea: Termitidae), with an extra-gastrointestinal mycobiome [59,62].

BSF prepupae do not feed, so the fat body acquired during larval development normally serves as an energy reserve, influencing metamorphosis and adult life-history traits [28,63]. Accordingly, low energy reserves in the NFR larvae may explain the negative effect on reproduction and low fertility, including lower egg clutch size and weight compared to the BAD diet. As an alternative to the fat body, females may also reabsorb oocytes and metabolize them to maintain respiration, which would reduce the egg clutch size [64]. Differences in reproductive success may also reflect microbe–insect interactions. In BSF, oviposition is mediated by bacteria in a conspecific manner [65]. Other dipteran species, including *Aedes aegypti* (Linnaeus, 1762; Diptera: Culicidae), also recognize specific bacterial compositions, indicating appropriate conditions for egg development and deposition [66]. *Bacillus* species isolated from BSF eggs reduced the ovipositional response of adult females by more than 50% when added to artificial egg traps [65]. *Bacillus* was an

abundant genus in both diets: 28.5–33% (NFR) and ≥99.8% (BAD). However, there were pronounced differences between the *Bacillus* species in the BAD and NFR groups. We found that ~68% of the reads in the BAD feed represented *B. coagulans*, whereas the NFR feed contained a more even distribution of *Bacillus* species, with *B. clausii* predominant at ~6% relative abundance (also found in all corresponding gut samples). It is possible that some of these species inhibited oviposition in the NFR group, resulting in fewer egg clutches. Moreover, the microbial community of the NFR feed probably features a higher bacterial load than the BAD feed because only the latter was autoclaved. Gut microbes are known to influence the behavior of several insects. In *Drosophila* species (Diptera: Drosophilidae), food-induced biases in adult mating behavior were prevented by antibiotic supplements, presumably by inhibiting the growth of sex pheromone-producing bacteria like *Lactobacillus plantarum* [67]. Such microbially-regulated hormonal changes may also affect BSF mating. It is not clear to what extent fungi play a role in this context. However, fungi can also modify insect host behavior, as shown by various entomopathogenic members of the order Entomophthorales [68].

4.3. Analysis of the Bacterial Communities Associated with BSF

Amplicon sequencing revealed major differences in the microbial communities of the NFR and BAD samples, probably reflecting the pretreatment of the feed. However, we also identified a potential BSF larval core microbiome present in all replicates of both diet groups, including the families *Enterobacteriaceae*, *Enterococcaceae*, *Actinomycetaceae*, *Lachnospiraceae*, and *Ruminococcaceae*.

Enterobacteriaceae, *Enterococcaceae* and *Actinomycetaceae* have already been recognized as core families in previous studies [7,69] and are prominent members in the guts of BSF larvae reared on chicken feed [7]. Furthermore, representatives of the families *Lachnospiraceae* and *Ruminococcaceae* are frequently found in BSF larval guts [9,69], although not in all samples, and the relative abundance of the *Ruminococcaceae* was low [7]. Our study is the first to demonstrate the significant enrichment of *Ruminococcaceae* (44–50% relative abundance) in the guts of BSF larvae due to dietary adaptations. Furthermore, *Lachnospiraceae* and *Actinomycetaceae* were significantly enriched in larvae reared on the BAD diet, indicating that pretreatment favored these groups. *Lachnospiraceae* and *Ruminococcaceae* are obligate anaerobic gut-associated families known for their ability to break down cellulose [70,71]. Therefore, whereas the NFR larval gut was dominated by the facultatively anaerobic *Enterobacteriaceae*, typically one of the most prominent families in BSF larvae [7,10,69,72], fermentation with *B. adusta* clearly resulted in a shift towards obligate anaerobic and cellulolytic bacteria.

Only a single draft genome sequence is available for *B. adusta* (IMG genome ID 2761201615), so we cannot fully evaluate its role in cellulose degradation. However, *B. adusta* is known to degrade gelatin, chitin, starch, pectin, tributyrin, and carboxymethyl-cellulose, but not fibrous cellulose, such as filter paper [73]. Therefore, we can assume that lignin, but not fibrous cellulose, was degraded during the pretreatment of EFB-PKM. The degradation of fiber-rich material by bacteria is largely restricted to biomass with a low lignin content [74], so lignin degradation by *B. adusta* probably facilitated subsequent anaerobic degradation of the remaining fibrous structures by cellulolytic bacteria in the gut. Once lignin is degraded, bacteria can attack cellulose and hemicellulose with multiple carbohydrate-active enzymes (CAZymes). Endoglucanases cleave cellulose chains internally, mainly from the amorphous region, and the released units are further degraded by exoglucanases (cellobiohydrolases) and β-glucosidases. Exoglucanases cleave cellobiose from the end of the polysaccharide chains, and these are subsequently hydrolyzed by β-glucosidases to produce glucose monomers [75]. Endoglucanase and β-glucosidase genes are more abundant in the taxa that were positively correlated with the BAD group, supporting the shift toward a cellulolytic community. Members of the families *Ruminococcaceae* (e.g., *Ruminiclostridium* and *Ruminococcaceae* UCG-014) and *Lachnospiraceae* (e.g., *Anaerocolumna*, *Anaerosporobacter*, and *Lachnoclostridium*) contained the largest complement of cellulose

degradation genes (Figure 7). The genera *Actinomyces* and *Salana* as well as the [*Eubacterium*] *fissicatena* group were also positively correlated with the larvae reared on the BAD diet and contained a large set of β-glucosidase genes, but none encoding endoglucanases (Supplementary Table S3).

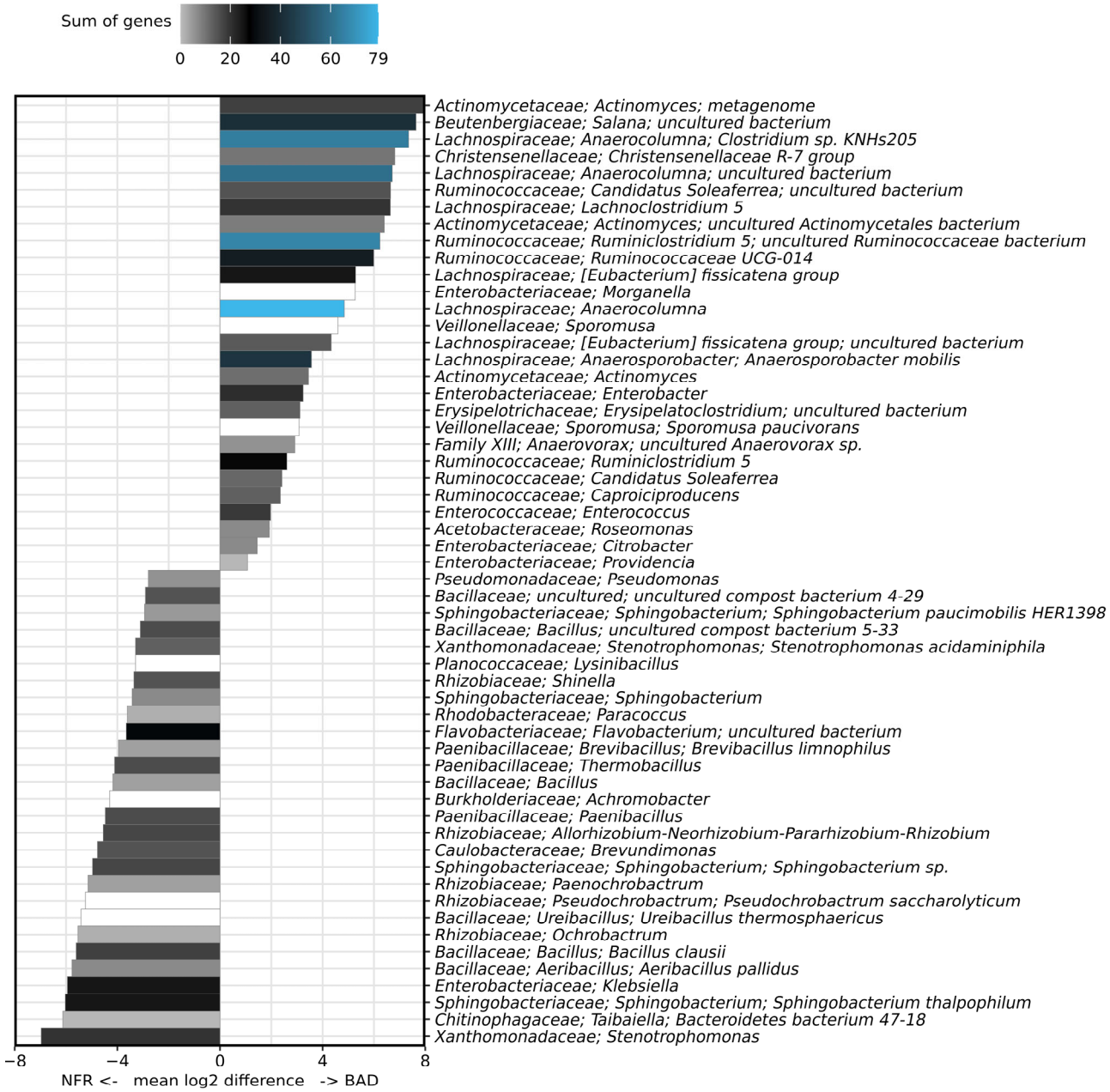

Figure 7. Sum of genes involved in cellulose degradation (endoglucanases EC 3.2.1.4 and β-glucosidases EC 3.2.1.21) found in the genomes of representatives of the 28 most positively and 28 most negatively correlated taxa (5 genomes per taxon) present in BSF larval guts based on the discrete false-discovery rate (DS-FDR). No genes for exoglucanases (EC: 3.2.1.91) were found. For better illustration, the raw data (Table S3) were transformed by multiplying all values by −1.

Fungal fermentation not only led to lignin degradation and more accessible cellulose in the feed, but probably also lowered the redox potential and created anaerobic conditions. The ingested anaerobic feed substrate may have therefore promoted colonization by obligate anaerobes of the families *Lachnospiraceae* and *Ruminococcaceae*, which require negative redox potential for energy metabolism in the gut [76].

The families *Rhizobiaceae*, *Sphingobacteriaceae*, *Bacillaceae*, and *Paenibacillaceae* were positively correlated with larvae in the NFR group. BSF larvae may acquire members of these families during feeding, given their presence in all replicate NFR feed samples. Although these families primarily feature soil/plant-associated species [77–79] the physiochemical conditions in the BSF larval gut nevertheless appear suitable. In contrast, soil/plant-associated microbes were rare in the BAD samples, which was anticipated because the EFB-PKM diet was autoclaved before fermentation. The BAD diet is probably not the major source of bacterial inoculation. Instead, *Ruminococcaceae* and *Lachnospiraceae* may be taken up from the eggs or the rearing environment. For example, *Lachnospiraceae* have been detected in egg samples with a relative abundance of ~11% [72]. The families *Lachnospiraceae* and *Ruminococcaceae* feature many spore-forming genera [80,81] and were present in all frass samples in this study and in a previous study [7], suggesting they survive on the pupal surface and are subsequently associated with the flies and their eggs.

Lachnospiraceae and *Ruminococcaceae* species produce hydrogen during the anaerobic breakdown of cellulose and associated fermentation processes [82]. Such conditions would support colonization by hydrogenotrophic methanogenic archaea, as previously reported in termites and cockroaches [83], but none of the ASVs was assigned to the domain Archaea. In previous studies, methanogenic archaea have been shown to be absent from BSF larval guts [7], present at a low relative abundance [12], or present at a high relative abundance [14]. Other studies have shown that methane emission from BSF farms is generally low and can vary between locations and feeding strategies [84–86]. Therefore, the growth of methanogenic archaea in the BSF larval gut and frass should be investigated in more detail when new feed sources are tested. Our data suggest that BSF larvae reared on NFR or BAD diets do not produce methane, which would be climate-friendly in the context of large-scale insect farming.

4.4. Analysis of the Fungal Communities Associated with BSF

Whereas the bacterial community in BSF larvae has been studied intensively over the last decade, the composition and function of the fungal community has been largely overlooked. We found that *Trichosporon asahii* (*Trichosporonaceae*) was dominant in all gut and frass samples regardless of the diet, in agreement with earlier reports [7,8], indicating that this yeast is closely associated with BSF larvae and is probably a core species. However, although the fungal microbiome in the NFR group was almost exclusively composed of *T. asahii* (92–96% relative abundance), the BAD group was additionally colonized with *Fusarium* species (35–41%), which were not detected in the NFR group nor in BSF larvae reared on chicken feed [7]. As discussed for the bacterial community, cellulose-degrading fungi may be favored by the fermented diet. Although the genus *Fusarium* includes several entomopathogenic species [87], larval growth performance was clearly not affected by the presence of *Fusarium* (not assigned to species level). And given that some *Fusarium* species can break down cellulose [88,89], the presence of these fungi may even boost growth performance by promoting more efficient digestion of the feed.

When new feed substrates are introduced, they must be evaluated for potentially hazardous microorganisms such as mycotoxin-producing fungi. The family *Aspergillaceae*, which produces aflatoxins and gliotoxins [90], was present at a high relative abundance in the NFR diet, but not in the guts of larvae in the NFR group. BSF does not accumulate aflatoxin B1 [91], but the monitoring of farmed BSF larvae for the accumulation of other mycotoxins would be a wise precaution. The same applies to *Fusarium* species, which synthesize a wide variety of mycotoxins [92] and were relatively abundant (>34%) in all larvae in the BAD group. *Fusarium* species are known to produce trichothecenes, such as deoxynivalenol, although species-dependent and strain-specific variations in metabolic profiles have already been described [93].

5. Conclusions

The palm oil industry produces millions of tons of waste biomass annually, which is usually incinerated [94]. Therefore, the bioconversion of these residues into insect protein and lipids can contribute to an environmentally sustainable circular economy with high added value.

We have shown that BSF larvae can be reared on palm oil side streams. The pretreatment of the EFB-PKM mixture by fermentation with *B. adusta* accelerated development and simultaneously enhanced larval weight compared to the untreated control diet. These observations can be explained by the increase in digestibility through the depolymerization of lignocellulose, the accumulation of fungal mycelia as a potential nutrient source, and the shift to a predominantly anaerobic microbial gut community which subsequently degrades cellulose. Our protocol for the fermentation of EFB and PKM therefore represents a promising approach for the efficient bioconversion of high-fiber side streams by BSF larvae, allowing the production of valuable insect protein that reduces environmental pollution.

6. Patents

Klüber P, Zorn H, Rühl M, Bakonyi D, Pfeiffer J, and Vilcinskas A. Process for the production of an insect substrate, insect substrate and uses thereof. Application number EP22152265.9.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/xxx/s1, Figure S1: Heat map showing the genus-level composition of the bacterial community characterized by amplicon sequencing of the 16S rRNA gene. Samples (feed, gut, and frass) of the non-fermented reference (NFR) and *B. adusta* fermented diet (BAD) groups are shown. Only the 38 most abundant classified genera are listed. Genera without a suitable classification (uncultivated, undefined, and not applicable) are excluded. The relative abundance (%) is shown as a color gradient that runs from light gray through black to blue, the latter representing the highest abundance. White areas indicate that the genus was not detected in the sample; Figure S2: Heat map showing the genus-level composition of the fungal community characterized by ITS amplicon sequencing. Samples (feed, gut, and frass) of the non-fermented reference (NFR) and *B. adusta* fermented diet (BAD) groups are shown. Only the 38 most abundant genera are listed. Genera without a suitable classification (uncultivated, undefined, and not applicable) are excluded. The relative abundance (%) is shown as a color gradient that runs from light gray through black to blue, the latter representing the highest abundance. White areas indicate that the genus was not detected in the sample; Table S1: Interactive spreadsheet showing the detailed composition of the bacterial community in the feed, BSF larval gut, and frass when larvae were reared on a non-fermented reference (NFR) diet or a *B. adusta* fermented (BAD) diet. Data are based on 16S rRNA gene sequencing and show the relative abundances of ASV counts at different taxonomic levels. The categories on the left allow switching between different taxonomic levels (2 = Phylum; 3 = Class; 4 = Order; 5 = Family; 6 = Genus; and 7 = Species); Table S2: Interactive spreadsheet showing the detailed composition of the fungal community composition in the feed, BSF larval guts, and frass when larvae were reared on a non-fermented reference (NFR) diet or a *B. adusta* fermented (BAD) diet. Data are based on ITS sequencing and show the relative abundances of ASV counts at different taxonomic levels. The categories on the left allow switching between different taxonomic levels (2 = Phylum; 3 = Class; 4 = Order; 5 = Family; 6 = Genus; and 7 = Species); Table S3: Number of genes involved in cellulose degradation (endoglucanases EC 3.2.1.4 and β-glucosidases EC 3.2.1.21) in the genomes of representatives of the 28 most positively and 28 most negatively correlated taxa found in BSF larval guts based on the discrete false-discovery rate (DS-FDR). Only genomes of representatives which show ≥91% sequence identity to the target sequence are included. Taxa that are not classified up to the family level are excluded.

Author Contributions: Conceptualization, P.K., H.Z., M.R. and D.T.; methodology, P.K., D.T. and J.P.; software, P.K., S.H. and D.T.; validation, P.K., D.T., S.H., H.Z. and M.R.; formal analysis, P.K. and D.T.; investigation, P.K., D.T. and J.P.; resources, H.Z., M.R. and A.V.; data curation, P.K., D.T. and S.H.; writing—original draft preparation, P.K., D.T. and S.H.; writing—review and editing, P.K., D.T., M.R. and H.Z.; visualization, P.K. and S.H.; supervision, P.K.; project administration, P.K.;

funding acquisition, H.Z., M.R. and A.V. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Hessian Ministry of Higher Education, Research and the Arts via the LOEWE Center for Insect Biotechnology, and by PT Alternative Protein Indonesia. We acknowledge access to resources financially supported by the BMBF grant FKZ 031A533 within the de.NBI network. The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Raw data for 16S rRNA gene and ITS amplicon sequencing are available via NCBI (accession number PRJNA723723).

Acknowledgments: We thank Bio S. Biogas for providing BSF larvae. We also thank Karina Brinkrolf (Justus Liebig University Giessen, Germany) for helpful suggestions on the experimental design, Stefan Janssen (Justus Liebig University Giessen, Germany) for valuable discussions about amplicon data analysis, Christopher Back for milling empty fruit bunches and screening fungal strains, and Richard M. Twyman for manuscript editing.

Conflicts of Interest: The authors declare no conflict of interest.

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