Dynamics and Cultivation of Gut Microbiota in *Hermetia illucens* Larvae

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Gießen, Hessen — Yina Alejandra Cifuentes Triana

“Experience is the name everyone gives to their mistakes.”

Lady Windermere's Fan (1892) act 3, Oscar Wilde

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Hypotheses

Hypothesis 1: *Hermetia illucens* larvae possess a specific and stable core gut microbiome that remains consistent across different developmental stages.

Hypothesis 2: The bacterial community composition within the feed residue of *H. illucens* larvae undergoes significant changes during the rearing process.

Hypothesis 3: Rearing *H. illucens* can contribute to a reduction of potentially pathogenic bacteria in the feed substrate.

Hypothesis 4: Within the stable core gut microbiome of *H. illucens* larvae, there exists a higher degree of strain-level diversity than previously recognized.

Hypothesis 5: Diverse cultivation strategies, especially those incorporating a dilution-to-extinction approach, enhance the genetic diversity of cultured bacteria isolated from the gut of *H. illucens* larvae.

Summary

Understanding the intricate interplay between *Hermetia illucens* larvae, and their gut microbiome is crucial for using their potential in applications such as waste management and animal feed production. This study investigated the composition and dynamics of bacterial communities within the larval gut and the feed residue during *H. illucens* larvae rearing. It was found that *H. illucens* larvae gut harbors a consistent and stable core microbiome, composed mainly of specific bacterial genera, including *Dysgonomonas*, *Morganella*, *Enterococcus*, *Providencia*, *Klebsiella*, and others. This potential core microbiome exhibits a high degree of strain-level diversity, suggesting a complex ecosystem within the larval gut that may contribute to the adaptability of *H. illucens* larvae to varying diets.

In contrast to the stable gut microbiome, the bacterial community in the feed residue undergoes significant alterations during rearing. This is likely due to the feeding and digestion processes of the larvae, which modify the composition of the residue, leading to a decrease in the abundance of certain bacterial taxa and an increase in others. Remarkably, a significant decrease was observed in the abundance of potential pathogens in the feed residue as rearing progressed. This indicates that *H. illucens* larvae rearing can contribute to a reduction in harmful bacteria within processed organic waste.

To further elucidate the interplay between the larval gut microbiome and the feed residue, this study also assessed the presence and prevalence of antibiotic and disinfectant resistance genes within both environments. For instance, the alternative penicillin binding protein coding gene *mecA*, mainly found in *Staphylococcus* species, was highly abundant in the feed residue at the initial stage but decreased significantly in the last stages. This pattern mirrored the relative abundance of *Staphylococcus* in the residue, which was also abundant at the beginning and declined over time. In contrast, the extended-spectrum beta-lactamase gene, bla_{SHV}, was consistently present in the gut microbiome of the *H. illucens* larvae and pupae throughout the rearing process. While bla_{SHV} genes were also detected in the feed residue in the first stages, they were not detected at the final stage.

The quaternary ammonium compound (QAC) resistance genes coding for transmembrane protein (*qacE/qacEΔ1)* were not detected in any of the larval gut samples but were found in the feed residue. Their abundance increased significantly from the initial to the final stage of

rearing. This finding implies that while the larvae themselves may not harbor these disinfectant resistance genes, the feed residue, potentially enriched with these genes, could pose a risk if applied as fertilizer. Despite significant shifts in bacterial community composition during *H. illucens* larvae rearing, the relative abundance of the tetracycline resistance gene (*tetM*) remained stable in both the larval gut and the feed residue. However, the relative abundance of the sulfonamide resistance gene (*sul2*) increased significantly in the feed residue, while remaining stable in the larval gut. The potential for the transfer of these antibiotic and disinfectant resistance genes from the feed residue to the environment, and possibly other organisms, demands more in-depth analysis.

Two different cultivation strategies, a dilution-to-extinction cultivation approach, and direct plating approach, were used to further characterize *H. illucens* larvae gut microbiome. Both methods successfully cultured a diverse array of bacterial species. The dilution-to-extinction approach yielded a total of 341isolates and the direct plating approach 138 isolates. A total of 18 different phylotypes based on 16S rRNA gene sequence analysis were identified. Six phylotypes were found exclusively in the dilution-to-extinction approach: *Pseudomonas*, *Alcaligenes*, *Providencia*, *Serratia*, *Brucella*, and *Micrococcus*. Only the *Mammaliicoccus* phylotype was unique to the direct plating method. Genomic fingerprinting further revealed a high degree of genetic diversity at the strain level within several phylotypes, particularly for *Enterobacteriaceae*, *Providencia*, *Enterococcus*, and *Morganella*. These findings highlight the importance of utilizing diverse cultivation techniques to gain a more comprehensive understanding of the complex microbial diversity present within the *H. illucens* larvae gut.

Zusammenfassung

Das Verständnis der komplexen Wechselwirkungen zwischen *Hermetia illucens*-Larven und ihrem Darmmikrobiom ist entscheidend für die Ausschöpfung ihres Potenzials in Anwendungsbereichen wie Abfallwirtschaft und Tierfutterproduktion. Diese Studie untersuchte die Zusammensetzung und Dynamik der bakteriellen Gemeinschaften im Larvendarm und im Futterrückstand während der Aufzucht von *H. illucens*-Larven. Es wurde festgestellt, dass der Darm von *H. illucens*-Larven ein konsistentes und stabiles Kerndarmmikrobiom beherbergt, das hauptsächlich aus spezifischen Bakteriengattungen wie *Dysgonomonas*, *Morganella, Enterococcus*, *Providencia*, *Klebsiella* und anderen besteht. Das Kerndarmmikrobiom zeichnet sich durch eine hohe Diversität auf Stammebene aus, was auf ein komplexes Ökosystem im Larvendarm hinweist. Dieses könnte einen wesentlichen Beitrag zur Anpassungsfähigkeit der *H. illucens*-Larven an verschiedene Diäten leisten.

Im Gegensatz zu dem stabilen Darmmikrobiom unterliegt die bakterielle Gemeinschaft im Futterrückstand während der Aufzucht erheblichen Veränderungen. Diese Veränderungen sind vermutlich auf die Fütterungs- und Verdauungsprozesse der Larven zurückzuführen, welche die Zusammensetzung des Rückstands beeinflussen und zu einer Abnahme der Häufigkeit bestimmter Bakterienarten sowie einer Zunahme anderer führen. Bemerkenswerterweise wurde ein signifikanter Rückgang der Häufigkeit potenzieller Krankheitserreger im Futterrückstand im Laufe der Aufzucht beobachtet. Dies deutet darauf hin, dass die Aufzucht von *H. illucens*-Larven möglicherweise zur Reduzierung schädlicher Bakterien im verarbeiteten organischen Abfall beitragen könnte.

Um die Wechselwirkungen zwischen dem Larvendarmmikrobiom und dem Futterrückstand weiter zu untersuchen, wurden in dieser Studie auch das Vorhandensein und die Häufigkeit von Antibiotika- und Desinfektionsmittelresistenzgenen in beiden Umgebungen bewertet. Zum Beispiel war das alternative Penicillin-Bindungsprotein-codierende Gen *mecA*, das hauptsächlich in *Staphylococcus* Arten nachgewiesen wird, zu Beginn der Aufzucht im Futterrückstand stark vertreten, nahm jedoch im Laufe der Zeit signifikant ab. Dieses Muster spiegelte sich in der relativen Häufigkeit von *Staphylococcus* im Rückstand wider, die ebenfalls zu Beginn hoch war und im Laufe der Zeit abnahm. Im Gegensatz dazu war das Gen für die

Extended-Spectrum Beta-Laktamase, *bla*$_{SHV}$, im Darmmikrobiom der *H. illucens*-Larven und Puppen während des gesamten Aufzuchtprozesses konstant vorhanden. Während *bla*$_{SHV}$ Gene auch in den initialen Stadien im Futterrückstand nachgewiesen wurden, erfolgte in der Endphase kein Nachweis mehr.

Die Resistenzgene für quaternäre Ammoniumverbindungen (QAV), die für Transmembranproteine (qacE/qacEΔ1) kodieren, wurden in keinem der Larvendarmproben nachgewiesen. Allerdings konnte ein Vorliegen dieser Gene im Futterrückstand bestätigt werden. Ihre Häufigkeit nahm von der Anfangs- zur Endphase der Aufzucht signifikant zu. Diese Erkenntnis deutet darauf hin, dass, obwohl die Larven selbst diese Desinfektionsmittelresistenzgene womöglich nicht beherbergen, der Futterrückstand, der möglicherweise mit diesen Genen angereichert ist, ein Risiko darstellen könnte, wenn er als Dünger verwendet wird.

Trotz signifikanter Veränderungen in der Zusammensetzung der bakteriellen Gemeinschaft während der Aufzucht von *H. illucens*-Larven blieb die relative Häufigkeit des Tetracyclin-Resistenzgens (*tetM*) sowohl im Larvendarm als auch im Futterrückstand stabil. Dagegen jedoch nahm die relative Häufigkeit des Sulfonamid-Resistenzgens (*sul2*) im Futterrückstand signifikant zu, während sie im Larvendarm stabil blieb. Das Potenzial für den Transfer dieser Antibiotika- und Desinfektionsmittelresistenzgene vom Futterrückstand in die Umwelt und möglicherweise auf andere Organismen erfordert eine eingehendere Analyse.

Zur weiteren Charakterisierung des Darmmikrobioms von *H. illucens*-Larven wurden zwei verschiedene Kultivierungsstrategien angewandt: eine Kultivierung durch Verdünnung bis zum Aussterben und eine direkte Plattierung. Die Anwendung beider Methoden führte zur erfolgreichen Kultivierung einer vielfältigen Reihe von Bakterienarten. Im Rahmen des Verdünnungs-zu-Aussterben-Ansatzes wurden insgesamt 341 Isolate generiert, während der Direktplattierungsansatz 138 Isolate ergab. Insgesamt wurden 18 verschiedene Phylotypen basierend auf der 16S rRNA Gen Sequenzanalyse identifiziert. Sechs Phylotypen wurden ausschließlich im Verdünnungs-zu-Aussterben-Ansatz gefunden: *Pseudomonas, Alcaligenes, Providencia, Serratia, Brucella* und *Micrococcus.* Lediglich der Phylotyp *Mammaliicoccus* war einzigartig für die Direktplattierungsmethode. Die Analyse genomischer Fingerabdrücke zeigte weiterhin einen hohen Grad an genetischer Vielfalt auf Stammebene innerhalb mehrerer Phylotypen, insbesondere bei *Enterobacteriaceae, Providencia, Enterococcus* und

Morganella. Diese Ergebnisse heben die Bedeutung der Verwendung vielfältiger Kultivierungstechniken hervor, um ein umfassenderes Verständnis der komplexen mikrobiellen Vielfalt im Darm der *H. illucens*-Larven zu gewinnen.

CHAPTER I

Review chapter: *Hermetia illucens* gut microbiota

Yina Cifuentes

Introduction

Insects, encompassing more than 50% of all described species, exhibit remarkable adaptability across diverse environments, including terrestrial, freshwater, and near-coastal marine habitats, as well as deserts and hot springs (Redak, 2023; Schowalter, 2022). They respond quickly to environmental changes, affecting other species and ecosystem parameters (Schowalter, 2022). Their diversity is reflected in various attributes such as feeding behavior, developmental patterns, ecological roles, habitat preferences, social structures, mouthparts, flight capabilities, coloration, size, lifespan, geographic distribution, and reproductive strategies (Capinera, 2008; Chapman & de Boer, 1995; Schowalter, 2022). This adaptability enables them to inhabit nearly every ecological niche (Schowalter, 2022).

A critical aspect of this adaptability lies in the insect microbiota, the community of microorganisms residing primarily in the gut (Girard et al., 2022; Jones et al., 2013). Scavengers like *H. illucens*, which consume a varied diet of decaying organic matter, exemplify this remarkable adaptability, where the gut microbiota helps digest various organic sources and detoxifies toxic compounds, which efficiently convert into valuable products through a process known as bioconversion (De Smet et al., 2018; Eke et al., 2023; Smetana et al., 2019). This process offers solutions for rising concerns about organic waste management and the need for sustainable protein sources (Bruno et al., 2019; Eke et al., 2023; Spranghers et al., 2017). The ability to thrive on many types of organic waste, including food scraps, agricultural byproducts, and manure, due to the contribution of its gut microbiota, underscores the potential for optimizing mass rearing and bioconversion of *H. illucens* for even large-scale processes (Chavan et al., 2022; Eke et al., 2023).

Microbial communities are strategically dispersed across various anatomical structures of insects, encompassing the exoskeleton, gut, blood cavity, salivary gland, and other organs. Remarkably, they constitute a substantial portion of the insect biomass, ranging from 1% to 10% (Zhao et al., 2022). Their presence and distribution play crucial roles in numerous features of insect biology and physiology, ultimately facilitating essential functions and contributing to the insect overall survival and adaptive capabilities (Douglas, 2015; Zhao et al., 2022).

Despite the well-recognized presence of bacteria in other organs, the gut is the most studied protagonist since bacteria are predominantly present in the digestive tract, where they can act as modulators of the diverse lifestyles of their insect host (Gupta & Nair, 2020). The gut microbiota is well recognized for facilitating feeding even on recalcitrant food and compensating for poor diets by supplying essential amino acids. Consequently, insects become highly dependent on their gut microbiota for survival and development (Douglas, 2015; Engel & Moran, 2013; Gupta & Nair, 2020).

Nutrient digestion and metabolism

The gut microbiota is vital in aiding insects with nutrient digestion and metabolism, especially given the limitations of their intrinsic digestive systems. Many insects lack the specific enzymes needed to break down complex carbohydrates like cellulose and hemicellulose found in plants. They have formed symbiotic relationships with gut bacteria to counteract this, significantly contributing to their nutritional needs and overall physiology (Douglas, 2009; Engel & Moran, 2013).

Insects with cellulose-rich diets also utilize bacterial cellulases to degrade these complex carbohydrates. *H. illucens* larvae illustrate this relationship by efficiently converting various organic substrates into protein-rich and fat-rich supplements, supporting the biosynthesis of polysaccharides, membrane transport, and energy metabolism attributed to a stable gut bacteria community, including *Actinomycetes*, *Dysgomonas, Enterococcus*, *Providencia*, and *Proteus* (Cifuentes et al., 2020, 2022; Klammsteiner et al., 2020; Shelomi et al., 2021; Tegtmeier, Hurka, Mihajlovic, et al., 2021; Xiao et al., 2018).

Nitrogen is an essential nutrient for insect growth and development. *H. illucens* larvae gut bacteria contribute to nitrogen metabolism in several ways, such as nitrogen fixation and urea hydrolysis, which is strongly correlated to bacteria such as *Klebsiella*, *Enterobacter, Proteus, and Providencia* (Behar et al., 2005; Cifuentes et al., 2022; Gold et al., 2020; Klammsteiner et al., 2020). In addition to carbohydrate and nitrogen metabolism, these members have been linked to the breakdown of sulfur-containing amino acids and other organic molecules (Cifuentes et al., 2020; Jiang et al., 2019).

Although several studies have focused on identifying bacterial taxa in *H. illucens* larvae gut, a comprehensive understanding requires going beyond taxonomy. The actual functions of these

microbes within the *H. illucens* larvae gut are often inferred from their known activities in other systems and require further experimental validation (Cifuentes et al., 2022; Eke et al., 2023).

Immune system modulation and development

Insects possess innate immunity without memory cells yet may exhibit a memory-like mechanism called immune priming, which microorganisms can regulate (Girard et al., 2022; Prakash & Khan, 2022). Insect immune priming showcases specificity similar to vertebrate adaptive immunity. For example, flour beetles exhibit priming specific to *Bacillus thuringiensis* strains, showing no survival advantage with different pathogen strains (Ferro et al., 2019; Khan et al., 2016; Prakash & Khan, 2022; Roth et al., 2010). This suggests that priming evolves against natural pathogens using strain-specific information. Understanding specific priming responses remains challenging due to limited insect immunity knowledge (Prakash & Khan, 2022). Traditionally, insect immunity was seen as broad, but recent findings show that antimicrobial peptides (AMPs) like Diptericins and Drosocin can offer complete protection against specific pathogens (Hanson et al., 2019; Prakash & Khan, 2022).
The microbiota significantly impacts insect immunity by regulating inflammatory pathways (Prakash & Khan, 2022; Thaiss et al., 2016), reducing oxidative damage (Belkaid & Hand, 2014; Prakash & Khan, 2022; Ray & Kidane, 2016), and potentially influencing immune strategies (Futo et al., 2017; Martínez et al., 2020; Prakash & Khan, 2022; Rodrigues et al., 2010). The microbiota can also induce physicochemical changes for pathogen resistance. For instance, *Kosakonia cowanii* in tsetse flies acidifies the midgut, inhibiting trypanosome growth and increasing survival post-*Serratia marcescens* infection (Schmidt & Engel, 2021; Weiss et al., 2019).

The gut microbiota and the immune system of *H. illucens* point to a complex interplay that contributes to the larvae ability to thrive in environments rich in diverse microorganisms, including potential pathogens (Cifuentes et al., 2020; C. Lalander et al., 2013; Vogel et al., 2018). Inhabiting decaying organic matter, a niche with a wide array of microbes, *H. illucens* larvae have developed a robust immune system to survive this challenging environment, likely bolstered by their gut microbiota (Cifuentes et al., 2022; Eke et al., 2023; Tegtmeier, Hurka, Mihajlovic, et al., 2021; Vogel et al., 2018).

While the specific mechanisms are not yet fully elucidated, it has been shown that the presence of a gut microbiota can indeed influence the expression of immune-related genes in *H. illucens*

larvae. The absence of a microbiome has been shown to trigger significant changes in the transcriptional profile of *H. illucens* larvae during larval development, suggesting that the interaction with microbes induces intense regulation of host functional genes, including those involved in immune responses (Auger et al., 2023; Eke et al., 2023). Specific bacterial and fungal species residing in the gut have been shown to exhibit antimicrobial activity against pathogens. For instance, *Trichosporon asahii*, a yeast species frequently found in *H. illucens* larvae, has been shown to have antimicrobial activity against pathogenic yeasts (Tegtmeier, Hurka, Klüber, et al., 2021). Additionally, *Bacillus* strains isolated from *H. illucens* larvae exhibited potent antimicrobial activity against *Staphylococcus aureus* (Eke et al., 2023; Zhang et al., 2022).

Furthermore, studies investigating microbial changes during the development of *H. illucens* larvae and the impact on the employed substrate have shown that certain pathogenic bacteria decrease while other bacterial abundances remain stable or increase (Cai, Ma, Hu, Tomberlin, Thomashow, et al., 2018; Cifuentes et al., 2020; Wynants et al., 2019). This phenomenon is mainly attributed to AMPs in *H. illucens.* Notably, *H. illucens* expresses approximately 50 genes encoding putative AMPs, contributing to their ability to modulate microbial populations (Park & Yoe, 2017; Van Moll et al., 2022; Vogel et al., 2018). Such observations highlight the need for further research to fully comprehend immunological responses. Considering the dynamics of the different bacterial genotype profiles, as it has been stated, the diversity of genotypes in isolated bacteria associated with *H. illucens* (Cifuentes et al., 2022) and potential ignored mechanisms where colonization resistance could contribute to its defense.

Overall host fitness

There is a strong connection between the gut microbiota and the overall fitness of *H. illucens* larvae, with impacts extending beyond digestion to immune function and potentially even detoxification. The gut microbiota can be viewed as a key contributor to *H. illucens* larvae success in utilizing a wide range of organic substrates and surviving in challenging environments.

A primary factor in *H. illucens* larvae fitness is their ability to convert organic waste into valuable biomass efficiently (Xiang et al., 2024). This efficient bioconversion relies heavily on the metabolic activities of their gut microbiota, since *H. illucens* larvae lack the necessary enzymes to break down complex molecules like cellulose and lignin, which are abundant in

many organic waste materials (Eke et al., 2023; Xiang et al., 2024). The gut microbiota fills this gap, providing diverse enzymes that contribute to the breakdown of complex polysaccharides, proteins, and lipids and influencing the downstream metabolic processes within the host (Eke et al., 2023; Y. Wang et al., 2024; Xiang et al., 2024). This symbiotic partnership enables *H. illucens* larvae to extract nutrients from various substrates, contributing to their remarkable dietary flexibility.

Emerging research suggests that the gut microbiota of *H illucens* larvae may contribute to the detoxification of harmful compounds present in organic waste, such as antibiotics and heavy metals (Cai, Ma, Hu, Tomberlin, Yu, et al., 2018; Eke et al., 2023; C. Liu et al., 2020, 2021). This detoxification capability further enhances *H. illucens* larvae fitness by enabling them to tolerate substrates that might be toxic to other organisms. The specific mechanisms involved in detoxification by the gut microbiota are still under investigation, but the potential benefits for both *H. illucens* larvae and the environment are significant.

Studies focused on identifying critical bacterial players in the development of *H. illucens*, have shed light on the importance of bacteria during different developmental stages (Cifuentes et al., 2020; Querejeta et al., 2022; Zheng et al., 2013). For instance, *Comamonas* is highly present in the egg stage of *H. illucens,* potentially aiding in lipid biosynthesis and supporting insect development (Querejeta et al., 2022). On the other hand, bacteria like *Brevundimonas* are abundant during the pupal stage and may be involved in oxidative protection due to their production of carotenoids (Cifuentes et al., 2020; Querejeta et al., 2022).

The complex interplay between *H. illucens* and its gut microbiota has significant implications for its overall fitness, allowing it to thrive in challenging environments and efficiently convert waste into valuable biomass. This symbiotic relationship not only benefits the insect, but also has the potential for biotechnological applications, such as the development of probiotics for improving insect rearing and the discovery of novel enzymes for industrial uses. Further exploration of these interactions is crucial for unlocking the full potential of *H. illucens* in various fields, including waste management, animal feed production, and biotechnology.

Gut Microbiota Composition in H. illucens Larvae: General Patterns and Variations

The gut microbiota of *H. illucens* larvae exhibits notable patterns, including a potential core microbiota, as well as significant variations influenced by factors like diet, developmental stage, and rearing environment.

Dominant Phyla and Genera

The gut microbiota of *H. illucens* larvae is predominantly composed of four bacterial phyla, which are *Pseudomonadota* (formerly Proteobacteria), *Bacillota* (formerly Firmicutes), *Actinomycetota* (formerly Actinobacteria), and *Bacteroidota* (formerly Bacteroidetes) (Cifuentes et al., 2020; Eke et al., 2023). These phyla encompass several prevalent bacterial genera, including *Morganella, Providencia, Dysgonomonas*, *Ignatzschineria, Enterobacter*, *Proteus*, *Enterococcus*, *Bacillus*, *Klebsiella*, *Citrobacter*, *Scrofimicrobium*, and *Actinomyces* (Cifuentes et al., 2020; Eke et al., 2023; Tegtmeier, Hurka, Mihajlovic, et al., 2021; Zheng et al., 2013). This prevalent bacteria has been suggested to be part of a potential core microbiota in *H. illucens* larvae gut.

Core Microbiota

Stable bacterial communities, transmitted through vertical mechanisms, often exhibit a core microbiota fundamental to community stability. However, the precise characterization of this core microbiota remains the subject of ongoing discussion within the scientific community. This ongoing debate is attributed to the fact that most compositional studies have traditionally focused on genus-level discrimination, with scant consideration for strain-specific variations (Berg et al., 2020).

In this context, the well-studied *H. illucens* larvae serve as an illustrative case study. Extensive research has explored the gut microbiota composition in these larvae, employing high-throughput sequencing to analyze various growth stages and rearing conditions, with findings suggesting the existence of a potential core microbiota (Callegari et al., 2020; Cifuentes et al., 2020; Gorrens et al., 2021; Jeon et al., 2011; Shelomi et al., 2021; Tegtmeier, Hurka, Mihajlovic, et al., 2021). Nevertheless, a more comprehensive analysis at the strain level, using fingerprinting techniques, reveals remarkable diversity within genera (Cifuentes et al., 2022).

The genus *Providencia*, which is postulated to constitute a part of the core microbiota, demonstrated the presence of at least two distinct phylotypes, each characterized by unique genotypic profiles as elucidated by their BOX-Polymerase Chain Reaction (BOX-PCR) patterns (Cifuentes et al., 2022). Similarly, the genus *Morganella* exhibited a single phylotype accompanied by multiple genotypes (Cifuentes et al., 2022). These observations highlight the intricate complexity involved in definitively characterizing the core microbiota of *H. illucens*, as mentioned by Eke et al. in 2023.

Other members of the potential core microbiota in *H. illucens* larvae include *Dysgonomonas*, *Ignatzschineria*, *Enterobacter*, *Proteus*, *Enterococcus*, *Bacillus*, *Klebsiella*, *Citrobacter*, *Scrofimicrobium*, and *Actinomyces*. Notably, while these genera are frequently found in *H. illucens* larval guts, no single genus has been shown to have 100% prevalence across studies (Bruno et al., 2019; Cifuentes et al., 2020, 2022; Eke et al., 2023; Klammsteiner et al., 2020). Furthermore, there is no evidence suggesting these microbes are vertically transmitted from parents to offspring (Eke et al., 2023).

Although the specific functions of many potential core microbiota members remain largely unknown, they are hypothesized to play a crucial role in the capability to digest complex organic materials. *H. illucens* larvae inherently lack the enzymatic machinery necessary for the efficient degradation of complex compounds such as cellulose and lignin (Eke et al., 2023). The gut microbiota compensates for this enzymatic deficiency. For example, *Dysgonomonas*, known for its polysaccharide-degrading abilities, likely facilitates the breakdown of lignocellulose within the digestive tract (Cifuentes et al., 2020; Eke et al., 2023; Querejeta et al., 2022; Xiang et al., 2024). *Enterococcus* may contribute to the production of immune-related antimicrobial peptides, engage in the degradation of plant polymers, and participate in nitrogen, hydrogen, and sulfur metabolism (Eke et al., 2023; Klammsteiner et al., 2020). *Morganella* might be involved in urea hydrolysis and phenol production (Eke et al., 2023). *Providencia* is thought to assist in protein and lipid conversion, antibiotic degradation, and may contribute to hemicellulose digestion via xylanase production (Eke et al., 2023; Xiang et al., 2024). Additionally, *Lactobacillus* may exert protective effects by detoxifying pesticides and xenobiotics, and promoting the expression of antimicrobial peptides which inhibit pathogenic bacterial colonization (Eke et al., 2023; Y. Wang et al., 2024).

A comprehensive characterization of the potential core microbiota in *H. illucens* larvae requires further rigorous research. The variability in experimental conditions, such as rearing temperatures, humidity levels, and substrate compositions, across different studies, complicates the comparative analysis and the extraction of definitive conclusions (Eke et al., 2023). Additionally, the reliance on short-read sequencing techniques limits taxonomic resolution and the accurate inference of biological functions (Cifuentes et al., 2022; Eke et al., 2023). The functional roles of core microbiota members require extensive experimental validation. Genomic analyses, in vitro phenotyping, and in vivo experiments are essential for elucidating the functional diversity and ecological roles of the gut microbiota in *H. illucens* larvae.

Diet and Environmental Conditions

The diet plays an essential role in modulating the gut microbiota. Empirical evidence has shown that dietary variations significantly impact the metabolic activities and structural composition of gut bacterial communities (Colman et al., 2012; Yun et al., 2014). While one part of the gut microbiome of *H. illucens* larvae exhibits stability during development under a consistent diet (Cifuentes et al., 2020), notable shifts in microbial composition occur with dietary changes. This phenomenon is evidenced by studies demonstrating significant microbiome alterations in *H. illucens* larvae reared on diverse substrates, including food waste, cooked rice, and calf forage (Jeon et al., 2011; Tegtmeier, Hurka, Klüber, et al., 2021), as well as vegetable or fish meal (Bruno et al., 2019; Tegtmeier, Hurka, Klüber, et al., 2021).

Recent studies, such as the one conducted by Bruno et al. (2019), have investigated the gut microbiota composition of *H. illucens* across various substrates with different nutrient contents. The results highlight the significant influence of diet, particularly in the midgut, on the composition of the gut microbiota. Diets rich in carbohydrates were associated with an increased abundance of bacteria like *Sphingobacterium* and *Dysgomonas*, indicating their potential role in polysaccharide degradation. On the other hand, diets based on fish led to an abundance of *Providencia* in the gut microbiota, suggesting a response to the specific dietary composition in *H. illucens* larvae.

The functional consequences of these dietary-driven microbial shifts extend beyond simple nutrient digestion C. Liu et al., 2021 suggest that the *H. illucens* larvae gut microbiota could

play a critical role in detoxifying harmful compounds in various organic wastes. For instance, larvae feeding on oxytetracycline-enriched diets exhibit increased antibiotic-resistant bacteria, suggesting a potential for bioremediation of pharmaceutical waste (Eke et al., 2023; C. Liu et al., 2021). Similarly, heavy metals like cadmium and copper found in animal manure have been shown to alter the gut microbiota composition, although without apparent negative effects on larval development (Eke et al., 2023; Wu et al., 2020).

Among the various factors influencing the gut microbiota of *H. illucens* larvae, rearing temperature stands out by altering the relative abundance of bacterial taxa (Raimondi et al., 2020). Increasing the rearing temperature has been shown to decrease the relative abundance of *Providencia* while increasing the abundance of other genera like *Bacillus, Proteus, Bordetella*, and *Alcaligenes* (Eke et al., 2023; Raimondi et al., 2020). These temperature-induced shifts could have implications for bioconversion efficiency, as well as the potential for pathogen multiplication (Eke et al., 2023).

Developmental Stage

Insect development includes several stages, each characterized by significant physiological and morphological changes, including the structure and its microenvironmental conditions, which are closely linked to the composition and function of the gut microbiota (Girard et al., 2022). These transformations significantly impact extracellular symbionts, which reside on the surface of tissues that change during development, compared to their intracellular counterparts (Girard et al., 2022; Hammer & Moran, 2019).

Insect development is a progressive transformation that culminates in the adult form. During this process, most organs undergo modifications in response to endocrine regulations. The digestive tract is a significant carrier of microbial diversity and density among these organs. The gut microbiota, in particular, experiences substantial changes due to the elimination of the gut epithelium and shifts in physicochemical conditions. These changes profoundly impact the microbial communities residing in the gut (Girard et al., 2022)

Insects that undergo complete metamorphosis experience an even more drastic change. During pupation, the gut is replaced, leading to a shift in bacterial composition from the larval stage to the adult stage (Girard et al., 2022; Manthey et al., 2023).

H. illucens, as a holometabolous insect, undergoes complete metamorphosis, including a pupal stage. Significant anatomical changes occur during this transformation, particularly in the digestive system (Bonelli et al., 2020; Nguyen et al., 2013; Querejeta et al., 2022). The bacterial community of *H. illucens* changes throughout its life cycle in a stage-specific manner, influenced by factors such as gut remodeling, dietary shifts, and host-microbe interactions (Cifuentes et al., 2020; Eke et al., 2023; Querejeta et al., 2022; Zheng et al., 2013). Despite these stage-specific variations, consistent evidence suggests the presence of a potential core microbiota that persists throughout the life cycle of *H. illucens*. This core set of abundant taxa remains relatively stable and is independent of the developmental stage (Cifuentes et al., 2020; Eke et al., 2023).

While most research focuses on bacterial diversity, a shift in the fungal composition of the *H. illucens* larvae gut microbiota across different life stages has been observed. For instance, *Trichosporon asahii,* from the family *Trichosporonaceae*, becomes significantly enriched in the larval gut in later stages (Tegtmeier, Hurka, Klüber, et al., 2021).

Gut Regionalization and Host Genetics

The *H. illucens* larval midgut is anatomically and functionally compartmentalized, with different regions exhibiting distinct pH levels and hosting different microbial communities (Bonelli et al., 2020; Bruno et al., 2019). The anterior midgut harbors a greater microbial diversity than the middle and posterior regions (Bruno et al., 2019). This regionalization emphasizes the need to investigate the microbiota composition at a finer scale, considering the specific conditions and functions of each gut region. Additionally, the genetic nature of the host can influence the composition of the gut microbiota, potentially through variations in immune responses or other physiological factors. Studies have shown that *H. illucens* strains originating from different geographical locations, and therefore possessing genetic variations, harbor distinct bacterial communities (Eke et al., 2023; Khamis et al., 2020).

Beyond Bacteria: Mycobiota and Virobiota

While the majority of research on the *H. illucens* gut microbiota centers around bacteria, few studies provide some information about mycobiota (fungi) and virobiota (viruses) (Klüber et al., 2022; Pienaar et al., 2022; Tegtmeier, Hurka, Klüber, et al., 2021). *H. illucens* hosts a

variety of fungi, mainly from the phylum *Ascomycota*, including genera like *Pichia, Candida, Diutina, Kluyveromyces, Trichosporon*, and *Fusarium*. While a core mycobiota has not been established, *Pichia* and *Candida* are frequently associated with the species (Eke et al., 2023; Klüber et al., 2022; Tegtmeier, Hurka, Klüber, et al., 2021). The mycobiota is hypothesized to contribute to detoxification processes, enzyme production, and provision of essential nutrients (Eke et al., 2023; Klüber et al., 2022). However, concerns exist regarding the potential presence of pathogenic fungi, like *Fusarium solani*, and the risk of mycotoxin production under specific conditions (Klüber et al., 2022; Schrögel & Wätjen, 2019).

The virobiota of *H. illucens* remains largely unexplored. In silico analysis suggests the presence of *Totiviridae* viruses, but the nature of these interactions is unknown (Pienaar et al., 2022). While *H. illucens* larvae have shown the ability to reduce viral loads in contaminated substrates (Eke et al., 2023; C. H. Lalander et al., 2015), more research is needed to understand the composition and structure of the virobiota, particularly in the context of potential risks associated with mass rearing.

Impact of Rearing on the Feed Residue Microbiome

Knowing the microbial composition of the residual substrate after *H. illucens* larvae bioconversion is crucial for several reasons, ranging from optimizing bioconversion efficiency to addressing safety concerns. Analyzing the microbial composition of the residual substrate can help researchers understand how effectively *H. illucens* larvae and their gut microbiota have broken down the organic matter (Y. Wang et al., 2024; Xiang et al., 2024). On the other hand, the residual substrate may harbor potentially pathogenic microorganisms, including bacteria, fungi, and viruses (Y. Wang et al., 2024). Knowing the microbial composition allows researchers to assess the safety risks associated with the residual substrate, particularly if it is intended for use as animal feed or fertilizer.

Various studies have reached different conclusions regarding the impact of *H. illucens* larvae on the microbial community of feed residues. Some investigations report no significant alteration in the bacterial community composition from the initial to the final phase of feeding (Bruno et al., 2019; Cifuentes et al., 2020). Conversely, other studies indicate a progressive modification of the microbial community, particularly noting a reduction in potential human pathogens within the residual substrate (Cai, Ma, Hu, Tomberlin, Thomashow, et al., 2018;

Cifuentes et al., 2020). These discrepancies highlight the complex and context-dependent nature of microbial interactions within the *H. illucens* rearing environment.

Antibiotic Resistance and Safety Considerations

The use of *H. illucens* larvae as a sustainable protein source for animal feed and potentially even human consumption is gaining traction. However, the concern about antibiotic resistance and other safety aspects related to the bacteria inhabiting the larval gut is growing. A variety of antibiotic resistant genes (ARGs) in both *H. illucens* larvae and their frass (excrement and substrate residue) has been detected. These genes confer resistance to major antibiotic classes, including tetracyclines, erythromycin, vancomycin, β-lactams, and aminoglycosides (Cifuentes et al., 2020; C. Liu et al., 2021; Milanović, Cardinali, et al., 2021; Milanović, Roncolini, et al., 2021).

Several studies have suggested that the composition of the larvae feed can significantly influence the prevalence of ARGs. For example, substrates enriched with the microalgae *Isochrysis galbana* were linked to a higher incidence of ARGs in larvae and their frass (Milanović, Roncolini, et al., 2021). This highlights the potential role of the feed in shaping the resistome of the larvae. Moreover, specific bacterial genera within the gut microbiome, such as *Morganella*, *Paenibacillus*, *Lysinibacillus*, and *Enterococcus*, have been identified as potential hosts for ARGs, particularly those conferring resistance to tetracyclines and erythromycin (Milanović, Cardinali, et al., 2021; Milanović, Roncolini, et al., 2021)

Beyond antibiotic resistance, the presence of potentially pathogenic bacteria in the gut microbiome of *H. illucens* larvae, including those belonging to the *Enterobacteriaceae* family, if not adequately controlled, could pose a risk to animal or human health (Cifuentes et al., 2020). A deeper understanding of strain-level variations within the gut microbiota is crucial to assess the functional implications of bacterial changes during larval development (Cifuentes et al., 2020, 2022). This includes identifying specific strains carrying ARGs and their potential for horizontal gene transfer.

The use of *H. illucens* larvae in applications like animal feed and waste management holds immense promise, but the potential risks posed by pathogenic bacteria and ARGs necessitate the development and implementation of effective decontamination strategies (Cifuentes et al.,

2020; Xiang et al., 2024). By optimizing rearing practices, such as using controlled environments and appropriate insect densities, the incidence of contamination can be reduced (Eke et al., 2023; R Caparros et al., 2024). Careful substrate selection, including pretreatments like composting, can minimize the introduction of harmful microbes (Eke et al., 2023; Klammsteiner et al., 2020; R Caparros et al., 2024; Y. Wang et al., 2024). Furthermore, manipulating feed composition to include balanced diets and natural antimicrobial compounds can foster a healthy gut microbiota that contributes to pathogen control and ARG degradation. Implementing post-harvest processing methods, such as heat treatment, fermentation, and extraction techniques, is crucial to ensure the safety and quality of *H. illucens* products (Callegari et al., 2020; Eke et al., 2023).

Methodological Approaches for Studying Insect Gut Microbiota

The first step in studying the gut microbiota of insects involves careful sampling and experimental design. This could include field studies, semi-field studies, or laboratory studies. The choice of study type depends on the research question and the insect species being studied (Dada et al., 2021; De Cock et al., 2019). Metadata collection is another essential aspect of studying insect gut microbiota. This involves collecting information about the habitat of the insect, diet, life stage, and other factors that could influence the gut microbiota (Dada et al., 2021).

Once the samples have been collected, they need to be processed to know the microbial composition, which could involve different approaches. In microbial community analysis, it has become increasingly evident that exploring diversity at the genus level is often insufficient, especially when searching for deeper classifications, such as at the strain level (S. Liu et al., 2022). The establishment of a core microbiota, for instance, exemplifies the necessity for analyses beyond the genus level (Shade & Handelsman, 2012).

Microbial communities exhibit inherent genetic diversity, and understanding this diversity at the level of community properties and functions is essential (Ackermann, 2015; S. Liu et al., 2022). This underexplored diversity holds the potential for unearthing novel biosynthetic pathways and previously unknown biochemical characteristics with applications in various industries (Overmann et al., 2017).

Cultivation-based approaches provide invaluable insights into the phenotypic and genotypic attributes of bacterial isolates, enabling taxonomic identification and classification at varying levels of resolution (Hahn et al., 2019). This becomes particularly crucial when metagenomic sequencing fails to distinguish closely related bacterial taxa (Lema et al., 2023). Characterizing bacteria or microbial communities at the genotypic level is of fundamental importance in diverse sectors, including medical, industrial, and environmental, offering insights into the ecology and taxonomy of microbiota (Emerson et al., 2008; Nocker et al., 2007).

Selecting the appropriate cultivation technique depends on the desired bacteria to be isolated. Traditional methods often rely on plate-based techniques, but it has been reported that the employed media may not accurately represent the natural environment. Consequently, some bacteria fail to thrive. Additionally, the presence of symbiosis between cells or viable but non-colony-forming cells can pose challenges. Notably, gut microbiota are anaerobic organisms, and they may require complex media in an anaerobic environment (Xu et al., 2024).

To address these challenges, culturomics has emerged as an approach where diverse culture conditions are employed for studying gut microbiota. However, one of the major drawbacks is its time-consuming nature (S. Wang et al., 2020; Xu et al., 2024). High-throughput droplet microfluidic systems, where bacteria are encapsulated within droplets, allow the study of a large number of colonies in a single experiment. These systems also facilitate the growth of slow growers and rare taxa. However, maintaining such systems can be challenging due to specialized equipment requirements (Watterson et al., 2020; Xu et al., 2024).

In situ cultivation is a technique that aims to replicate the natural growth conditions of bacteria. This method offers advantages over traditional lab techniques because it closely resembles the environments where these microorganisms thrive. However, challenges remain in designing equipment that can effectively mimic these environments and in purifying bacterial colonies from these complex setups (Xu et al., 2024). Another approach, known as dilution-to-extinction cultivation, has been successfully employed to isolate and study a wide variety of bacteria. This method, pioneered by Button et al. (1993), was initially used to culture slow-growing marine bacteria that were difficult to isolate using conventional methods (Button et al., 1993; Cifuentes et al., 2022). Dilution-to-extinction involves repeatedly diluting a sample of bacteria in a low-nutrient medium until no further growth is observed (Button et al., 1993; Cifuentes et al., 2022). This process ensures that only a small number of cells are present in each culture well,

increasing the likelihood of obtaining pure cultures of individual bacterial species (Button et al., 1993; Cifuentes et al., 2022).

This technique has proven particularly useful in isolating bacteria that were previously considered "unculturable." Button et al. (1993) achieved success in isolating *Candidatus Pelagibacter ubique* (SAR11) using this approach (Button et al., 1993). *Candidatus Pelagibacter ubique* is a highly abundant marine bacterium that plays a significant role in ocean ecosystems but was notoriously difficult to culture using standard laboratory techniques (Button et al., 1993). The success in culturing *Candidatus Pelagibacter ubique* highlighted the potential of dilution-to-extinction cultivation in uncovering the hidden diversity of microbial life. Building on these successes, researchers have adapted dilution-to-extinction cultivation to study the human gut microbiota, a complex community of microorganisms that plays a crucial role in human health (Lagier et al., 2018).

The utility of the dilution-to-extinction technique was evaluated in the study of the gut microbiota of *H. illucens* larvae (Cifuentes et al., 2022). This method facilitated the isolation of a wide variety of bacteria from the larvae gut, including genera such as *Alcaligenes*, *Providencia*, *Serratia*, *Brucella*, *Micrococcus*, and *Enterococcus* (Cifuentes et al., 2022). Significantly, even specific strains of *Pseudomonas*, a bacterium typically considered easy to cultivate, suggesting that dilution-to-extinction can uncover a higher level of diversity within bacterial genera (Cifuentes et al., 2022). This finding underscores the importance of this technique in revealing the full spectrum of microbial diversity.

Implications and Future Directions

The use of *H. illucens* larvae to convert organic waste into animal feed is a promising approach to address sustainability challenges. However, potential risks associated with the transfer of ARGs and pathogenic bacteria need to be addressed.

One implication of the findings is the need for a comprehensive risk assessment to ensure the safety of *H. illucens* larvae and their residue as a food source. This assessment should consider various factors, such as the types and abundance of ARGs present, their potential for transfer, and the risks associated with pathogenic bacteria. While *H. illucens* larvae effectively reduce the levels of certain pathogenic bacteria, the presence of ARGs raises concerns. The widespread

use of disinfectants in animal husbandry may increase the risk associated with these genes. Therefore, decontamination technologies should be considered to eliminate or reduce hazards in the final product.
Future research should focus on elucidating the dynamics of ARG transfer within the gut microbiome and between the larvae and their environment. This includes understanding the mechanisms of horizontal gene transfer, the factors influencing transfer rates, and the persistence of ARGs in the surrounding environment. Studying the impact of different rearing conditions and substrate types on ARG profiles would provide valuable insights into mitigating these risks.

Genomic studies of *H. illucens* gut microbes are crucial for comprehending their diversity, shared genetic traits, and adaptations to the larval gut environment. This includes analyzing the metabolic functions of various strains, their roles in nutrient digestion, waste degradation, and their impact on the larval resistome. Building extensive strain collections is essential for thorough genomic analysis and understanding species-specific traits.

Recognizing a potential core microbiome that can be manipulated to enhance performance is vital. Promoting beneficial bacteria could improve nutrient digestion and pathogen suppression, thus boosting rearing practices and larval growth. The strain-level diversity within this potential core microbiome underscores the importance of considering strain-specific traits, as different strains might possess unique functional capacities that influence digestive processes and interactions with their environment.

However, the difficulty in cultivating certain core taxa, like *Lachnospiraceae* and *Actinomyces*, underscores the need for developing and refining cultivation techniques. Expanding the range of culturable bacteria would enable researchers to perform more detailed functional studies, uncovering novel metabolic pathways and interactions within the gut ecosystem, potentially leading to the discovery of new enzymes or bioactive compounds.

Finally, exploring the intricate interactions between different bacterial taxa within the *H. illucens* gut microbiome and their influence on the overall ecosystem dynamics is essential. Network analysis and co-culture experiments could reveal the synergistic or antagonistic relationships between microbes, providing a deeper understanding of the complexity and functionality of the gut microbiome.

Bibliography

Ackermann, M. (2015). A functional perspective on phenotypic heterogeneity in microorganisms. *Nature Reviews Microbiology*, *13*(8), 497–508.

Auger, L., Bouslama, S., Deschamps, M.-H., Vandenberg, G., & Derome, N. (2023). Absence of microbiome triggers extensive changes in the transcriptional profile of Hermetia illucens during larval ontogeny. *Scientific Reports*, *13*(1), 2396.

Behar, A., Yuval, B., & Jurkevitch, E. (2005). Enterobacteria-mediated nitrogen fixation in natural populations of the fruit fly Ceratitis capitata. *Molecular Ecology*, *14*(9), 2637–2643.

Belkaid, Y., & Hand, T. W. (2014). Role of the microbiota in immunity and inflammation. *Cell*, *157*(1), 121–141.

Berg, G., Rybakova, D., Fischer, D., Cernava, T., Vergès, M.-C. C., Charles, T., Chen, X., Cocolin, L., Eversole, K., & Corral, G. H. (2020). *Microbiome definition re-visited: old concepts and new challenges. Microbiome 8: 103*.

Bonelli, M., Bruno, D., Brilli, M., Gianfranceschi, N., Tian, L., Tettamanti, G., Caccia, S., & Casartelli, M. (2020). Black soldier fly larvae adapt to different food substrates through morphological and functional responses of the midgut. *International Journal of Molecular Sciences*, *21*(14), 4955.

Bruno, D., Bonelli, M., De Filippis, F., Di Lelio, I., Tettamanti, G., Casartelli, M., Ercolini, D., & Caccia, S. (2019). The intestinal microbiota of Hermetia illucens larvae is affected by diet and shows a diverse composition in the different midgut regions. *Applied and Environmental Microbiology*, *85*, e01864–18. https://doi.org/10.1128/AEM.01864-18

Button, D. K., Schut, F., Quang, P., Martin, R., & Robertson, B. R. (1993). Viability and isolation of marine bacteria by dilution culture: theory, procedures, and initial results. *Applied and Environmental Microbiology*, *59*(3), 881–891.

Cai, M., Ma, S., Hu, R., Tomberlin, J. K., Thomashow, L. S., Zheng, L., Li, W., Yu, Z., & Zhang, J. (2018). Rapidly mitigating antibiotic resistant risks in chicken manure by Hermetia illucens bioconversion with intestinal microflora. *Environmental Microbiology*, *20*(11), 4051–4062. https://doi.org/10.1111/1462-2920.14450

Cai, M., Ma, S., Hu, R., Tomberlin, J., Yu, C., Huang, Y., Zhan, S., Li, W., Zheng, L., yu Ziniu, & Zhang, J. (2018). Systematic characterization and proposed pathway of tetracycline degradation in solid waste treatment by Hermetia illucens with intestinal microbiota. *Environmental Pollution*, *242*. https://doi.org/10.1016/j.envpol.2018.06.105

Callegari, M., Jucker, C., Fusi, M., Leonardi, M. G., Daffonchio, D., Borin, S., Savoldelli, S., & Crotti, E. (2020). Hydrolytic Profile of the Culturable Gut Bacterial Community Associated With Hermetia illucens. *Frontiers in Microbiology*, *11*, 1965. https://doi.org/10.3389/fmicb.2020.01965

Capinera, J. L. (2008). *Encyclopedia of entomology*. Springer Science & Business Media.

Chapman, R. F., & de Boer, G. (1995). *Regulatory mechanisms in insect feeding*. Springer Science & Business Media.

Chavan, S., Yadav, B., Atmakuri, A., Tyagi, R. D., Wong, J. W. C., & Drogui, P. (2022). Bioconversion of organic wastes into value-added products: A review. *Bioresource Technology*, *344*, 126398. https://doi.org/10.1016/J.BIORTECH.2021.126398

Cifuentes, Y., Glaeser, S. P., Mvie, J., Bartz, J. O., Müller, A., Gutzeit, H. O., Vilcinskas, A., & Kämpfer, P. (2020). The gut and feed residue microbiota changing during the rearing of Hermetia illucens larvae. *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology*, *113*(9), 1323–1344. https://doi.org/10.1007/s10482-020-01443-0

Cifuentes, Y., Vilcinskas, A., Kämpfer, P., & Glaeser, S. P. (2022). Isolation of Hermetia illucens larvae core gut microbiota by two different cultivation strategies. *Antonie van Leeuwenhoek*. https://doi.org/10.1007/s10482-022-01735-7

Colman, D. R., Toolson, E. C., & Takacs-Vesbach, C. D. (2012). Do diet and taxonomy influence insect gut bacterial communities? *Molecular Ecology*, *21*(20), 5124–5137. https://doi.org/10.1111/j.1365-294X.2012.05752.x

Dada, N., Jupatanakul, N., Minard, G., Short, S. M., Akorli, J., & Villegas, L. M. (2021). Considerations for mosquito microbiome research from the Mosquito Microbiome Consortium. *Microbiome*, *9*, 1–16.

De Cock, M., Virgilio, M., Vandamme, P., Augustinos, A., Bourtzis, K., Willems, A., & De Meyer, M. (2019). Impact of sample preservation and manipulation on insect gut microbiome profiling. A test case with fruit flies (Diptera, Tephritidae). *Frontiers in Microbiology*, *10*, 460687.

De Smet, J., Wynants, E., Cos, P., & Van Campenhout, L. (2018). Microbial Community Dynamics during Rearing of Black. *Applied and Environmental Microbiology*, *84*(9), 1–17.

Douglas, A. E. (2009). The microbial dimension in insect nutritional ecology. *Functional Ecology*, *23*(1), 38–47.

Douglas, A. E. (2015). Multiorganismal insects: diversity and function of resident microorganisms. *Annual Review of Entomology*, *60*, 17–34.

Eke, M., Tougeron, K., Hamidovic, A., Tinkeu, L. S. N., Hance, T., & Renoz, F. (2023). Deciphering the functional diversity of the gut microbiota of the black soldier fly (Hermetia illucens): recent advances and future challenges. *Animal Microbiome*, *5*(1), 40.

Emerson, D., Agulto, L., Liu, H., & Liu, L. (2008). Identifying and Characterizing Bacteria in an Era of Genomics and Proteomics. *BioScience*, *58*(10), 925–936. https://doi.org/10.1641/B581006

Engel, P., & Moran, N. A. (2013). The gut microbiota of insects - diversity in structure and function. *FEMS Microbiology Reviews*, *37*(5), 699–735. https://doi.org/10.1111/1574-6976.12025

Ferro, K., Peuß, R., Yang, W., Rosenstiel, P., Schulenburg, H., & Kurtz, J. (2019). Experimental evolution of immunological specificity. *Proceedings of the National Academy of Sciences*, *116*(41), 20598–20604.

Futo, M., Sell, M. P., Kutzer, M. A. M., & Kurtz, J. (2017). Specificity of oral immune priming in the red flour beetle Tribolium castaneum. *Biology Letters*, *13*(12), 20170632.

Girard, M., Luis, P., Moro, C. V., & Minard, G. (2022). Crosstalk between the microbiota and insect postembryonic development. *Trends in Microbiology*.

Gold, M., von Allmen, F., Zurbrügg, C., Zhang, J., & Mathys, A. (2020). Identification of Bacteria in Two Food Waste Black Soldier Fly Larvae Rearing Residues. *Frontiers in Microbiology*, *11*(November). https://doi.org/10.3389/fmicb.2020.582867

Gorrens, E., Van Moll, L., Frooninckx, L., De Smet, J., & Van Campenhout, L. (2021). Isolation and Identification of Dominant Bacteria From Black Soldier Fly Larvae (Hermetia illucens) Envisaging Practical Applications. *Frontiers in Microbiology*, *12*(May). https://doi.org/10.3389/fmicb.2021.665546

Gupta, A., & Nair, S. (2020). Dynamics of Insect–Microbiome Interaction Influence Host and Microbial Symbiont. *Frontiers in Microbiology*, *11*, 1357. https://doi.org/10.3389/fmicb.2020.01357

Hahn, M. W., Koll, U., & Schmidt, J. (2019). Isolation and Cultivation of Bacteria. In C. J. Hurst (Ed.), *The Structure and Function of Aquatic Microbial Communities* (pp. 313–351). Springer International Publishing. https://doi.org/10.1007/978-3-030-16775-2_10

Hammer, T. J., & Moran, N. A. (2019). Links between metamorphosis and symbiosis in holometabolous insects. *Philosophical Transactions of the Royal Society B*, *374*(1783), 20190068.

Jeon, H., Park, S., Choi, J., Jeong, G., Lee, S. B., Choi, Y., & Lee, S. J. (2011). The intestinal bacterial community in the food waste-reducing larvae of Hermetia illucens. *Current Microbiology*, *62*(5), 1390–1399. https://doi.org/10.1007/s00284-011-9874-8

Jiang, C., Jin, W., Tao, X., Zhang, Q., Zhu, J., Feng, S., Xu, X., Li, H., Wang, Z., & Zhang, Z. (2019). Black soldier fly larvae (Hermetia illucens) strengthen the metabolic function of food waste biodegradation by gut microbiome. *Microbial Biotechnology*, *12*. https://doi.org/10.1111/1751-7915.13393

Jones, R. T., Sanchez, L. G., & Fierer, N. (2013). A cross-taxon analysis of insect-associated bacterial diversity. *PLoS One*, *8*(4), e61218.

Khamis, F. M., Ombura, F. L. O., Akutse, K. S., Subramanian, S., Mohamed, S. A., Fiaboe, K. K. M., Saijuntha, W., Van Loon, J. J. A., Dicke, M., & Dubois, T. (2020). Insights in the global genetics and gut microbiome of black soldier fly, Hermetia illucens: implications for animal feed safety control. *Frontiers in Microbiology*, *11*, 1538.

Khan, I., Prakash, A., & Agashe, D. (2016). Divergent immune priming responses across flour beetle life stages and populations. *Ecology and Evolution*, *6*(21), 7847–7855.

Klammsteiner, T., Walter, A., Bogataj, T., Heussler, C. D., Stres, B., Steiner, F. M., Schlick-Steiner, B. C., Arthofer, W., & Insam, H. (2020). The Core Gut Microbiome of Black Soldier Fly (Hermetia illucens) Larvae Raised on Low-Bioburden Diets . In *Frontiers in Microbiology* (Vol. 11, p. 993). https://www.frontiersin.org/article/10.3389/fmicb.2020.00993

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

Lagier, J.-C., Dubourg, G., Million, M., Cadoret, F., Bilen, M., Fenollar, F., Levasseur, A., Rolain, J.-M., Fournier, P.-E., & Raoult, D. (2018). Culturing the human microbiota and culturomics. *Nature Reviews Microbiology*, *16*(9), 540–550.

Lalander, C., Diener, S., Magri, M., Zurbrügg, C., Lindström, A., & Vinnerås, B. (2013). Faecal sludge management with the larvae of the black soldier fly (Hermetia illucens) - From a hygiene aspect. *The Science of the Total Environment*, *458-460C*, 312–318. https://doi.org/10.1016/j.scitotenv.2013.04.033

Lalander, C. H., Fidjeland, J., Diener, S., Eriksson, S., & Vinnerås, B. (2015). High waste-to-biomass conversion and efficient Salmonella spp. reduction using black soldier fly for waste recycling. *Agronomy for Sustainable Development*, *35*, 261–271.

Lema, N. K., Gemeda, M. T., & Woldesemayat, A. A. (2023). Recent Advances in Metagenomic Approaches, Applications, and Challenges. *Current Microbiology*, *80*(11), 347.

Liu, C., Yao, H., Chapman, S. J., Su, J., & Wang, C. (2020). Changes in gut bacterial communities and the incidence of antibiotic resistance genes during degradation of antibiotics by black soldier fly larvae. *Environment International*, *142*, 105834. https://doi.org/https://doi.org/10.1016/j.envint.2020.105834

Liu, C., Yao, H., & Wang, C. (2021). Black soldier fly larvae can effectively degrade oxytetracycline bacterial residue by means of the gut bacterial community. *Frontiers in Microbiology*, *12*, 663972.

Liu, S., Moon, C. D., Zheng, N., Huws, S., Zhao, S., & Wang, J. (2022). Opportunities and challenges of using metagenomic data to bring uncultured microbes into cultivation. *Microbiome*, *10*(1), 76.

Manthey, C., Johnston, P. R., Nakagawa, S., & Rolff, J. (2023). Complete metamorphosis and microbiota turnover in insects. *Molecular Ecology*, *32*(23), 6543–6551.

Martínez, B. A., Hoyle, R. G., Yeudall, S., Granade, M. E., Harris, T. E., Castle, J. D., Leitinger, N., & Bland, M. L. (2020). Innate immune signaling in Drosophila shifts anabolic lipid metabolism from triglyceride storage to phospholipid synthesis to support immune function. *PLoS Genetics*, *16*(11), e1009192.

Milanović, V., Cardinali, F., Aquilanti, L., Maoloni, A., Garofalo, C., Zarantoniello, M., Olivotto, I., Riolo, P., Ruschioni, S., & Isidoro, N. (2021). Quantitative assessment of transferable antibiotic resistance genes in zebrafish (Danio rerio) fed Hermetia illucens-based feed. *Animal Feed Science and Technology*, *277*, 114978.

Milanović, V., Roncolini, A., Cardinali, F., Garofalo, C., Aquilanti, L., Riolo, P., Ruschioni, S., Corsi, L., Isidoro, N., & Zarantoniello, M. (2021). Occurrence of antibiotic resistance genes in Hermetia illucens larvae fed coffee silverskin enriched with Schizochytrium limacinum or Isochrysis galbana microalgae. *Genes*, *12*(2), 213.

Nguyen, T.-X., Tomberlin, J., & Vanlaerhoven, S. (2013). Influence of Resources on Hermetia illucens (Diptera: Stratiomyidae) Larval Development. *Journal of Medical Entomology*, *50*, 898–906. https://doi.org/10.1603/ME12260

Nocker, A., Burr, M., & Camper, A. K. (2007). Genotypic Microbial Community Profiling: A Critical Technical Review. *Microbial Ecology*, *54*(2), 276–289. https://doi.org/10.1007/s00248-006-9199-5

Overmann, J., Abt, B., & Sikorski, J. (2017). Present and future of culturing bacteria. *Annual Review of Microbiology*, *71*, 711–730.

Park, S.-I., & Yoe, S. M. (2017). Defensin-like peptide3 from black solder fly: Identification, characterization, and key amino acids for anti-Gram-negative bacteria. *Entomological Research*, *47*(1), 41–47. https://doi.org/10.1111/1748-5967.12214

Pienaar, R. D., Gilbert, C., Belliardo, C., Herrero, S., & Herniou, E. A. (2022). First evidence of past and present interactions between viruses and the black soldier fly, Hermetia illucens. *Viruses*, *14*(6), 1274.

Prakash, A., & Khan, I. (2022). Why do insects evolve immune priming? A search for crossroads. *Developmental & Comparative Immunology*, *126*, 104246.

Querejeta, M., Hervé, V., Perdereau, E., Marchal, L., Herniou, E. A., Boyer, S., & Giron, D. (2022). Changes in Bacterial Community Structure Across the Different Life Stages of Black Soldier Fly (Hermetia illucens). *Microbial Ecology*, 1–14.

R Caparros, M., JK, T., & CD, M. (2024). *A worldwide overview of the status and prospects of edible insect production*.

Raimondi, S., Spampinato, G., Macavei, L. I., Lugli, L., Candeliere, F., Rossi, M., Maistrello, L., & Amaretti, A. (2020). Effect of rearing temperature on growth and microbiota composition of Hermetia illucens. *Microorganisms*, *8*(6), 902.

Ray, D., & Kidane, D. (2016). Gut microbiota imbalance and base excision repair dynamics in colon cancer. *Journal of Cancer*, *7*(11), 1421.

Redak, R. (2023). Introduction to and Importance of Insects. In J. D. Allison, T. D. Paine, B. Slippers, & M. J. Wingfield (Eds.), *Forest Entomology and Pathology: Volume 1: Entomology* (pp. 1–17). Springer International Publishing. https://doi.org/10.1007/978-3-031-11553-0_1

Rodrigues, J., Brayner, F. A., Alves, L. C., Dixit, R., & Barillas-Mury, C. (2010). Hemocyte differentiation mediates innate immune memory in Anopheles gambiae mosquitoes. *Science*, *329*(5997), 1353–1355.

Roth, O., Joop, G., Eggert, H., Hilbert, J., Daniel, J., Schmid-Hempel, P., & Kurtz, J. (2010). Paternally derived immune priming for offspring in the red flour beetle, Tribolium castaneum. *Journal of Animal Ecology*, *79*(2), 403–413.

Schmidt, K., & Engel, P. (2021). Mechanisms underlying gut microbiota–host interactions in insects. *Journal of Experimental Biology*, *224*(2), jeb207696.

Schowalter, T. D. (2022). *Insect ecology: an ecosystem approach*. Academic press.

Schrögel, P., & Wätjen, W. (2019). Insects for food and feed-safety aspects related to mycotoxins and metals. *Foods*, *8*(8), 288.

Shade, A., & Handelsman, J. (2012). Beyond the Venn diagram: the hunt for a core microbiome. *Environmental Microbiology*, *14*(1), 4–12.

Shelomi, M., Wu, M. K., Chen, S. M., Huang, J. J., & Burke, C. G. (2021). Microbes associated with black soldier fly (Diptera: Stratiomiidae) degradation of food waste. *Environmental Entomology*, *49*(2), 405–411. https://doi.org/10.1093/EE/NVZ164

Smetana, S., Schmitt, E., & Mathys, A. (2019). Sustainable use of Hermetia illucens insect biomass for feed and food: attributional and consequential Life Cycle Assessment. *Resources Conservation and Recycling*. https://doi.org/10.1016/j.resconrec.2019.01.042

Spranghers, T., Ottoboni, M., Klootwijk, C., Ovyn, A., Deboosere, S., Meulenaer, B., Michiels, J., Eeckhout, M., De Clercq, P., & De Smet, S. (2017). Nutritional composition of black soldier fly (Hermetia illucens) prepupae reared on different organic waste substrates. *Journal of the Science of Food and Agriculture*, *97*, 2594–2600. https://doi.org/10.1002/jsfa.8081

Tegtmeier, D., Hurka, S., Klüber, P., Brinkrolf, K., Heise, P., & Vilcinskas, A. (2021). Cottonseed press cake as a potential diet for industrially farmed black soldier fly larvae triggers adaptations of their bacterial and fungal gut microbiota. *Frontiers in Microbiology*, *12*, 634503.

Tegtmeier, D., Hurka, S., Mihajlovic, S., Bodenschatz, M., Schlimbach, S., & Vilcinskas, A. (2021). Culture-Independent and Culture-Dependent Characterization of the Black Soldier Fly Gut Microbiome Reveals a Large Proportion of Culturable Bacteria with Potential for Industrial Applications. *Microorganisms*, *9*(8), 1642.

Thaiss, C. A., Zmora, N., Levy, M., & Elinav, E. (2016). The microbiome and innate immunity. *Nature*, *535*(7610), 65–74.

Van Moll, L., De Smet, J., Paas, A., Tegtmeier, D., Vilcinskas, A., Cos, P., & Van Campenhout, L. (2022). In vitro evaluation of antimicrobial peptides from the black soldier fly

(Hermetia illucens) against a selection of human pathogens. *Microbiology Spectrum*, *10*(1), e01664-21.

Vogel, H., Müller, A., Heckel, D. G., Gutzeit, H., & Vilcinskas, A. (2018). Nutritional immunology: Diversification and diet-dependent expression of antimicrobial peptides in the black soldier fly Hermetia illucens. *Developmental & Comparative Immunology*, *78*, 141–148. https://doi.org/https://doi.org/10.1016/j.dci.2017.09.008

Wang, S., Wang, L., Fan, X., Yu, C., Feng, L., & Yi, L. (2020). An insight into diversity and functionalities of gut microbiota in insects. *Current Microbiology*, *77*, 1976–1986.

Wang, Y., Quan, J., Cheng, X., Li, C., & Yuan, Z. (2024). Relationship of black soldier fly larvae (BSFL) gut microbiota and bioconversion efficiency with properties of substrates. *Waste Management*, *180*, 106–114. https://doi.org/https://doi.org/10.1016/j.wasman.2024.03.030

Watterson, W. J., Tanyeri, M., Watson, A. R., Cham, C. M., Shan, Y., Chang, E. B., Eren, A. M., & Tay, S. (2020). Droplet-based high-throughput cultivation for accurate screening of antibiotic resistant gut microbes. *ELife*, *9*, e56998. https://doi.org/10.7554/eLife.56998

Weiss, B. L., Maltz, M. A., Vigneron, A., Wu, Y., Walter, K. S., O’Neill, M. B., Wang, J., & Aksoy, S. (2019). Colonization of the tsetse fly midgut with commensal Kosakonia cowanii Zambiae inhibits trypanosome infection establishment. *PLoS Pathogens*, *15*(2), e1007470.

Wu, N., Wang, X., Xu, X., Cai, R., & Xie, S. (2020). Effects of heavy metals on the bioaccumulation, excretion and gut microbiome of black soldier fly larvae (Hermetia illucens). *Ecotoxicology and Environmental Safety*, *192*, 110323.

Wynants, E., Frooninckx, L., Crauwels, S., Verreth, C., De Smet, J., Sandrock, C., Wohlfahrt, J., Van Schelt, J., Depraetere, S., Lievens, B., Van Miert, S., Claes, J., & Van Campenhout, L. (2019). Assessing the Microbiota of Black Soldier Fly Larvae (Hermetia illucens) Reared on Organic Waste Streams on Four Different Locations at Laboratory and Large Scale. *Microbial Ecology*, *77*(4), 913–930. https://doi.org/10.1007/s00248-018-1286-x

Xiang, F., Zhang, Q., Xu, X., & Zhang, Z. (2024). Black soldier fly larvae recruit functional microbiota into the intestines and residues to promote lignocellulosic degradation in domestic biodegradable waste. *Environmental Pollution*, *340*, 122676. https://doi.org/https://doi.org/10.1016/j.envpol.2023.122676

Xiao, X., Mazza, L., Yu, Y., Cai, M., Zheng, L., Tomberlin, J. K., Yu, J., van Huis, A., Yu, Z., & Fasulo, S. (2018). Efficient co-conversion process of chicken manure into protein feed

and organic fertilizer by Hermetia illucens L.(Diptera: Stratiomyidae) larvae and functional bacteria. *Journal of Environmental Management*, *217*, 668–676.

Xu, M.-Q., Pan, F., Peng, L.-H., & Yang, Y.-S. (2024). Advances in the isolation, cultivation, and identification of gut microbes. *Military Medical Research*, *11*(1), 34. https://doi.org/10.1186/s40779-024-00534-7

Yun, J.-H., Roh, S. W., Whon, T. W., Jung, M.-J., Kim, M.-S., Park, D.-S., Yoon, C., Nam, Y.-D., Kim, Y.-J., & Choi, J.-H. (2014). Insect gut bacterial diversity determined by environmental habitat, diet, developmental stage, and phylogeny of host. *Applied and Environmental Microbiology*, *80*(17), 5254–5264.

Zhang, Y., Xiao, X., Elhag, O., Cai, M., Zheng, L., Huang, F., Jordan, H. R., Tomberlin, J. K., Sze, S., & Yu, Z. (2022). Hermetia illucens L. larvae–associated intestinal microbes reduce the transmission risk of zoonotic pathogens in pig manure. *Microbial Biotechnology*, *15*(10), 2631–2644.

Zhao, M., Lin, X., & Guo, X. (2022). The role of insect symbiotic bacteria in metabolizing phytochemicals and agrochemicals. *Insects*, *13*(7), 583.

Zheng, L., Crippen, T. L., Singh, B., Tarone, A. M., Dowd, S., Yu, Z., Wood, T. K., & Tomberlin, J. K. (2013). A Survey of Bacterial Diversity From Successive Life Stages of Black Soldier Fly (Diptera: Stratiomyidae) by Using 16S rDNA Pyrosequencing. *Journal of Medical Entomology*, *50*(3), 647–658. https://doi.org/10.1603/ME12199

CHAPTER II

The gut and feed residue microbiota changing during the rearing of *Hermetia illucens* larvae

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Contributions:

SG, JM, YC designed the study, AM and HOG provided samples, YC, JM, J-OB, and AM performed research, YC, JM, and J-OB analysed data, SG, YC wrote the paper which was proofed by all co-authors, AV and PK received the funding.

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ORIGINAL PAPER

The gut and feed residue microbiota changing during the rearing of *Hermetia illucens* larvae

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Abstract Larvae of *Hermetia illucens,* commonly known as black soldier fly, efficiently convert organic waste into nutrient-rich supplements for different applications. Here we performed a preliminary experiment to investigate the dynamics of the *H. illucens* gut microbiota and changes in the composition of the bacterial community in the residue of the larval feed during rearing. We furthermore quantified the presence of antibiotic resistance and disinfectant genes in the gut and feed microbiota during the rearing process to elucidate if rearing leads to a reduction, increase, and/or transfer of resistance genes from the feed to larvae and vice versa. We found that the gut and feed residue bacterial communities were distinct throughout the rearing process. The gut microbiome remained more stable compared to the feed residue microbiome varying in both bacterial abundance and community structure during rearing. Antibiotic-resistance genes were present in both, gut and feed residues, with a significant increase in pupae and residue samples taken at the end of the rearing process. Disinfectant-resistance genes were present in the feed residue and even increased during the rearing process but were not transferred to the gut microbiome. We conclude that *H. illucens* larvae have a stable gut microbiome that does not change significantly over the course of larval development, whereas bacterial communities in the feed residue are strongly affected by rearing. If the

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presence of antibiotics and disinfectants during rearing, can promote the spread of antibiotic/disinfectant-resistance genes among feed and larvae needs to be evaluated in further experiments.

Keywords Antibiotic resistance genes · Black soldier fly · Disinfectant resistance genes · Feed residue microbiome · Gut microbiome · *Hermetia illucens* · Microbiome analysis · Quantitative PCR

Introduction

The larvae of *Hermetia illucens* (Diptera: Stratiomyidae), known as black soldier fly, can feed on decaying vegetables, waste from restaurants or biogas fermentation plants (Spranghers et al. 2017), swine, or dairy animals (Erickson et al. 2004; Beskin et al. 2018; Rehman et al. 2017; Xiao et al. 2018), and even seaweed (Liland et al. 2017). They can efficiently convert these diverse organic waste substrates into protein-rich and fat-rich supplements for animals (Xiao et al. 2018). It can also be expected that larvae are able to degrade non-biodegradable waste substances such as polyethylene and polystyrene as reported for other insect larvae (Yang et al. 2015; Przemieniecki et al. 2020). The *H. illucens* larvae accumulate large quantities of nutrients during rearing, with the nutrient composition depending on their feed substrate (Barroso et al. 2017; Liland et al. 2017; Nguyen et al. 2013; Oonincx et al. 2015; Spranghers et al. 2017).

The gut microbiome plays a significant role in digestion, host physiology and immunity, and the prevention of gut colonization by pathogens (Engel and Moran 2013; Vogel et al. 2017). Initial studies of the gut microbiome of *H. illucens* larvae fed on different diets revealed that the makeup of the bacterial community is diet dependent, but a core microbiome is also present (Jeon et al. 2011). The analysis of bacterial communities in whole *H. illucens* larvae at different growth stages indicated that a core community might be retained through successive life stages, and that particular bacterial species may enhance the ability of the larvae to digest waste and deactivate waste-associated pathogens by the production of antimicrobial compounds (Zheng et al. 2013). More recent studies have addressed the gut microbiome and the microbial community present in feed residues at the same time (Bruno et al. 2019; Wynants et al. 2019; Jiang et al. 2019). De Smet et al. (2018) found that feed-associated bacteria can affect growth and development of *H. illucens* larvae either positively or negatively. *H. illucens* larvae do not develop rapidly on sterile substrates, suggesting that some bacteria are beneficial and even essential for nutrient utilization. On the other hand, bacteria present in feed substrates could also compete with *H. illucens* larvae for nutrients. Organic waste substrates such as manure contain many pathogenic bacteria, some of which appear to be eliminated during the rearing process based on the results of spiking experiments with food-borne pathogens such as *Escherichia coli* and *Salmonella* spp. (Erickson et al. 2004; Liu et al. 2008; Lalander et al. 2013), whereas others such as *Enterococcus* spp. are not affected (Kolb et al. 2003). De Smet et al. (2018) discussed the need for a detailed analysis of changes in the gut microbiome according to the bacterial community present in the feed substrate.

The antimicrobial activity of *H. illucens* has been attributed to the presence of antimicrobial peptides that are expressed in a diet-dependent manner (Vogel et al. 2018). This reflects the need of larvae to adapt their host-associated core microbiome and the environmental microbial communities to accommodate diverse diets (Vogel et al. 2018). Thus far, the ability of *H. illucens* larvae to deal with antibiotic-resistant bacteria has not been addressed. However, this raises an important safety issue in livestock rearing and aquaculture if *H. illucens* larvae are used to prepare feed meal, because it provides a potential route for the transfer of antibiotic/disinfectant-resistance genes (Glaeser et al. 2016; Heuer et al. 2011); *H. illucens* larvae have been shown to reduce the abundance of tetracycline linked to the presence of tetracycline-resistance genes (Cai et al. 2018). A detailed study of house flies (*Musca domestica*) revealed that the antibiotic-resistance profile of swine manure was significantly altered during bioconversion (Wang et al. 2016). The use of biocides for disinfection is common in insect farming (Committee ES 2015) but the impact of this practice on the transmission of disinfectant-resistance genes in *H. illucens* larvae has not been assessed. Pathogens with resistance to

disinfectants are often co-resistant to antibiotics, thus significantly contributing to the spread of antibiotic resistance in the absence of antibiotic pressure (Mulder et al. 2018). The driving hypothesis of our study was that the rearing process (larvae digestion) must have a strong impact on bacterial assemblages present in the rearing substrate (feed), while the gut microbiota may be more stable due to the host adaptation. During feed digestion bacteria present in the feed and gut are coming in direct contact and may get mixed and/or exchange antibiotic resistance and disinfectant genes. We performed a preliminary experiment to get first answers to those hypotheses.

We investigate the composition of the *H. illucens* gut microbiome and the microbial communities in the feed residue during rearing, in order to map changes in total microbial abundance, composition, and diversity, and determined the presence of antibiotic/disinfectant-resistance genes. We conducted a simple experiment with a *H. illucens* larvae population reared on commercially available chicken feed, and analysed gut samples at three larval development states and in pupae, together with samples of the residual feed substrate. The experimental strategy is summarized in Supplementary Fig. 1.

Materials and methods

H. illucens larvae rearing and sample collection

H. illucens larvae were supplied by Prof. Gutzeit (Technical University Dresden) and the Bio S Biogas GmbH and were reared at the JLU Giessen in a pot (10 cm in length and width, 5 cm height) on commercial chicken feed at 25 °C. The larval activity continuously mixed the substrate within the pot. No special disinfectants were used for sterilization. Three individual insects were collected at different points of the pot at three rearing stages (S1, S2, and S3) and following pupation (P). Larvae and pupae were transferred to sterile tubes. Three independent samples of the feed residue (approximately 5 g) were collected from different places of the pot at the three larval growth stages (Supplementary Fig. 1). The samples were stored at − 20 °C.

Gut dissection

Larvae and pupae were surface sterilized with 12% (w/v) sodium hypochlorite and washed in autoclaved pure water. After washing, dissections were carried out under a stereomicroscope on sterile glass slides. The extracted gut tissue was cleaned to remove fat, washed in pure water, and transferred to sterile 2-mL reaction tubes. Before DNA extraction the weight of each gut sample was measured.

DNA extraction and quality control

Gut and feed residue samples were extracted using the DNeasy Blood & Tissue Kit (Qiagen). The samples were mixed with 180 µL tissue lysis buffer and 20 µL proteinase K (as provided in the kit), vortexed thoroughly and incubated overnight at 56 °C before following the manufacturer's protocol. Finally DNA was eluted in 100 µL PCR-grade water from the purification column. The concentration of extracted DNA was measured using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific) and its quality was tested by amplifying the bacterial 16S rRNA gene using primers 339F (5′-ACTCCTACGG-GAGGCAGCAGT-3′) and 907R (5′-CCGTCAATTCMTTTGAGTTT-3′) (Teske et al. 1996).

Detection of extended spectrum beta-lactamase (ESBLs) coding genes by multiplex-PCR

The presence of the three most prominent ESBL genes mainly detected in *Enterobacteriaceae*, $bla_{CTX\text{-}M}$, bla_{TEM}, and bla_{SHV} coding for CTX-M, TEM, and SHV β-lactamases, were evaluated by a multiplex PCR approach according to Schauss et al. (2015).

Quantification of target genes

Quantitative PCR (qPCR) was used to estimate the total bacterial abundance by targeting the 16S rRNA gene and to quantify antibiotic and disinfectant resistance genes. Four antibiotic resistance genes (ARBs) were quantified, the most abundant ESBL gene bla_{SHV}, the tetracycline resistance protein TetM coding gene *tetM*, the sulfonamide resistance gene *sul2*, and the alternative penicillin binding protein coding gene *mecA* as well as quaternary ammonium

compound (QAC) resistance genes coding for transmembrane protein (*qacE/qacEΔ1*). Analyses were performed in a total volume of 10 µL. Antibiotic resistance genes were determined by a SybrGreenI-based assays using the SsoFast EvaGreen Kit (BioRad) comprising 5 µL 2 × SsoFast EvaGreen supermix, 0.2 µM of each primer, and 1 µL DNA template (5 ng μL^{-1}). The *qacE/qacEΔ1* gene was detected using a TaqMan assay comprising 2 × TaqMan Gene Expression Master Mix (Applied Biosystems), 0.3 µM of each primers, 0.25 µM TaqMan probe, and 1 µL DNA (5 ng μL^{-1}). Standard curves were produced using purified PCR amplified gene fragments containing internally the qPCR primer binding sides flanked by additional nucleotides. Standards were prepared either using (1) gene specific primers flanking the qPCR target or (2) DNA fragments amplified with the qPCR primer system which was reamplified with plasmid primers after molecular cloning in chemical competent *E. coli* using the pJET1.2/blunt vector (Thermo Fisher Scientific). The DNA concentration of standard DNA fragments of known length were measured using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific) and the concentration of target copies per µL was calculated according to Kolb et al. (2003). Reference strains and primer systems used for qPCR and the generation of qPCR standards are listed in Supplementary Table 1. The gene copy number of the standards were in ranged from 10^1 to 10^9 targets μL^{-1}. This concentration was plotted against Ct values to generate a standard curve. Primer systems, amplification efficiencies, and qPCR programs are listed in Supplementary Table 1. Beside the absolute quantification in copy numbers per µg gut or residue, antibiotic/detergent-resistance gene copy numbers were normalized to the number of 16S rRNA gene copies. Significant differences among samples were tested in SigmaPLOT v12.5 (Systat Software) by one way analysis of variance (ANOVA) using the Tukey test (Post Hoc test), the Shapiro–Wilk normality test, and the Brown Forsythe equal variance test.

16S rRNA gene amplicon sequencing and bacterial community analysis

Bacterial communities of the gut and feed residue were analysed by 16S rRNA gene amplicon sequencing. The 16S rRNA gene fragments were amplified using primers 341F (5′-CCTACGGGNGGCWGCAG-3′) and 785R (5′-GACTACHVGGGTATCTAAKCC-3′) (Klindworth et al. 2012). PCR amplification and Illumina 300-bp paired-end read sequencing was carried out by LGC Genomics using the Illumina MiSeq v3 system and analysed using the SILVAngs pipeline (https://www.arb-silva.de/ngs/) as described in detail by Aydogan et al. (2018). Archaeal, chloroplast, mitochondria, and "No Relative" reads were excluded from further analysis, leaving only those reads assigned to Bacteria. Operational taxonomic units (OTUs) were determined at a 98% similarity level. OTUs were clustered to phylogenetic groups which represent a genus-level resolution. The amplicon sequences obtained in this study were deposited in the GenBank short-read archive (SRA) with bio project accession number PRJNA578547 and bio sample accessions SRX7029577 to SRX7029597, respectively.

The composition of the bacterial communities was analysed at the level of phyla and phylogenetic groups (genus level). Analyses were performed in PAST v3.11 (Hammer et al. 2001). The alpha diversity was studied at the genus-level considering the number of phylogenetic groups and the number of sequences per phylogenetic group. Following Alpha diversity parameters were determined, richness (Chao-1), evenness, dominance, and Shannon index (Harper 1999). Relative abundance patterns of phyla and phylogenetic groups were used for Beta diversity analysis. Hierarchical clustering (unweighted pair group method with arithmetic mean) and non-metric multidimensional scaling (NMDS) plots (Taguchi and Oono 2004) were calculated based on pairwise similarities determined with the Bray–Curtis similarity index (Bray and Curtis 1957). ANOSIM (Clarke 1993) and PERMANOVA tests (Anderson 2001) were applied to test for significant differences among the a priori defined groups (gut versus feed residue samples taken at different rearing stages). MC permutations were used given the low number of possible permutations, and *p* values were calculated using type III sums of squares and 999 permutations. To determine whether significant ($p < 0.05$) PERMANOVA results were based on location or dispersion effects, homogeneity of dispersion (PERMDISP) was applied on calculated distances to centroids (Anderson et al. 2008). PERMANOVA analysis was conducted using PRIMER 7 (PRIMER-E) including the

PERMANOVA + add-on package (Anderson et al. 2008; Clarke and Gorley 2015). Rank distances of bacterial communities within sample replicates and between different samples were compared to determine the variability or stability of the communities among biological replicates. Similarity percentage breakdown (SIMPER) analysis (Clarke 1993) focused on phylogenetic groups with a relative abundance of at least 0.5% and were used to identify phylogenetic groups that were primarily responsible for the observed differences between larvae and feed residue communities at different growth stages. Alpha-diversity parameters and relative abundance patterns of individual phyla or genera were tested for significant differences using ANOVA analysis as described above.

Results

Bacterial abundance during rearing remains stable in the gut but increases significantly in the feed residue

The abundance of *Bacteria* in the guts of larvae and early stage pupae and the feed residue was estimated by qPCR based quantification of the bacterial 16S rRNA gene sequences per gram of gut material or feed residue (Fig. 1). The concentration of bacterial 16S rRNA gene copies ranged from 10^9 (larvae) and 10^{10} (pupae) copies per gram (fresh weight) of gut material with no significant difference during development (ANOVA, $p > 0.05$). In the feed residue, the concentration of bacterial 16S rRNA gene copies was in the same range (10^9–10^{10} copies per gram fresh weight) but a significant increase was observed between larva at stage S2 and S3 (ANOVA $p < 0.05$).

Broad phylogenetic analysis of the bacterial communities in the gut and feed residue

Changes in the diversity and phylogenetic composition of bacterial communities in the gut and feed residue were studied by 16S rRNA gene amplicon sequencing. We generated 505,989 combined sequences from where 489,260 sequences (96.7%) could be assigned to *Bacteria* (212–57,148 sequences per sample) including 198,925 from gut samples and 290,335 from residue samples. The remainder were assigned to *Archaea* (0.1%; 5 sequences), chloroplasts (0.6%; 2926 sequences), mitochondria (2.5%; 12,888 sequences), and "no relatives" (0.2%; 910 sequences), and were excluded for further analysis. The *Bacteria* sequences were set to 100% and considered as the gut and feed residue associated microbial communities for subsequent experiments. A total of 56,978 OTUs were defined and assigned to 516 different phylogenetic groups which represents a genus level resolution. Because not all phylogenetic groups could be assigned to described genera we remained phylogenetic groups instead of genera all over the text. Of the 516 detected phylogenetic groups, 163 were determined in gut and feed residue samples, 116 were solely found in the gut, and 237 solely in the feed residue samples. We found that 78 of the shared groups, 5 of the gut-specific groups and 6 of the residue-specific groups occurred in at least one sample with a relative abundance of at least 1%.

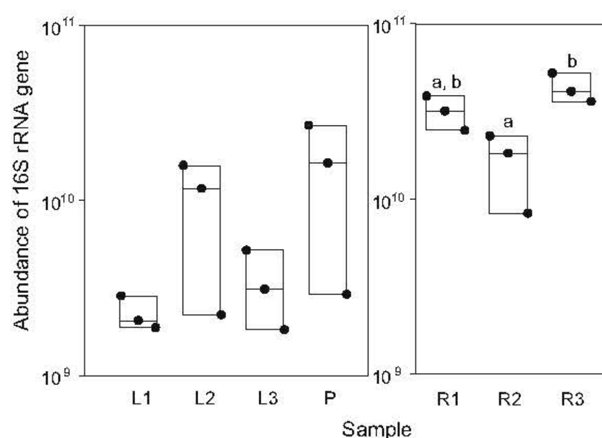

Fig. 1 Concentrations of bacterial 16S rRNA gene copies in guts of *H. illucens* larvae and pupae and residues of the employed substrate investigated at three different growth stages (R1 to R3). Box-plots with data of three independent biological replicates are depicted. Statistically significant differences between gut samples and residue samples were tested by one-way ANOVA based on the Tukey test (SigmaPlot). Different letters at top of the box-plots represent significant differences ($p < 0.05$). L: Larvae; P: pupae; R: residue; 1, 2, 3: different studied growth stages (see also Supplementary Fig. 1)

Alpha diversity during rearing remained stable in the gut but not in the feed residue microbiome

The diversity of the gut microbiota measured using the Shannon index did not change significantly over time. We observed a slight but non-significant increase in diversity between larvae at stage S1/S2 and S3/pupae (Fig. 2a). The estimated number of phylogenetic

groups (Chao-1 index) ranged from 35 to 99 in larval gut samples with non-significant differences among the different growth stages. A slight but non-significant increase in the taxonomic richness (83–101) was observed for pupae compared to the three larval stages (Fig. 2b). No significant changes were observed in terms of community evenness or dominance (Fig. 2c, d).

In contrast to the gut microbiome, the diversity of *Bacteria* in the feed residue changed significantly during the different growth stages. A significant increase in diversity was observed between rearing stages S1 and S2, followed by a decrease in stage S3, although the diversity at stage S3 was still higher than at stage S1. *Bacteria* in the feed residue at stage S2 were more evenly distributed (Fig. 2c) owing to the equal abundance of individual phylogenetic groups indicated by the low dominance values compared to stage S1 and S3 (Fig. 2d).

Overall, the diversity of the gut microbiome was higher than that of the feed residue except at stage S2. Richness increased over time in the feed residue, whereas there were slight but non-significant changes in the gut microbiome. The gut microbiome of the

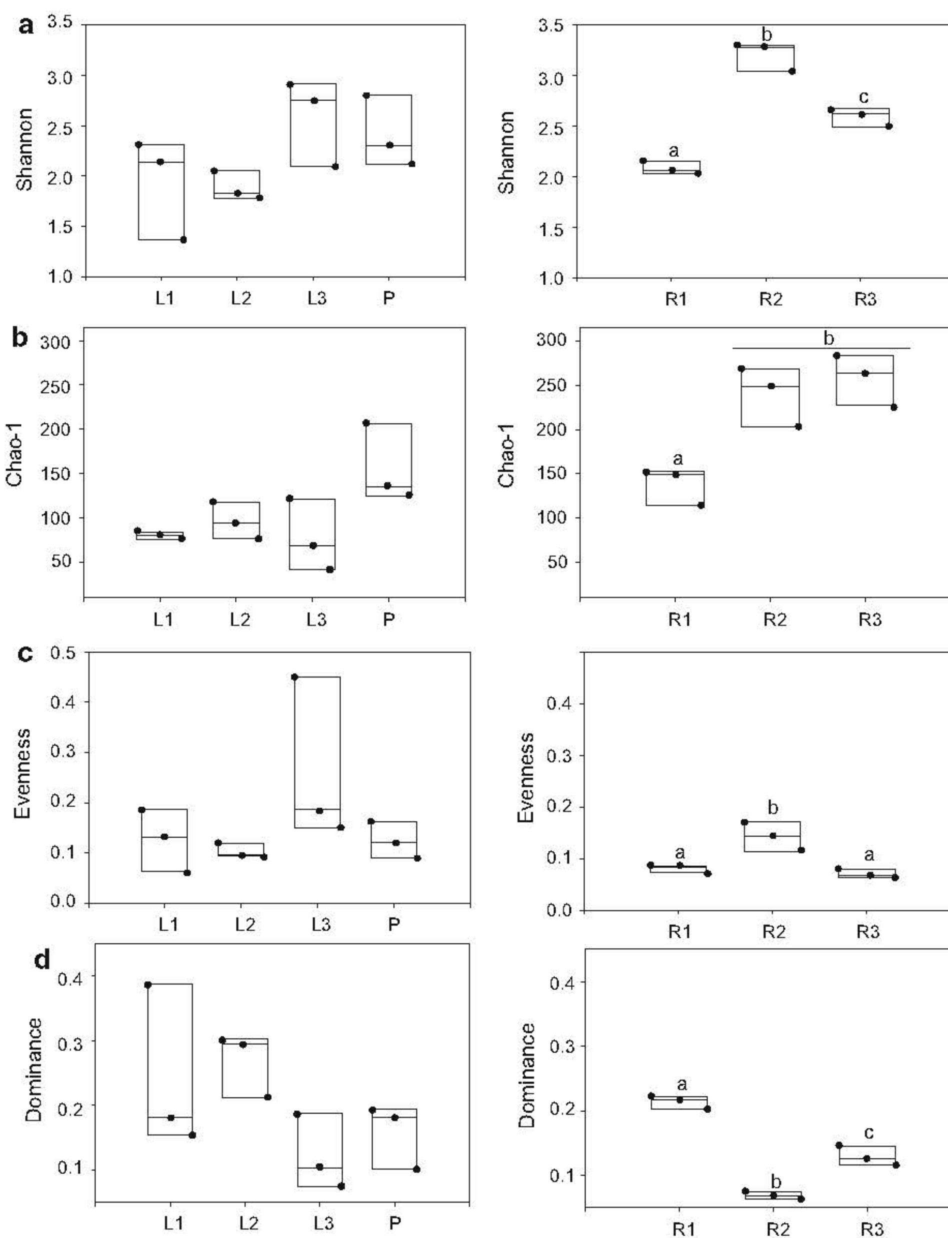

Fig. 2 Alpha diversity measurements of the bacterial assemblages detected by the 16S rRNA gene amplicon sequencing approach in *H. illucens* gut samples (larvae, pupae) and substrate residues determined at three different growth stages. Boxplots display the diversity estimation/Shannon index values (**a**), community richness/chao1 values (**b**), community evenness (**c**), and dominance values (**d**). One-way ANOVA based on the Tukey's multiple comparison test was used to investigate the samples for significant differences. Different letters at top of the bars represent significant differences ($p < 0.05$)

larvae/pupae showed higher values for evenness and lower values for dominance, indicating a more equal distribution of phylogenetic groups. In contrast, the evenness values in the feed residue remained lower, indicating the dominance of certain phylogenetic groups.

The gut and residual feed contained distinct bacterial communities that vary according to the rearing stage

The bacterial communities in the gut and feed residue samples were clearly separated in NMDS plots if studied at a genus level resolution (Fig. 3). Relative abundance patterns of phylogenetic groups were thereby compared for different samples with the Bray–Curtis similarity index. Significant differences between the gut and feed residue samples were confirmed by one-way analysis of similarities (ANOSIM; global scale $p = 0.001$; R = 0.7707), permutational multivariate analysis (PERMANOVA; $p < 0.001$) and pairwise comparisons using Monte Carlo (MC) permutations (p-MC < 0.05). The bacterial community profiles of larval guts at all three stages were positioned close together albeit without direct overlaps in the NMDS plots, with non-significant differences (p-MC > 0.05; Table 1). In contrast, the bacterial community profiles in the feed residue at the same three stages showed clear separation supported by low MC p values (p-MC = 0.001–0.003) indicating significant differences (Table 1). The high variability of the gut microbiome of individual larvae at each stage explained the lack of significant differences among individual gut samples whereas the bacterial community in the feed residue showed low variability.

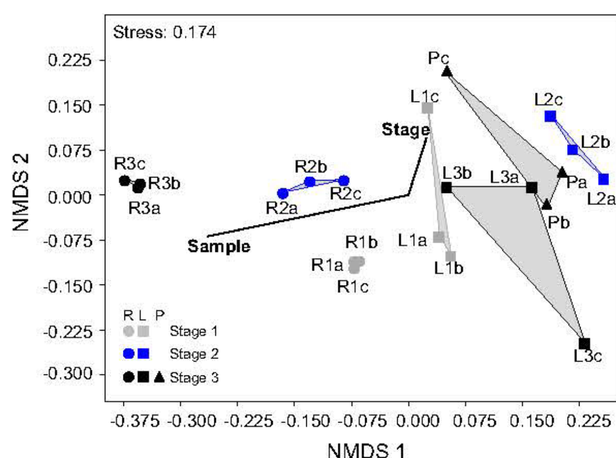

Fig. 3 Visualization of differences in gut and residue bacterial assemblages present at the studied rearing stages. Non-metric multidimensional scaling (NMDS) plots illustrate differences of the bacterial community compositions between gut and residue microbiota studied at the three stages of the rearing process

Differences in the composition of the bacterial communities of the gut and feed residue at the phylum level

The 516 phylogenetic groups we identified were assigned to 17 phyla. Most abundant in all samples

Table 1 Global and pairwise ANOSIM and PERMANOVA analysis, and adjusted Monte Carlo p values (p-MC) of the relative abundance profiles of phylogenetic groups in the gut and feed residue bacterial communities at different growth stages

Groups	ANOSIM	PERMANOVA		
	p	t	p	p-MC
Global	**0.001**		**0.001**	**0.001**
Pairwise				
L1–L2	0.105	1.9051	0.071	0.061
L1–L3	0.097	1.3024	0.11	0.22
L1–P	0.094	1.461	0.092	0.142
L2–L3	0.207	1.3166	0.102	0.185
L2–P	0.301	0.93978	0.584	0.49
L3–P	0.707	0.85456	0.905	0.563
R1–R2	0.107	4.0357	0.094	**0.003**
R1–R3	0.117	13.323	0.113	**0.001**
R2–R3	0.117	5.0539	0.115	**0.004**
L1–R1	0.102	2.6858	0.097	**0.015**
L1–R2	0.098	2.3476	0.107	**0.017**
L1–R3	0.088	3.2176	0.114	**0.008**
L2–R1	0.109	3.7559	0.102	**0.007**
L2–R2	0.103	3.1231	0.112	**0.006**
L2–R3	0.098	4.0683	0.108	**0.003**
L3–R1	0.095	2.1829	0.106	**0.031**
L3–R2	0.122	1.9029	0.12	0.051
L3–R3	0.086	2.5294	0.108	**0.013**
P–R1	0.094	2.4345	0.088	**0.022**
P–R2	0.11	2.2415	0.101	**0.028**
P–R3	0.104	2.7211	0.09	**0.017**

Significant results ($p < 0.05$) are highlighted in bold

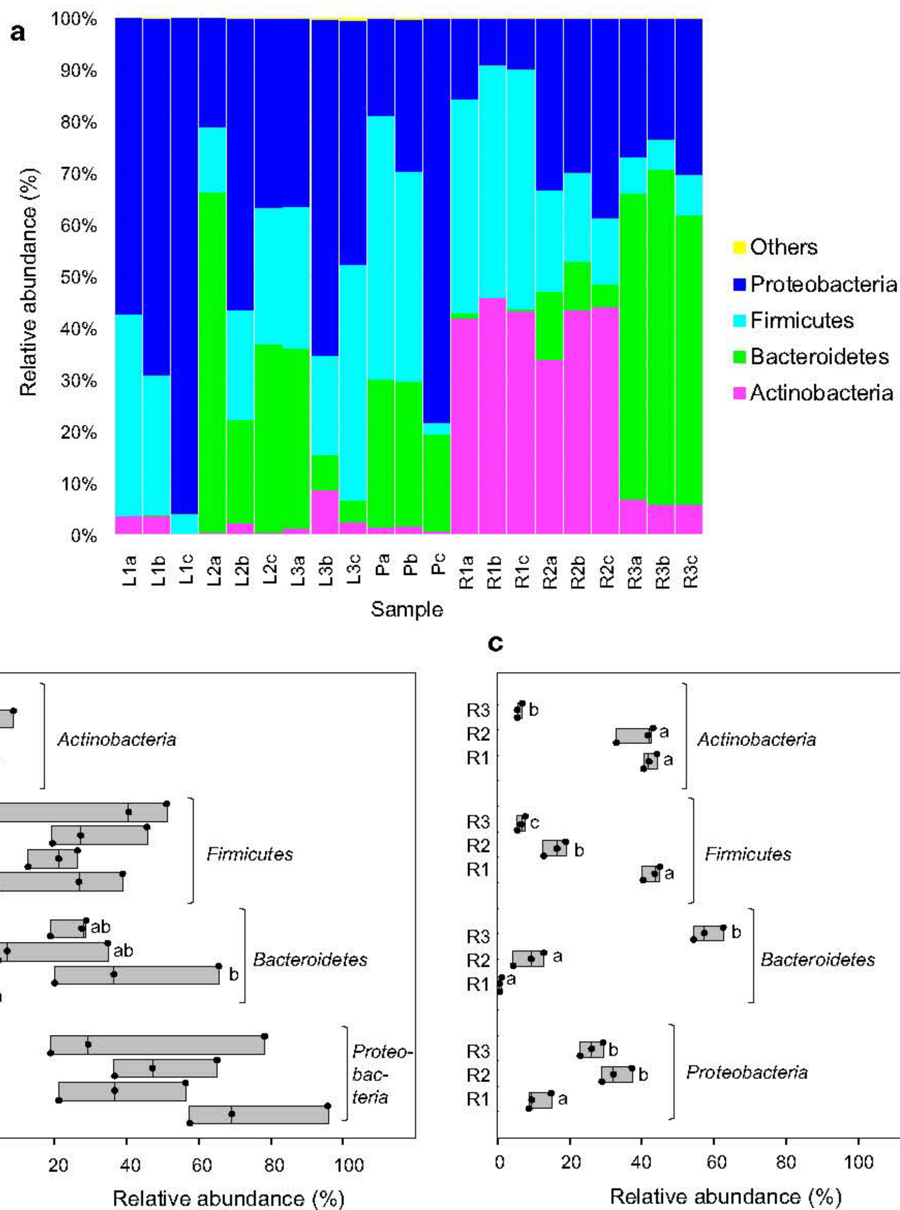

Fig. 4 Phylogenetic composition of bacterial communities of the gut and feed residue analysed at the level of phyla. **a** Relative abundance profiles of bacterial phyla present in the gut samples of *H. illucens* larvae and pupae and substrate residue harvested at three different rearing stages (1–3). Illustration of changes in the relative abundance of most abundant phyla among: **b** gut samples of larvae in different development stages and pupae and **c** among the substrate samples harvested at the same time points as larvae-pupae. The differences between the samples were calculated using one-way ANOVA based on the Tukey's multiple comparison test (SigmaPlot). Different letters represent significant differences ($p < 0.05$). L: Larvae; P: pupae; R: residue; 1, 2, 3: different studied growth stages (see also Supplementary Fig. 1). A: Letters a, b, and c correspond to biological replicates

were *Actinobacteria, Firmicutes*, *Bacteroidetes*, and *Proteobacteria* (Fig. 4a). The gut microbiome showed slight changes in phylum-level composition during development (Fig. 4b). At stage S1, the gut microbiome was dominated by *Proteobacteria* (57–96%). At stage S2, it was still dominated by *Proteobacteria* but was also significantly enriched by *Bacteroidetes* (10–36%). At stage S3 larvae, it was dominated by *Proteobacteria* (36–47%) and *Firmicutes* (27–46%). The pupal gut microbiome was also dominated by these phyla, but with different relative proportions (19–78% *Proteobacteria*, 2–51% *Firmicutes*). In contrast, the phylum-level composition of the feed residue bacterial community showed significant

changes during development. At the S1 stage, both, *Firmicutes* (41–47%) and *Actinobacteria* (42–46%), were highly abundant, whereas *Proteobacteria* were enriched at the S2 stage (30–39%) and *Bacteroidetes* in stage S3 (56–65%) (Fig. 4c). The abundance of *Firmicutes* and *Actinobacteria* during rearing therefore declined in the feed residue but slightly increased in the gut (Fig. 4b, c).

Genus-level differences in the composition of the bacterial communities of the gut and feed residue

More detailed analysis of changes in the phylogenetic composition of the gut and feed residue bacterial communities during the rearing process were performed at the genus level. We focused thereby on the most abundant phylogenetic groups. Among the 516 phylogenetic groups we identified, 108 occurred with a relative abundance of at least 0.5% in at least one sample (Fig. 5). This subset of phylogenetic groups represented 95–100% of the phylogenetic groups detected in the individual samples and could thereby be considered as the predominant groups in the gut and feed residue bacterial communities.

The core microbiome of the gut and stage-specific variations

The most abundant phylogenetic groups in the larval and pupal gut microbiome were *Morganella* (T1), *Klebsiella* (T3), *Providencia* (T4), *Citrobacter* (T89), *Enterobacter* (T13), and *Proteus* (T21), representing the family *Enterobacteriaceae* (class *Gammaproteobacteria*); *Ignatzschineria* (T5) and *Stenotrophomonas* (T6), representing the family *Xanthomonadaceae* (class *Gammaproteobacteria*); *Dysgonomonas* (T54) representing the phylum *Bacteriodetes*; *Enterococcus* (T2), *Bacillus* (T11), uncultured *Lachnospiraceae* (T12), and *Clostridium* sensu stricto group 1 (T31), representing the phylum *Firmicutes*; and *Desulfovibrio* (T25) representing the class *Deltaproteobacteria*. Seven of these groups (*Morganella*, *Klebsiella, Providencia, Enterobacter, Enterococcus, Bacillus*, and uncultured *Lachnospiraceae*) were shared with no significant differences in abundance by all larvae stages and pupae, and were therefore defined as the core microbiome of the gut (Fig. 6a). Furthermore, *Actinomyces* (T14; *Actinobacteria*) were shared by all gut samples with a relative abundance of $\sim 2\%$, and *Dysgonomonas* (T54; *Bacteroidetes*) were abundant in the gut at all stages except S1, and these were also assigned to the core gut microbiome.

Changes in the relative abundance of phylogenetic groups assigned to the core gut microbiome accounted to most of the differences between different rearing stages (SIMPER analysis). *Dysgonomonas* (T54, *Bacteroidetes*) was scarce at the S1 stage (0.007% relative abundance), but was the most abundant phylogenetic group in the gut at stages S2 and S3 and in pupae (among 13.6–35.8%), showing the highest relative abundance of 35.8% at stage S2 (Fig. 6a). *Morganella* (T1), *Enterococcus* (T2), and *Klebsiella* (T3) together accounted for a total mean relative abundance of 55.2% at stage S1, but their relative abundance declined at later stages. *Providencia* (T4) was abundant at stage S1 before declining, but then bounced back at the pupal stage to reach a high of 12.5%. Similarly, the abundance of *Bacillus* (T11) and *Lachnospiraceae* (T12) was low at stages S1 and S2 (0.6–1.4%) but increased to 8.3% and 7.1% respectively in S3 larvae and to 3.7% and 10.6% respectively in pupae (Fig. 6a).

Non-core taxa also contributed to the stage-specific changes in the gut microbiome because they were among the most abundant taxa (at least 5% relative abundance) at specific rearing stages. Those prevalent at stage S2 included *Sedimentibacter* (T75) and *Desulvovibrio* (T25), and those prevalent at stage S3 including *Clostridium* sensu stricto group 7 (T31) and three phylogenetic groups of the family *Enterobacteriaceae*: *Citrobacter* (T89), *Enterobacter* (T13), and *Proteus* (T21).

The changing bacterial community in the feed residue and key stage-specific variations

Parallel analysis of the bacterial communities in the feed residues during rearing (Fig. 6a) revealed that *Dysgonomonas* (T54), uncultured *Lachnospiraceae* (T12), and *Actinomyces* (T14) were not present in any samples with a relative abundance greater than 0.5%. *Enterococcus* (T2) and *Bacillus* (T11) were abundant at stage S1 (2.2–3.8%) but then declined, falling to $< 0.1\%$ by stage S3. Four of the core taxa, namely *Morganella* (T1), *Klebsiella* (T3), *Enterobacter* (T13), and *Providencia* (T4), were abundant in the

Phyla	Class	Family	Genus	L1a	L1b	L1c	L2a	L2b	L2c	L3a	L3b	L3c	Pa	Pb	Pc	R1a	R1b	R1c	R2a	R2a	R2b	R3a	R3b	R3c	Av. dissim	Contrib. %
Actinobacteria		Actinomycetaceae	Actinomyces	0.7	1.6	0.1	0.5	0.9	0.5	0.8	0.6		1.1	0.7	0.3	0.3	0.2	0.3	0.1	0.1	0.2	0.0	0.0	0.0	0.24	0.28
			Flaviflexus								0.0				0.0				0.8	1.0	0.4	0.1	0.0	0.0	0.11	0.12
		Corynebacteriaceae	Corynebacterium 1	2.7	1.7	0.1	0.0	0.0		0.1	2.2		0.0	0.2	0.0	25.7	31.0	22.4	6.7	6.4	5.9	1.1	0.7	0.6	4.45	5.21
		Dietziaceae	Dietzia								0.1					0.0	0.0		1.1	1.3	1.8	0.1	0.1	0.1	0.20	0.24
		Beutenbergiaceae	Salana	0.1	0.1	0.1	0.0	1.2	0.0	0.5	5.4		0.1	0.4		0.0	0.0	0.1	0.2	0.3	0.4	0.2	0.1	0.2	0.36	0.42
		Bogoriellaceae	Georgenia	0.0															0.8	1.0	0.2	0.3	0.2	0.2	0.12	0.14
		Brevibacteriaceae	Brevibacterium	0.1	0.0						0.1			0.1	0.0	5.3	4.3	5.6	3.6	4.0	3.8	0.4	0.3	0.3	1.10	1.28
		Demequinaceae	Demequina														0.0		0.2	0.2	0.0	0.9	0.7	1.4	0.15	0.18
		Dermabacteraceae	Brachybacterium						0.0		0.0			0.1	0.0	9.3	8.9	12.2	10.1	16.8	15.0	0.2	0.2	0.2	2.98	3.49
			Helcobacillus															0.0	2.5	0.2	4.8	0.0	0.0	0.0	0.36	0.42
		Microbacteriaceae	Leucobacter								0.0			0.0	0.2	0.1	0.1	0.1	0.8	0.9	0.8	0.3	0.3	0.3	0.15	0.18
			Microbacterium		0.1								0.0	0.0	0.0	0.9	1.0	2.2	3.5	5.2	8.3	1.2	0.7	1.0	0.99	1.16
		Micrococcaceae	Enteractinococcus													0.0	0.0	0.0	0.5	0.6	0.2	0.0	0.0	0.0	0.07	0.08
			Micrococcus			0.0			0.0			0.5	0.0	0.0			0.0		0.0		0.0		0.0	0.0	0.02	0.03
		Ruaniaceae	Haloactinobacterium																0.8	1.1	0.4	1.4	1.1	0.8	0.23	0.27
		Propionibacteriaceae	Propionibacterium	0.0	0.1	0.1		0.0		0.0	0.0	1.9	0.1	0.2	0.0	0.0	0.0	0.0	0.0	0.1	0.1	0.0	0.0	0.0	0.11	0.13
		Cellulomonadaceae	Actinotalea													0.0			0.5	0.2	0.4	0.2	0.3	0.1	0.07	0.09
		Streptomycetaceae	Streptomyces			0.0										0.0	0.0	0.1	0.1	1.8	0.0	0.0	0.0	0.0	0.10	0.12
Bacteriodetes		Bacteroidaceae	Bacteroides	0.0	0.0		14.3	0.4						3.6	0.0			0.0	0.0	0.0	0.0	0.0			0.85	1.00
		Flavobacteriaceae	Aequorivita																			2.6	2.4	2.4	0.35	0.41
			Myroides											0.1	12.9	0.1		0.0	0.2	0.4	0.6	0.0	0.0	0.0	0.67	0.79
			Flavobacterium		0.1	0.0	0.0				0.1	0.5			0.0	0.0		0.0	0.8	0.4	0.0	0.1	0.1	0.1	0.08	0.10
			Ulvibacter						0.0		0.1								3.0	0.8	0.4	17.3	17.0	15.6	2.51	2.94
			uncultured								0.1				0.0				0.0	0.0		22.4	24.5	20.5	3.22	3.76
		Chitinophagaceae	Vibrionimonas				0.0	0.0				1.9						0.0							0.09	0.11
			Proteiniphilum									0.5													0.02	0.03
		Porphyromonadaceae	Porphyromonas								0.2	0.9	0.0									0.0			0.05	0.06
			uncultured						0.0					0.5											0.02	0.03
			Dysgonomonas	0.0		0.0	51.4	19.7	36.4	34.8	6.1		28.5	23.9	5.0				0.0	0.0	0.1	0.1	0.0	0.0	7.99	9.36
		Cytophagaceae	uncultured									0.5													0.02	0.03
		Sphingobacteriaceae	Sphingobacterium											0.0	0.9	0.6	0.0	0.1					0.0	0.0	0.08	0.09
		Shingobacteriaceae	uncultured								0.1			0.0	0.0				9.1	7.9	3.2	16.7	20.8	17.2	3.25	3.80
Firmicutes		Bacillaceae	Amphibacillus					0.0					0.0	0.0			0.0	0.0	2.0	1.2	0.2	0.9	0.7	0.9	0.25	0.29
			Bacillus	1.3	2.1	0.7	0.9	0.8	0.1	3.6	2.9	18.4	1.2	8.2	1.6	1.4	3.2	2.0	0.4	0.4	0.5	0.1	0.1	0.2	1.65	1.93
			Oceanobacillus	0.0				0.0			0.0					0.3	0.3	0.2	1.8	1.6	0.5	0.9	0.8	1.1	0.29	0.34
			Pseudogracilibacillus				0.0		0.0							0.0	0.1	0.0	4.0	2.2	0.4	3.0	2.2	3.3	0.63	0.74
			Natronobacillus																0.9	0.0	0.0	0.0	0.0		0.04	0.05
			Gracilibacillus					0.0		0.0	0.0		0.0	0.1			0.0	0.0	0.6	0.5	0.1	0.0	0.0	0.0	0.06	0.07
			uncultured								0.0			0.1	0.0				4.4	2.3	0.5	1.2	1.2	1.2	0.47	0.55
		Staphylococcaceae	Staphylococcus	0.3	0.2	0.0	0.0	0.1	0.0	0.1	1.2		0.0	0.4	0.0	34.3	33.5	37.7	1.9	3.2	6.6	0.1	0.0	0.1	5.42	6.35
		Veillonellaceae	Veillonella						0.0	0.0		0.5										0.0			0.02	0.03
			Sporomusa									0.9													0.04	0.05
		Aerococcaceae	Globicatella	0.5	0.3	0.0				0.0	0.0					0.2	1.7	0.4	0.1	0.2	0.1	0.1	0.1	0.1	0.15	0.17
		Carnobacteriaceae	Atopostipes											0.0	0.0	0.0	0.1	0.0	1.3	3.3	1.5	0.0	0.0	0.0	0.30	0.35
			uncultured	12.9	0.1	0.0	0.0	0.2			0.0		0.0	0.0		0.0	0.1	0.0	0.1	0.2	0.1		0.0	0.0	0.64	0.75
		Enterococcaceae	Enterococcus	21.7	22.4	2.1	0.7	6.7	0.4	4.5	10.8		4.2	9.0	0.1	3.2	3.3	4.9	0.4	0.8	1.3	0.0	0.0	0.0	3.26	3.81
		Lactobacillaceae	Lactobacillus	0.0	0.0	0.0	0.0	0.0		0.0						1.2	0.6	0.6	0.0	0.0	0.0	0.0			0.12	0.14
		Clostridiaceae 1	Clostridium sensu stricto 16			0.0			0.0	0.0		2.8													0.14	0.16
			Clostridium sensu stricto 3	0.0		0.0		0.0			0.0	0.9		0.0	0.0			0.0							0.05	0.06
			Clostridium sensu stricto 7	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.2	19.8	0.0	0.1	0.0			0.0				0.0			0.97	1.13
		Leuconostocaceae	Oenococcus			0.0						0.5													0.02	0.03
		Clostridiales;Family XI	Sedimentibacter			0.0	7.2	7.8	11.7		0.0		14.2	9.1							0.0	0.0			2.11	2.48
			No identified				0.9		3.1				0.0		0.3										0.20	0.24
		Lachnospiraceae	[Eubacterium] fissicatena group				0.2	0.1	0.0				0.5	0.2						0.0	0.0				0.04	0.05
			Lachnoclostridium 5				0.0	0.9	2.9	1.0	0.2		0.5	1.1	0.0							0.0			0.28	0.33
			Lachnoclostridium				0.0	2.3	3.8		0.2		0.1	2.0			0.0				0.0	0.0			0.37	0.44
			uncultured	2.0	1.4	0.7	0.5	1.3	0.4	18.0	3.3		26.7	3.2	0.0	0.0	0.0	0.0	0.1	0.1	0.2	0.0	0.0	0.0	2.51	2.94
		Peptostreptococcaceae	Peptoclostridium					0.1	0.0				0.0	1.2						0.0	0.0	0.0	0.0		0.07	0.08
			Ruminiclostridium 5				1.8	0.4	0.5				0.3	0.5					0.0	0.0	0.0	0.0		0.0	0.15	0.18
		Ruminococcaceae	uncultured					0.1					0.9												0.05	0.05
			Ruminococcaceae UCG-005									1.4													0.07	0.08
		Acidaminococcaceae	Phascolarctobacterium						3.3		0.0			4.9					0.0	0.0	0.0	0.0	0.0	0.0	0.37	0.44
Proteobacteria	Alphaprotobacteria	Caulobacteraceae	Brevundimonas	0.4	0.2	0.1	0.0		0.1	0.0		1.4	1.0	0.1	3.6	0.0	0.0	0.0			0.0		0.0		0.30	0.36
		Rhodospirillales Incertae Sedis	Reyranella									0.9													0.04	0.05
		Bradyrhizobiaceae	Bradyrhizobium		0.0	0.0			0.0	0.0		1.9		0.0	0.0										0.09	0.11
		Brucellaceae	Paenochrobactrum										0.0	0.2	3.4	0.0	0.0		0.1	0.1	0.1	0.0			0.18	0.21
			Pseudochrobactrum											0.0	6.3	0.1		0.0							0.30	0.36
		Rhodobacteraceae	Pannonibacter									0.5													0.02	0.03
			Pontibaca												0.0				0.6	0.5	0.2	0.3	0.2	0.2	0.09	0.10
			Paracoccus		0.0						0.0				0.0			0.0	0.2	0.1	0.1	1.8	1.4	1.3	0.23	0.27
		Sphingomonadaceae	Sphingobium									0.9													0.04	0.05
			Sphingomonas	3.3	2.6	0.8	0.2	0.1	0.1	0.3	0.5		0.2	1.9	1.2	0.2	0.3	0.1	0.0	0.0	0.0	0.0		0.0	0.44	0.52
	Betaproteobacteria	Alcaligenaceae	Alcaligenes											0.0	1.8	0.0		0.0	0.1	0.1	0.1	0.0	0.0	0.0	0.10	0.11
			Bordetella											0.1	8.0	0.0	0.0	0.0		0.0	0.0	0.0	0.0		0.39	0.46
			Achromobacter		0.3	0.0									0.5		0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.1	0.04	0.05
			Paenalcaligenes											0.1	10.3	0.0	0.0	0.0	0.1	0.1	0.1	0.0	0.0	0.0	0.51	0.60
			Pusillimonas								0.0			0.0	0.1				0.6	0.1	0.0	1.3	0.8	1.0	0.17	0.20
		Burkholderiaceae	Limnobacter									1.4										0.0		0.0	0.07	0.08
			Ralstonia		0.1		0.0			0.0	0.0	5.7	0.0				0.0								0.27	0.32
			Pelomonas	0.0	0.0	0.0	0.0	0.0	0.0	0.0		9.0	0.0	0.0				0.0		0.0					0.43	0.50
		Comamonadaceae	Comamonas			0.0					0.0	0.9				0.1	0.0	0.0			0.0				0.05	0.06
			Pseudorhodoferax	0.7	0.9	0.7	0.1	0.0	0.0	0.5	0.2	1.9	0.1	3.5	0.1	0.1	0.6	0.0			0.0	0.0			0.36	0.43
			Variovorax		1.2									0.0											0.06	0.07
		Oxalobacteraceae	Oxalobacter		0.0		0.6	0.2	0.2		0.0			1.1	0.2							0.0			0.10	0.12
	Deltaproteobacteria	Bradymonadaceae	No identified																			6.9	6.8	7.8	1.12	1.32
			Bradymonas				0.0															1.3	1.8	1.7	0.23	0.27
		Desulfovibrionaceae	Desulfovibrio	0.1	0.1	0.1	9.5	4.5	4.3	0.1	0.1	10.8	0.3	1.4	0.1			0.0	0.0	0.0	0.0	0.0			1.34	1.57
	Epsilonproteobacteria	Campylobacteraceae	Campylobacter				0.2	0.0	0.9	0.1	1.2		0.0	0.1				0.0							0.11	0.13
	Gammaproteobacteria	Enterobacteriaceae	Buttiauxella				0.0				2.2				0.0				0.0	0.1	0.0	0.0	0.0		0.11	0.13
			Citrobacter	0.0			3.3	1.6	1.6	4.5	14.0	0.5	0.8	2.5	0.8	0.0	0.0	0.1	3.0	5.4	9.1	0.0	0.0	0.0	1.72	2.02
			Enterobacter	1.9	1.2	0.2	0.6	0.1	0.4	12.5	6.3		6.0	0.3	0.0	0.2	0.1	0.1	0.4	1.0	2.2	0.0	0.0	0.0	1.30	1.52
			Escherichia-Shigella	0.0	0.0	0.0	0.0	0.0	0.0		0.0		0.2		0.1	0.2	0.1	0.0	0.3	0.6	0.5	0.0	0.0	0.0	0.08	0.10
			Klebsiella	31.2	5.3	8.8	1.8	0.1	0.1	3.8	8.6		2.4	0.1	0.6	2.2	2.0	5.1	4.5	7.1	11.4	0.0	0.0	0.0	3.17	3.72
			Morganella	12.5	5.6	65.9	0.7	49.2	0.8	0.4	2.0	0.9	0.2	0.3	1.2	0.8	0.6	0.8	0.5	0.9	1.6	0.3	0.1	0.1	5.57	6.52
			Pantoea	0.8	0.2	0.4					3.6		0.0			0.1	0.0	0.1	0.0	0.1	0.2				0.25	0.29
			Proteus	0.2	0.3	0.2	0.1	0.0	0.9	2.1	9.7	3.6	0.4	0.0	0.2	0.2	0.5	0.4	0.4	0.8	1.8	0.0	0.0		0.85	0.99
			Providencia	2.1	2.8	25.8	0.4	0.2	24.1	1.2	9.5		0.2	0.2	37.1	2.6	1.5	1.8	3.5	6.7	9.7	0.1	0.1	0.0	4.62	5.41
		Alcanivoracaceae	Alcanivorax																0.2	0.0	0.0	0.6	0.4	3.5	0.22	0.26
		Halomonadaceae	Halomonas											0.0					17.7	5.6	0.5	7.7	7.7	8.9	2.07	2.42
		Pasteurellaceae	Aggregatibacter	0.0				0.0			0.0	0.9													0.05	0.05
		Orbaceae	uncultured		0.1	0.3	2.3	0.4	0.8	10.3	2.4		5.3	8.1	0.1	0.1	0.1	0.1	0.0	0.0	0.0	0.0	0.0	0.0	1.24	1.46
		Alteromonadaceae	Marinobacter															0.0	0.2	0.1	0.0	0.7	0.7	0.8	0.11	0.13
		Moraxellaceae	Acinetobacter								0.0	0.5	0.0	0.0	0.0	0.7	0.0	0.3	0.0	0.0		0.0			0.07	0.08
		Aeromonadaceae	Aeromonas	0.4	0.5	0.3	0.1	0.0	0.0	0.1	0.2		0.2	0.9	0.1	0.3	0.4	0.0			0.0	0.0	0.0	0.1	0.12	0.14
		Pseudomonadaceae	Pseudomonas	3.2	5.0	1.5	0.2	0.1	0.1	0.4	3.3	4.7	0.8	7.7	1.3	7.1	2.4	0.2	0.2	0.1	0.1	2.6	2.6	3.8	1.31	1.54
			Ignatzschineria	0.1	23.4	0.6	0.9	0.0	2.2				0.3	0.0	0.0	0.1	0.3	0.3	0.0	0.0	0.0	0.0	0.0	0.0	1.29	1.51
		Xanthomonadaceae	Pseudoxanthomonas									0.5												0.0	0.02	0.03
			Stenotrophomonas		19.1		0.0		0.0					0.2	0.0	0.1	0.0	0.0	0.0						0.93	1.09

Colour scale: 0, 0.5, 1, 2.5, 5, 10

◀**Fig. 5** Heat map representing the relative abundance profiles of the most abundant phylogenetic groups determined in the gut of BSF *H. illucens* larvae and pupae and residue samples harvested at three different rearing stages (1–3). Contribution percentages were calculated in different groups A Residue, B larvae, and C all the samples. L: Larvae; P: pupae; R: residue; 1, 2, 3: different studied growth stages; a, b, c correspond to individual biological replicates (see also Supplementary Fig. 1). The feed residue (R) was harvested in parallel to larvae/pupae (R1–R3)

feed residue at stage S2 but were less abundant at stage S1 and had fallen to $< 0.1\%$ by stage S3 (Fig. 6a). The largest number of shared phylogenetic groups between the gut and feed residue was observed at stage S1. Five of the nine shared phylogenetic groups at stages S1 and S2 corresponded to the gut core microbiome. The number of shared groups fell to zero at the S3 and pupal stages if focusing solely on the core gut microbiome.

The most abundant bacteria in the feed residue changed significantly during rearing. Only 10 phylogenetic groups with a mean relative abundance of at least 0.5% were identified in the residue at stage S1, whereas this increased to 29 at stage S2 and 20 at stage S3. Few phylogenetic groups were thereby shared by stages S1 and S2 whereas a greater number were shared by stages S2 and S3. The most abundant phylogenetic groups were most often stage specific, and thus made a strong contribution to the differences in the feed microbiome over time (SIMPER analysis, Fig. 5). The phylogenetic groups with the highest relative abundance at stage S1 were *Staphylococcus* (T22) (*Firmicutes*), *Corynebacterium* group 1 (T10), *Brachybacterium* (T125), and *Brevibacterium* (T30), all representing *Actinobacteria* (5–35%). However, the relative abundance of these groups declined sharply over time, reaching 0.1–0.8% at stage S3 (Fig. 6b). Their relative abundance in the gut microbiome was also low ($< 1.5\%$). The two phylogenetic groups shared in all residue samples (T10, T35) were abundant at the first two stages (L1 = 26.3–1.4%, L2 = 6.3–5.7%) but declined to $\sim 1\%$ at stage S3 (Fig. 6b). Stage S2 was not only characterized by the greatest number of phylogenetic groups with a relative abundance of at least 0.5% (29 groups) but 18 of these were unique to this stage. *Klebisella* (T3), *Providencia* (T4), and *Citrobacter* (T89), all representing the family *Enterobacteriaceae* (*Gammaproteobacteria*), and *Microbacterium* (T35; *Actinobacteria*) were the most abundant groups at this stage (5.6–7.6%) making the strongest contribution to the stage-specific bacterial communities. Uncultured *Flavobacteriaceae* (T225), uncultured *Sphingobacteriaceae* (T233), and *Ulvibacter* (T223), all representing the phylum *Bacteroidetes*, *Halomonas* (T488; *Gammaproteobacteria*), and uncultured *Bradymonadales* (T441; *Deltaproteobacteria*) were the most abundant groups (relative abundance of at least 5%) at stage S3. None of these phylogenetic groups were detected in gut microbiome of larvae or pupae (Fig. 5).

Rearing showed different effects on individual resistance and disinfectant gene abundance in gut and residue

Gut and feed residue samples were screened for the presence of extended-spectrum β-lactamase (ESBL) genes including bla_{SHV}, $bla_{CTX\text{-}M}$, and bla_{TEM}, using a standard multiplex PCR assay (Table 2). Strong PCR products were detected for bla_{SHV} and weaker PCR products for $bla_{CTX\text{-}M}$ in all gut samples from stage S1, as well as individual replicates of samples from stage S2 and pupae (Supplementary Fig. 2). Two of the S3 gut samples also generated weak PCR products for $bla_{CTX\text{-}M}$. We did not detect bla_{TEM} genes in any of the gut samples. Similarly, strong bla_{SHV} and weaker $bla_{CTX\text{-}M}$ PCR products were detected in all feed residue samples from stage S1, in two of three samples from stage S2, but in none of the samples from stage S3 larvae or pupae.

The abundance of bla_{SHV} genes was then determined by qPCR. This gene family was most abundant in the gut microbiome at stage 1 with 2.5 ($\pm$ 2.8) $\times 10^6$ copies per gram of gut tissue and decreased during the rearing process to reach 2.2 ($\pm$ 3.7) $\times 10^4$ copies g^{-1} in the pupae (Table 3). In the feed residue bla_{SHV} gene was present at stage S1 and S2, but not detected at stage S3. To compare the abundance of resistance genes across samples and stages, the copy number of the bla_{SHV} gene was normalized to that of the bacterial 16S rRNA genes when both targets were quantified in parallel. This

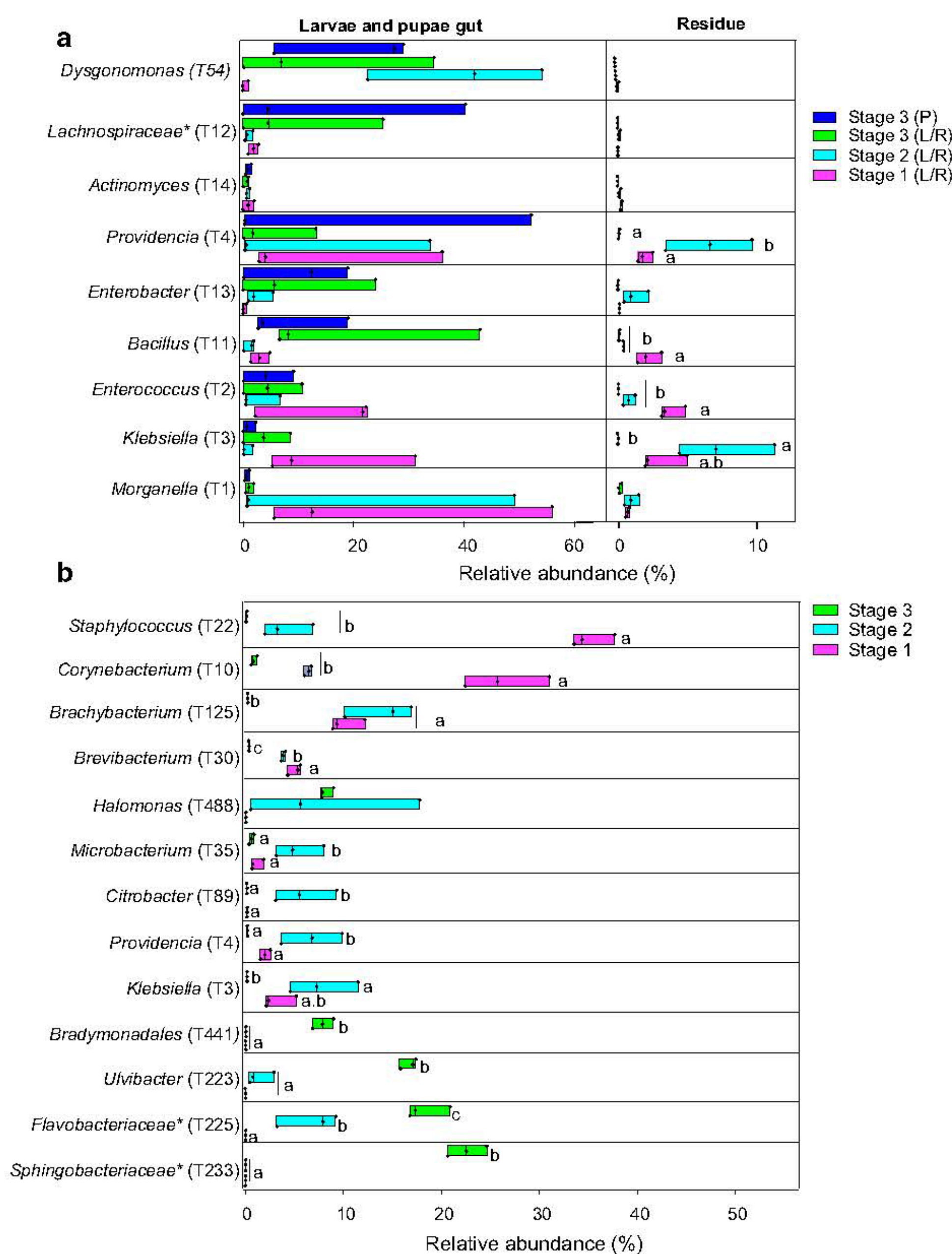

Fig. 6 Relative abundances of the gut core microbiota in gut samples of larvae and pupae and residues of the applied substrate studied at three different rearing stages. Box plots base on values of three biological replicates. Differences between the samples were calculated using one-way ANOVA based on the Tukey's multiple comparison test (SigmaPlot). Different letters represent significant differences. L: Larvae; P: pupae; R: residue; 1, 2, 3: different studied growth stages (see also Supplementary Fig. 1)

revealed no significant differences when comparing the larval and pupal gut microbiomes or the microbial community in the feed residue at stages S1 and S2 (Fig. 7a).

The qPCR based analysis of the tetracycline (*tetM*) and sulfonamide (*sul2*) resistance genes showed the presence of both genes in all gut and feed residue samples. The abundance of *tetM* was in the range of 10^7–10^8 copies g^{-1}, and the abundance of *sul2* in the range of 10^7–10^9 copies g^{-1} (Table 3). As above, we normalized the copy number of these genes to that of the bacterial 16S rRNA genes when both targets were quantified in parallel, revealing that the normalized abundance of *tetM* remained stable in the gut and feed residue samples (ANOVA; $p > 0.05$) (Fig. 7b), whereas the normalized abundance of *sul2* in the larval gut remained stable, but increased significantly from stage S2 to S3 of the feed residue samples (ANOVA $p < 0.05$) (Fig. 7c).

Table 2 Endpoint detection of bla_{TEM}, $bla_{CTX\text{-}M}$, and bla_{SHV} in a multiplex-PCR approach

	L1			L2			L3			P			R1			R2			R3		
	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c
bla_{TEM}	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−
$bla_{CTX\text{-}M}$	+	−	+	+	−	−	−	−	−	+	−	−	−	−	+	+	−	+	−	−	−
bla_{SHV}	+	+	+	+	−	−	−	−	−	+	−	−	+	+	+	+	−	+	−	−	−

+: DNA band of the respective PCR product visible in the agarose gel after ethidium bromide staining. −: no visible PCR Product detected. a, b, c: biological replicates, e.g. individual larvae/pupae or substrate spots

Table 3 Abundance of the 16S rRNA and antibiotic-resistance genes (bla_{SHV}, *tetM, sul2, and mecA*) and a disinfectant-resistance gene (*qacE*) in the larval and pupal gut and in feed residues at three different rearing stages

Sample	16S rRNA	*blaSHV*	*tetM*	*sul2*	*mecA*	*qacE*
L1	$2.3 (\pm 0.5) \times 10^{9}$	$2.5 (\pm 2.8) \times 10^{6}$	$1.8 (\pm 0.3) \times 10^{7}$	$3.6 (\pm 5.3) \times 10^{7}$	–	–
L2	$9.9 (\pm 7.0) \times 10^{9}$	$2.5 (\pm 4.1) \times 10^{4}$	$0.8 (\pm 1.0) \times 10^{8}$	$2.1 (\pm 2.2) \times 10^{7}$	–	–
L3	$3.4 (\pm 1.7) \times 10^{9}$	$1.6 (\pm 2.8) \times 10^{3}$	$7.2 (\pm 5.8) \times 10^{7}$	$4.3 (\pm 6.2) \times 10^{6}$	–	–
P	$1.5 (\pm 1.2) \times 10^{10}$	$2.2 (\pm 3.7) \times 10^{4}$	$2.5 (\pm 3.4) \times 10^{8}$	$6.4 (\pm 4.6) \times 10^{7}$	–	–
R1	$3.2 (\pm 0.7) \times 10^{10}$	$2.8 (\pm 1.6) \times 10^{6}$	$2.7 (\pm 0.2) \times 10^{7}$	$1.5 (\pm 1.0) \times 10^{7}$	$1.3 (\pm 0.1) \times 10^{8}$	$2.1 (\pm 0.3) \times 10^{7}$
R2	$1.7 (\pm 0.8) \times 10^{10}$	$2.8 (\pm 2.2) \times 10^{6}$	$9.0 (\pm 3.0) \times 10^{7}$	$3.3 (\pm 2.1) \times 10^{8}$	$2.5 (\pm 0.8) \times 10^{6}$	$8.8 (\pm 5.2) \times 10^{8}$
R3	$4.4 (\pm 0.9) \times 10^{10}$	–	$2.6 (\pm 2.8) \times 10^{8}$	$2.9 (\pm 0.3) \times 10^{9}$	$8.0 (\pm 1.9) \times 10^{6}$	$5.0 (\pm 1.5) \times 10^{9}$

Concentrations are shown as gene copies per gram fresh weight of gut tissue or feed residue. Data are means ± standard deviations of n = 3 biological replicates (three insects or three independent blobs of feed residue)

The major methicillin-resistance gene in staphylococci and enterococci (*mecA*) was not detected in gut samples but $\sim 10^{8}$ copies g^{-1} were detected in the feed residue at stage S1, falling to $\sim 10^{6}$ copies per gram at stages S2 and S3 (Table 3). Normalized to the bacterial 16S rRNA gene copy number, the *mecA* gene was significantly less abundant at stages S2/S3 compared to stage S1 (Fig. 7d). The disinfectant-resistance gene *qacE/qacEΔ1* was not detected in any of the gut samples but $\sim 10^{7}$ copies per gram were detected in the feed residue at stage S1 increasing to 10^{9} copies per gram by stage S3. Normalized to the bacterial 16S rRNA gene copy number, the *qacE/qacEΔ1* gene became significantly more abundant during rearing from stage S1 to S3 (ANOVA $p < 0.05$) (Fig. 7e).

Discussion

State of the art in comparative analysis of gut and feed residue bacterial communities during *H. illucens* rearing

Little is still known about the dynamic in the phylogenetic composition of the bacterial communities in the gut and feed residue during the rearing of *H. illucens* larvae, including the antibiotic/detergent-resistance gene profiles. However, both are important

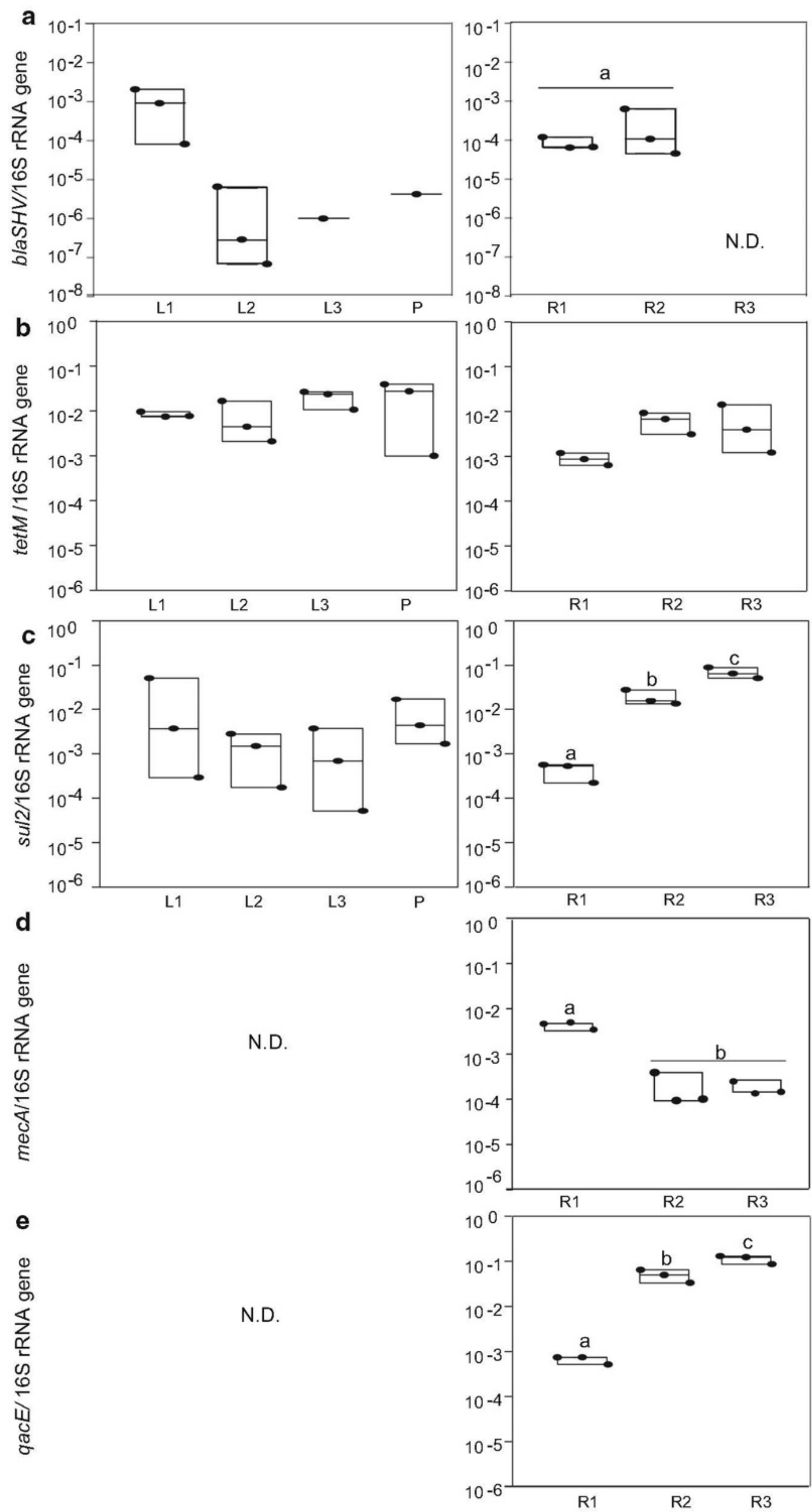

Fig. 7 Relative abundance of antibiotic resistance (*blaSHV*, *tet*M, *sul*2, *mecA*) and disinfectant (*qacE*) genes in gut samples of *H. illucens* larvae and pupae and residue samples of the employed substrate at the different rearing stages. The concentrations of genes (copy numbers g^{-1}) determine by qPCR were normalized to concentration of 16S rRNA genes determined for the same samples. Three independent biological replicates were analyzed. Statistically significant differences between gut and residue samples were tested by one-way ANOVA based on the Tukey test (SigmaPlot). Different letters at top of the box-plots represent significant differences ($p < 0.05$). L: Larvae; P: pupae; R: residue; 1, 2, 3: different studied growth stages (see also Supplementary Fig. 1). *mecA* and *qacE* were not detectable in gut samples

safety factors when evaluating the use of *H. illucens* larvae to convert organic waste, potentially loaded with pathogenic and antibiotic-resistant bacteria, into feed supplements for livestock and fish. We therefore designed a conceptually simple experiment to determine the impact of the rearing process. We analysed the gut and feed residue bacterial communities at three larval developmental stages as well as in pupae which are the final product of the rearing process for use as e.g. animal feed.

Only few previous studies have investigated bacterial communities in both, the gut and the feed residue of *H. illucens* rearing. Changes in the gut microbiota have been studied in the context of different feed substrates (Bruno et al. 2019; Jeon et al. 2011; Wynants et al. 2019), different growth stages (Zheng et al. 2013; Jiang et al. 2019), and different rearing cycles (Wynants et al. 2019). These studies variously considered whole larvae (Zheng et al. 2013), the entire gut, as well as midgut and hindgut sections (Jeon et al. 2011; Cai et al. 2018; Jiang et al. 2019; Wynants et al. 2019), or different parts of the midgut (Bruno et al. 2019). Only Bruno et al. (2019) and Wynants et al. (2019) also studied the microbial community in the initial feed substrate and final residue, whereas only Jiang et al. (2019) tested the feed residue throughput the rearing process.

The core microbiome of the *H. illucens* larval gut

Most previous studies have described a recurring set of abundant taxa in the *H. illucens* larval gut during rearing, regardless of the composition of the samples or the rearing conditions. Likewise, we determined the most abundant phylogenetic groups in the guts of larvae and pupae, which included *Dysgonomonas* (0–35.8%), *Morganella* (0.6–24.7%), *Enterococcus* (2.6–15.4%), *Providencia* (3.6–13.0%), *Klebsiella* (0.7–15.1%), uncultured *Lachnospiraceae* (0.7–11%), *Clostridium* sensu stricto 7 (0–6.7%), *Stenotrophomonas* (0–6.4%), *Bacillus* (0.6–8.3%), and *Bacteroides* (0–4.9%). Zheng et al. (2013) also reported *Providencia, Bacteroides,* and *Dysgonomonas* among the most abundant taxa (> 50% relative abundance) across all *H. illucens* life cycles. Furthermore, they found that *Enterobacteriaceae* was an abundant family at all development stages, raising safety concerns given the pathogenicity of several members of this family. Cai et al. (2018) found that *Morganella*, *Enterococcus*, *Dysgonomonas*, *Ignatzschneria,* and *Providencia* were the most abundant taxa in the gut of larvae reared for 12 days on chicken manure. *Morganella*, *Providencia*, *Enterococcus*, *Pseudomonas,* and *Bacillus* were also the most abundant genera of gut symbionts identified by Wynants et al. (2019). The same authors also identified three abundant OTUs groups as *Dysgonomonas*. Finally, *Dysgonomonas, Bacillus, Lactobacillales, Enterococcus,* and *Ureibacillus* were the most abundant taxa in the gut of the larvae after 10 days of vermicomposting (Jiang et al. 2019).

The functions of these recurring taxa in the larval gut are unknown, prompting Wynants et al. (2019) to exclude them from the definition of a "core microbiome" because this concept is based on functional definitions in other systems. Indeed, few studies have discussed the potential function of individual reoccurring abundant gut taxa. Co-occurrence network analysis of the gut and feed substrate bacteria identified as *Dysgonomonas* by Jiang et al. (2019) revealed this group as a hub taxon, indicating a central role in the gut microbiome interacting with other, less-abundant taxa. *Dysgonomonas* may facilitate the degradation of complex polysaccharides by *H. illucens* larvae, although other bacteria assigned to the *Bacteroidetes* may also fulfil this function (Bruno et al. 2019). They have also been detected as abundant bacteria in the guts of other larvae, including those of the red palm weevil *Rhynchophorus ferrugineus* Olivier (Tagliavia et al. 2014), the bamboo snout beetle *Cyrtotrachelus buqueti* (Luo et al. 2019), various cockroaches, as well as lower and higher wood-feeding and fungus-feeding termites (Otani et al. 2014).

Providencia and *Enterococcus* were described as abundant taxa in the gut of *H. illucens* larvae but were also enriched and metabolically active in the feed residue during vermicomposting (Jiang et al. 2019). The authors linked those taxa to carbohydrate-active enzymes, hydrogen metabolism, the nitrogen cycle, and sulphur compound metabolism. Putative CAZy genes required for lignocellulose degradation, including glycoside hydrolase and carbohydrate esterase, have been detected in the *Dysgonomonas* and *Enterococcus* strains isolated from larval guts of the beetle *C. buqueti* (Luo et al. 2019). Given its presence in all stages of *H. illucens* development from eggs to pupae, *Providencia* was proposed to be vertically transmitted (Zheng et al. 2013; Bruno et al. 2019). Given their

likely positive role in efficient substrate digestion, both *Providencia* and *Enterococcus,* include human pathogens (O'Hara et al. 2000; Ike 2017) and the former also includes insect pathogens (Galac and Lazzaro 2012; Msaad et al. 2018).

Comparing the gut microbiome of S3 larvae and pupae revealed a transformation in the composition of the bacterial community. Ten different phylogenetic groups showed a high relative abundance (> 1%) specifically in the pupal gut, but were not detected in the larval gut or feed residue. Such rapid changes in the gut microbiota are likely to reflect the renewal of the gut and other organs during metamorphosis, although the roles of the bacterial taxa in this process are unclear (Johnston and Rolff 2015).

It is important to note that we analysed total gut samples, which made it difficult to match relative abundances to specific functions. As previously shown by Bruno et al. (2019), different gut sections are colonized by different bacterial species, which react differently to the feed substrate and the environmental bacteria contained therein. However, our data help to determine the overall abundance of potentially pathogenic bacteria, which as discussed above is an important safety factor when *H. illucens* larvae or pupae are used as animal feed supplements. Comparison of bacterial abundances between previously reported studies may yield certain differences in values, since the difference in treating the sample varies in each study. As well Bruno et al. (2019), reported that the use of different diets can affect the bacterial composition of the gut.

Effects of *H. illucens* larvael rearing on bacterial communities in the feed residue

Bruno et al. (2019) and Wynants et al. (2019) studied the feed substrate/residue communities at the beginning and end of the rearing of *H. illucens* larvae. Wynants et al. (2019) concluded that differences in rearing conditions, rearing locations, and the bacterial community in the initial substrate could all affect the dynamic phylogenetic profile of the feed residue (De Smet et al. 2018; Wynants et al. 2019).

We determined the phylogenetic profile of the feed residue during rearing by recording significant changes in (1) the abundance of bacterial 16S rRNA genes and (2) the alpha and (3) beta diversity (community composition) determined by 16S rRNA gene amplicon sequencing. This allowed us to identify trends during the rearing process as well as changes specific for each rearing stage. We revealed a significant increase in diversity and taxonomic richness in the final residue compared to the original feed substrate. In contrast, Wynants et al. (2019) and Jiang et al. (2019) reported a decrease in richness, whereas Bruno et al. (2019) did not observe any significant effects on the taxonomic richness of the substrate residue. Different parameters were elucidated in previous studies which can have an impact on the taxonomic richness and bacterial community dynamic during rearing (Wynants et al. 2019; Jiang et al. 2019; Bruno et al. 2019). The type of the rearing process, continuous or two phase processes, and the type of the substrate with different initial bacterial loads can have a strong impact on community dynamics. Beside, changing intrinsic factors during rearing, as water activity, moisture content, and pH of substrate and residue, can also have a different impact on the taxonomic richness and dynamic of bacterial communities in the *H. illucens* rearing systems. Here, in contrast to the other studies a stationary rearing process was applied. This hindered a direct comparison of our results on taxonomic richness to those obtained in the other studies (Wynants et al. 2019; Jiang et al. 2019; Bruno et al. 2019). Because intrinsic factors were not determined for substrate and residue samples during rearing we have no information on the impact of intrinsic factors on the changes of the taxonomic richness observed in our experiment yet.

As a general trend, we found that the most abundant bacterial taxa in the feed at stage S1 had declined sharply in stage S3. This included several Gram-positive taxa corresponding to potential pathogens, such as the genera *Staphylococcus* (declining from 34–38% to 0%), *Corynebacterium* (from 22–30% to 1%), *Brevibacterium* (from 4.3–5.3% to 0%), *Enterococcus* (from 4–6% to 0%), and *Bacillus* (from 1–3% to 0%). *Enterobacteriaceae* (containing several genera with pathogens, including *Salmonella*) were only abundant at stage S2, and got significantly depleted thereafter in stage 3. In contrast, Wynants et al. (2019) showed that potential pathogens such as *Salmonella* spp. and *Bacillus cereus* were present in some feed residue samples and also in larvae and pupae, thus raising safety concerns for the production of supplements for livestock and fish. Differences in the obtained results and time point specific increased

relative abundances of potential pathogens (*Enterobacteriaceae* in our case) during rearing demonstrates the need to study the dynamic phylogenetic profile during the rearing process, to understand in detail the impact on *H. illucens* larvae on bacterial communities present in the feed.

In contrast to our study, Bruno et al. (2019) found that *H. illucens* larvae had no significant effect on the bacterial community in the feed between the initial substrate and the endpoint residue. However, Jiang et al. (2019) reported progressive changes in the bacterial community of the feed residue, and Cai et al. (2018) found, in agreement with our results, that the rearing process of *H. illucens* larvae caused a significant depletion of the highly abundant taxa present in the original substrate, especially potential human pathogens. The depletion of pathogens has also been demonstrated in spiking experiments with *E. coli* and *Salmonella* spp. (Erickson et al. 2004; Lalander et al. 2013; Liu et al. 2008), but not when feed was spiked with *Enterococcus faecalis*, which was not depleted during rearing (Lalander et al. 2013).

The production of antimicrobial compounds by *H. illucens* larvae or their gut bacteria may contribute to the depletion of potentially pathogenic bacteria in the feed. Park and Yoe (2017a) studied a peptide derived from the *H. illucens* larval gut which showed selective activity against Gram-negative bacteria such as *E. coli, Pseudomonas aeruginosa* and *Enterobacter aerogenes*, but not against Gram-positive bacteria such as *Staphylococcus aureus*, especially methicillin-resistant strains (MRSA), and *Staphylococcus epidermidis*. However, another peptide from the same source was active against both Gram-negative and Gram-positive bacteria (Park and Yoe 2017b). Water-soluble extracts from *H. illucens* larvae were previously shown to be active against both, Gram-negative and Gram-positive bacteria (Park et al. 2014), whereas methanol extracts were only active against Gram-negative species (Park et al. 2014; Choi and Jiang 2014). However, acidic methanol extracts obtained from immunized larvae were found to be active against both Gram-negative and Gram-positive species (Park et al. 2014). The ability of *H. illucens* larvae to (1) produce antimicrobial compounds with different activity ranges and (2) modulate the phylogenetic profile of bacteria in the feed residue during the rearing process suggests that the two phenomena may be causally linked. More detailed studies are needed to understand the nature of the interactions between *H. illucens* larvae, their gut microbiome, and bacteria present in the feed substrate and residues.

The abundance of antibiotic/disinfectant-resistance genes during the *H. illucens* rearing process

An important safety aspect neglected in most of the *H. illucens* studies discussed above is the potential transfer of antibiotic-resistant bacteria from the feed substrate to the larvae, which in turn could transmit the bacteria to livestock or fish. The profile of antibiotic-resistance genes during the rearing of *H. illucens* larvae has been addressed infrequently. Cai et al. (2018) reported the more efficient depletion of tetracycline-resistance and integrase (*int*) genes in a spiked feed substrate during the rearing of non-sterile compared to sterile larvae, suggesting the gut microbiota played an important role. However, the underlying mechanisms have not been investigated and the impact of *H. illucens* larvae on further antibiotic/disinfectant-resistance genes is unknown.

A more detailed study was conducted on common housefly larvae during the vermicomposting of manure, revealing that larval rearing depleted a broad range of antibiotic-resistance genes and was more efficient than traditional composting (Wang et al. 2015, 2016). However, the authors reported that some antibiotic-resistance genes (*ampC* and $bla_{\text{CTX-M}}$), not detected in the original manure, were present in the residual feed, and there was a higher rate of horizontal gene transfer to the gut microbiome, thus raising safety issues about the use of *H. illucens* to prepare feed supplements for livestock. The depletion of antibiotic-resistance genes during *H. illucens* rearing was correlated with the dynamic phylogenetic changes in the microbial community (Wang et al. 2016).

To gain more insight into the effect of *H. illucens* larvae on antibiotic/disinfectant-resistance genes, we determined the abundance of genes conferring resistance to tetracyclines (*tetM*), sulphonamides (*sul2*), cephalosporin (bla_{SHV}), methicillin (*mecA*) and quaternary ammonium compounds (*qacE*) and compared changes in their abundance (normalized to the presence of bacterial 16S rRNA gene copies) to changes in the relative abundance of potential carriers in the microbial communities of the gut and feed residue. Neither *tetM* nor *sul2* showed a significant decrease in

total abundance (copies per gram) or relative abundance (normalized to bacterial 16S rRNA gene copies) in the gut during the rearing process. In contrast, Cai et al. (2018) reported the depletion of *tet* genes during the rearing of *H. illucens* larvae. Both genes have been detected in a range of Gram-negative and Gram-positive bacteria, and because we were unable to determine which phylogenetic group in our communities carried these genes, it is not possible to determine why the results of our study differed from the results reported by Cai et al. (2018). We did not investigate the presence of antibiotics in our feed residues, whereas Cai et al. (2018) found that the presence of tetracycline in feed substrates significantly increased the abundance of different tetracycline-resistance genes including *tetM* in the gut of *H. illucens* larvae. The analysis of the gut microbiota in larvae exposed to tetracycline demonstrated that some of the gut core taxa increased in relative abundance (e.g., *Dysgonomonas, Morganella, Bacteroides,* and *Bacillus*) whereas others were slightly depleted (e.g., *Pseudomonas, Klebsiella, Providencia*, and *Enterococcus*) (Cai et al. 2018). The high abundance of antibiotic-resistance genes was recently noted when profiling a *Dysgonomonas* strain isolated from the faeces of a healthy human. We also detected other bacteria that have been reported to carry *tetM*, including *Enterococcus* (Channaiah et al. 2010) and *Pseudomonas* (Wang et al. 2016) in the gut microbiome. The presence of *sul2* was previously attributed to the genera *Pseudomonas* and *Bacillus* (Wang et al. 2014), which were also abundant in our rearing system.

We also determined the abundance of genes conferring resistance to cephalosporins and methicillin, which provided a better correlation with the phylogenetic profiles. The bla_{SHV} and $bla_{CTX\text{-}M}$ genes are carried on plasmids by several *Enterobacteriaceae* which were abundant in both the gut and feed residues we tested, including *Morganella*, *Klebsiella*, *Citrobacter*, *Proteus*, *Providencia,* and *Enterobacter* (Schauss et al. 2015; Sanjit Singh et al. 2017). *Enterobacteriaceae* and bla_{SHV} genes were continuously present in the gut microbiome of the *H. illucens* larvae and pupae, but both the abundant *Enterobacteriaceae* and the bla_{SHV} genes were depleted in the feed residue during the last stages of rearing. Unlike the other genes we analysed, *mecA* is predominantly found in *Staphylococcus* spp., and accordingly we found that the abundance of the *mecA* gene in our system showed a strong correlation to the relative abundance of staphylococci during the rearing process. Both were highly abundant in the feed residue at the S1 stage and then showed a parallel decline during the subsequent rearing stages.

The *qacE/qacEΔ1* genes confer resistance to quaternary ammonium compounds, which are widely used as disinfectants in the livestock industry (Mulder et al. 2018). Interestingly, *qacE* genes were not detected in the gut microbiome but their abundance in the feed residue increase significantly during the rearing process as *sul2* also did, both belonging to the class 1 Integrons. The *qacE/qacEΔ1* genes have been detected in many Gram-positive and Gram-negative bacteria, including the genera *Staphylococcus, Pseudomonas, Corynebacterium,* and *Aeromonas*, as well as various members of the *Enterobacteriaceae*, and they are often linked to antibiotic-resistance genes on plasmids (Gaze et al. 2005; Nardelli et al. 2012; Jechalke et al. 2014; Mulder et al. 2018). The bacteria carrying these important genes in our rearing system have yet to be identified. Our data so far showed that however *qac* genes were abundant and even increased in abundance in the feed residue they were not transferred to the gut microbiome. However, in our study disinfectants were not used. It needs to be elucidated in further studies if the presence of disinfectants in the feed can induce the transfer of *qac* genes to the gut microbiome. Specific spiking experiments are planned to investigate that in detail.

This initial study of *H. illucens* rearing was performed in the absence of added antibiotics. Although some studies report natural resistance to a broad spectrum of antibiotics without the need for exposure, as in the case of the *Helaeomiyia petrolei* larva due to the presence of *Providencia* strains (Knöppel et al. 2017) we also have to consider the detection of pseudogenes may be possible as Davis et al. (2011) reports in his study. On the other hand it is known that several bacteria (Ayuso-Sacido and Genilloud 2005; Svercel et al. 2009; Mora et al. 2011) and *H. illucens* itself produce antimicrobial peptides (Elhag et al. 2017; Park et al. 2014; Park and Yoe 2017a, b), the link between the increment of some antimicrobial-resistant genes can be attributed due to the levels of the antimicrobial peptides during the rearing process as Knöppel et al. (2017) suggest with

the presence of cross-resistance and/or collateral sensitivity.

Conclusion

Our study showed that the rearing of *H. illucens* larvae has a strong impact on the bacterial communities in the feed residue whereas the gut microbiome remains more stable, with a set of abundant taxa that are persistent throughout development and may be considered as the gut core microbiome. However, the functions of different taxa in the gut microbiome are yet not understood in detail. Importantly, several of these abundant taxa include pathogenic species, which raises safety concerns if *H. illucens* larvae are used to prepare animal feed supplements. As already pointed out by Wynants et al. (2019) the detection of potential foodborne pathogens such as *Salmonella* and *Bacillus cereus* in some *H. illucens* larvae and/or residue samples, indicated the necessity to emphasize decontamination technologies. Furthermore, the prevalence of antibiotic/disinfectant-resistance genes revealed by our tests indicates that this is another safety factor that must be evaluated carefully. The risk posed by antibiotic/disinfectant-resistance genes is likely to be determined by the presence of antibiotics and corresponding resistant bacterial strains in the feed substrate, and the widespread use of disinfectants in animal husbandry practices. The feed substrates and rearing practices used with *H. illucens* should therefore be considered in order to ensure that the process can be used to convert organic waste into safe and nutritious feed supplements. We performed in this study just a preliminary rearing experiment. More experiments with defined concentrations of antibiotics and disinfections are required to elucidate the consequences of the presence of those compounds during rearing in more detail. Here commercial chicken feed a common *H. illucens* rearing substrate, was used to get a first impression on the dynamics of the feed and gut microbiota. It was not expected that this substrate contains or may just contain minor amounts of pathogens and antibiotic/disinfectant resistance bacteria. Further rearing experiments will be performed with manure which contains high loads of pathogens and antimicrobial and disinfectant resistant bacteria.

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Authors' contributions SG, JM, YC designed the study, AM and HOG provided samples, YC, JM, J-OB, and AM performed research, YC, JM, and J-OB analysed data, SG, YC wrote the paper which was proofed by all co-authors, AV and PK received the funding.

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Compliance with ethical standards

Conflict of interest There are no conflict of interests.

References

Anderson MJ (2001) A new method for non-parametric multivariate analysis of variance. Austral Ecol 26:32–46. https://doi.org/10.1111/j.1442-9993.2001.01070.pp.x

Anderson M, Gorley R, Clarke K (2008) "PERMANOVA + for PRIMER: Guide to software and statistical methods" in PRIMER-E. PRIMER-E, Plymouth

Aydogan EL, Moser G, Müller C, Kämpfer P, Glaeser SP (2018) Long-term warming shifts the composition of bacterial communities in the phyllosphere of *Galium album* in a permanent grassland field-experiment. Front Microbiol 9:144

Ayuso-Sacido A, Genilloud O (2005) New PCR primers for the screening of NRPS and PKS-I systems in actinomycetes: detection and distribution of these biosynthetic gene sequences in major taxonomic groups. Microb Ecol 49:10–24. https://doi.org/10.1007/s00248-004-0249-6

Barroso FG, Sánchez-Muros MJ, Segura M, Morote E, Torres A, Ramos R, Guil JL (2017) Insects as food: enrichment of larvae of *Hermetia illucens* with omega 3 fatty acids by means of dietary modifications. J Food Compost Anal 62:8–13. https://doi.org/10.1016/j.jfca.2017.04.00

Beskin KV, Holcomb CD, Cammack JA, Crippen TL, Knap AH, Sweet ST, Tomberlin JK (2018) Larval digestion of different manure types by the black soldier fly (Diptera: Stratiomyidae) impacts associated volatile emissions. Waste Manag 74:213–220. https://doi.org/10.1016/j.wasman.2018.01.019

Bray JR, Curtis JT (1957) An ordination of the upland forest communities of Southern Wisconsin. Ecol Monogr 27:325–349. https://doi.org/10.2307/1942268

Bruno D, Bonelli M, De Filippis F, Di Lelio I, Tettamanti G, Casartelli M, Ercolini D, Caccia S (2019) The intestinal microbiota of *Hermetia illucens* larvae is affected by diet and shows a diverse composition in the different midgut regions. Appl Environ Microbiol 85:e01864-18. https://doi.org/10.1128/AEM.01864-18

Cai M, Ma S, Hu R, Tomberlin J, Yu C, Huang Y, Zhan S, Yu Z, Zhang J (2018) Systematic characterization and proposed pathway of tetracycline degradation in solid waste treatment by *Hermetia illucens* with intestinal microbiota. Environ Pollut 242:634–642. https://doi.org/10.1016/j.envpol.2018.06.105

Channaiah LH, Subramanyam B, McKinney LJ, Zurek L (2010) Stored-product insects carry antibiotic-resistant and potentially virulent enterococci. FEMS Microbiol Ecol 74:464–471. https://doi.org/10.1111/j.1574-6941.2010.00949.x

Choi WH, Jiang M (2014) Evaluation of antibacterial activity of hexanedioic acid isolated from *Hermetia illucens* larvae. J Appl Biomed 12:179–189. https://doi.org/10.1016/j.jab.2014.01.003

Clarke KR (1993) Non-parametric multivariate analyses of changes in community structure. Aust J Ecol 18:117–143. https://doi.org/10.1111/j.1442-9993.1993.tb00438.x

Clarke KR, Gorley RN (2015) PRIMER v6: user manual/tutorial (plymouth routines in multivariate ecological research). PRIMER-E, Plymouth

Committee ES (2015) Risk profile related to production and consumption of insects as food and feed. EFSA J 13:4257. https://doi.org/10.2903/j.efsa.2015.4257

Davis MA, Besser TE, Orfe LH, Baker KNK, Lanier AS, Broschat SL, New D, Call DR (2011) Genotypic-phenotypic discrepancies between antibiotic resistance characteristics of *Escherichia coli* isolates from calves in management settings with high and low antibiotic use. Appl Environ Microbiol 77:3293–3299. https://doi.org/10.1128/AEM.02588-10

De Smet J, Wynants E, Cos P, Van Campenhout L (2018) Microbial community dynamics during rearing of black soldier fly larvae *Hermetia illucens* and impact on exploitation potential. Appl Environ Microbiol 84:e02722-17. https://doi.org/10.1128/AEM.02722-17

Elhag O, Zhou D, Song Q, Soomro AA, Cai M, Zheng L, Yu Z, Zhang J (2017) Screening, expression, purification and functional characterization of novel antimicrobial peptide genes from *Hermetia illucens* (L.). PLoS ONE 2:e0169582. https://doi.org/10.1371/journal.pone.0169582

Engel P, Moran NA (2013) The gut microbiota of insects—diversity in structure and function. FEMS Microbiol Rev 37:699–735. https://doi.org/10.1111/1574-6976.12025

Erickson M, Sheppard C, Liao J, Doyle M (2004) Reduction of *Escherichia coli* O157:H7 and *Salmonella enterica* serovar enteritidis in chicken manure by larvae of the black soldier fly. J Food Prot 67:685–690. https://doi.org/10.4315/0362-028X-67.4.685

Galac MR, Lazzaro BP (2012) Comparative genomics of bacteria in the genus Providencia isolated from wild *Drosophila melanogaster*. BMC Genom 13:612. https://doi.org/10.1186/1471-2164-13-612

Gaze WH, Abdouslam N, Hawkey PM, Wellington EMH (2005) Incidence of class 1 integrons in a quaternary ammonium compound-polluted environment. Antimicrob Agents Chemother 49:1802–1807. https://doi.org/10.1128/AAC.49.5.1802-1807.2005

Glaeser SP, Sowinsky O, Brunner JS, Dott W, Kämpfer P (2016) Cultivation of vancomycin-resistant enterococci and methicillin-resistant staphylococci from input and output samples of German biogas plants. FEMS Microbiol Ecol 92:fiw010. https://doi.org/10.1093/femsec/fiw010

Hammer O, Harper D, Ryan P (2001) PAST: paleontological statistics software package for education and data analysis. Palaeontol Electron 4:1–9

Harper DAT (ed) (1999) Numerical palaeobiology. Wiley, Hoboken

Heuer H, Schmitt H, Smalla K (2011) Antibiotic resistance gene spread due to manure application on agricultural fields. Curr Opin Microbiol 14:236–243. https://doi.org/10.1016/j.mib.2011.04.009

Ike Y (2017) Pathogenicity of Enterococci. Nihon Saikingaku Zasshi. Rev Jpn 72:189–211. https://doi.org/10.3412/jsb.72.189

Jechalke S, Schreiter S, Wolters B, Dealtry S, Heuer H, Smalla K (2014) Widespread dissemination of class 1 integron components in soils and related ecosystems as revealed by cultivation-independent analysis. Front Microbiol 4:420

Jeon H, Park S, Choi J, Jeong G, Lee SB, Choi Y, Lee SJ (2011) The intestinal bacterial community in the food waste-reducing larvae of *Hermetia illucens*. Curr Microbiol 62:1390–1399. https://doi.org/10.1007/s00284-011-9874-8

Jiang C, Jin W, Tao X, Zhang Q, Zhu J, Feng S, Xu X, Li H, Wang Z, Zhang Z (2019) Black soldier fly larvae (*Hermetia illucens*) strengthen the metabolic function of food waste biodegradation by gut microbiota. Microb Biotechnol 12:528–543. https://doi.org/10.1111/1751-7915.13393

Johnston PR, Rolff J (2015) Host and symbiont jointly control gut microbiota during complete metamorphosis. PLoS Pathog 11:e1005246. https://doi.org/10.1371/journal.ppat.1005246

Klindworth A, Pruesse E, Schweer T, Peplies J, Quast C, Horn M, Glöckner FO (2012) Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Res 41:e1. https://doi.org/10.1093/nar/gks808

Knöppel A, Näsvall J, Andersson DI (2017) Evolution of antibiotic resistance without antibiotic exposure. Antimicrob Agents Chemother 61:e01495-17. https://doi.org/10.1128/AAC.01495-1

Kolb S, Knief C, Stubner S, Conrad R (2003) Quantitative detection of methanotrophs in soil by novel pmoA-targeted real-time PCR assays. Appl Environ Microbiol 69:2423–2429. https://doi.org/10.1128/AEM.69.5.2423-2429.2003

Lalander C, Diener S, Magri M, Zurbrügg C, Lindström A, Vinnerås B (2013) Faecal sludge management with the larvae of the black soldier fly (*Hermetia illucens*)—from a hygiene aspect. Sci Total Environ 458–460:312–318. https://doi.org/10.1016/j.scitotenv.2013.04.033

Liland NS, Biancarosa I, Araujo P, Biemans D, Bruckner CG, Waagbø R, Torstensen B, Lock EJ (2017) Modulation of nutrient composition of black soldier fly (*Hermetia illucens*) larvae by feeding seaweed-enriched media. PLoS

ONE 12:e0183188. https://doi.org/10.1371/journal.pone.0183188

Liu Q, Tomberlin JK, Brady JA, Sanford MR, Yu Z (2008) Black soldier fly (Diptera: Stratiomyidae) larvae reduce *Escherichia coli* in dairy manure. Environ Entomol 37:1525–1530. https://doi.org/10.1603/0046-225X-37.6.1525

Luo C, Li Y, Chen Y, Fu C, Long W, Xiao X, Liao H, Yang Y (2019) Bamboo lignocellulose degradation by gut symbiotic microbiota of the bamboo snout beetle *Cyrtotrachelus buqueti*. Biotechnol Biofuels 12:70. https://doi.org/10.1186/s13068-019-1411-1

Mora I, Cabrefiga J, Montesinos E (2011) Antimicrobial peptide genes in *Bacillus* strains from plant environments. Int Microbiol 14(4):213–223. https://doi.org/10.2436/20.1501.01.151

Msaad M, Djobbi W, Charaabi K, Hamden H, Fadhl S, Marzouki W, Dhaouedi F, Chevrier C (2018) Evaluation of *Providencia rettgeri* pathogenicity against laboratory Mediterranean fruit fly strain (*Ceratitis capitata*). PLoS ONE 13:e0196343. https://doi.org/10.1371/journal.pone.0196343

Mulder I, Siemens J, Sentek V, Amelung W, Smalla K, Jechalke S (2018) Quaternary ammonium compounds in soil: implications for antibiotic resistance development. Rev Environ Sci Bio 17:159–185. https://doi.org/10.1007/s11157-017-9457-7

Nardelli M, Scalzo PM, Ramírez MS, Quiroga MP, Cassini MH, Centrón D (2012) Class 1 integrons in environments with different degrees of urbanization. PLoS ONE 7:e39223. https://doi.org/10.1371/journal.pone.0039223

Nguyen TX, Tomberlin J, Vanlaerhoven S (2013) Influence of resources on *Hermetia illucens* (Diptera: Stratiomyidae) larval development. J Med Entomol 50:898–906. https://doi.org/10.1603/ME12260

O'Hara C, Brenner FW, Miller JM (2000) Classification, identification, and clinical significance of Proteus, Providencia, and Morganella. Clin Microbiol Rev 13:534–536. https://doi.org/10.1128/cmr.13.4.534-546.2000

Oonincx D, Huis A, van Loon J (2015) Nutrient utilisation by black soldier flies fed with chicken, pig, or cow manure. J Insects Food Feed 1:131–139. https://doi.org/10.3920/JIFF2014.0023

Otani S, Mikaelyan A, Nobre T, Hansen LH, Koné NA, Sørensen SJ, Aanen DK, Boomsma JJ, Brune A, Poulsen M (2014) Identifying the core microbial community in the gut of fungus-growing termites. Mol Ecol 23:4631–4644. https://doi.org/10.1111/mec.12874

Park SI, Yoe SM (2017a) A novel cecropin-like peptide from black soldier fly, *Hermetia illucens*: isolation, structural and functional characterization. Entomol Res 47:115–124. https://doi.org/10.1111/1748-5967.12226

Park SI, Yoe SM (2017b) Defensin-like peptide3 from black solder fly: identification, characterization, and key amino acids for anti-Gram-negative bacteria. Entomol Res 47:41–47. https://doi.org/10.1111/1748-5967.12214

Park SI, Chang BS, Yoe SM (2014) Detection of antimicrobial substances from larvae of the black soldier fly, *Hermetia illucens* (Diptera: Stratiomyidae). Entomol Res 44:58–64. https://doi.org/10.1111/1748-5967.12050

Przemieniecki SW, Kosewska A, Ciesielski S, Kosewska O (2020) Changes in the gut microbiome and enzymatic profile of *Tenebrio molitor* larvae biodegrading cellulose, polyethylene and polystyrene waste. Environ Pollut 256:113265. https://doi.org/10.1016/j.envpol.2019.113265

Rehman K, Cai M, Xiao X, Zheng L, Wang H, Soomro AA, Zhou Y, Li W, Yu Z, Zhang J (2017) Cellulose decomposition and larval biomass production from the co-digestion of dairy manure and chicken manure by mini-livestock (*Hermetia illucens* L.). J Environ Manag 196:458–465. https://doi.org/10.1016/j.jenvman.2017.03.047

Sanjit Singh A, Lekshmi M, Prakasan S, Nayak BB, Kumar S (2017) Multiple antibiotic-resistant, extended spectrum-β-lactamase (ESBL)-producing enterobacteria in fresh seafood. Microorganisms 5:53. https://doi.org/10.3390/microorganisms5030053

Schauss T, Glaeser SP, Gütschow A, Dott W, Kämpfer P (2015) Improved detection of extended spectrum beta-lactamase (ESBL)-producing *Escherichia coli* in input and output samples of German biogas plants by a selective pre-enrichment procedure. PLoS ONE 10:e0119791. https://doi.org/10.1371/journal.pone.0119791

Spranghers T, Ottoboni M, Klootwijk C, Ovyn A, Deboosere S, Meulenaer B, Michiels J, Eeckhout M, De Clercq P, De Smet S (2017) Nutritional composition of black soldier fly (*Hermetia illucens*) prepupae reared on different organic waste substrates. J Sci Food Agric 97:2594–2600. https://doi.org/10.1002/jsfa.8081

Svercel M, Christen D, Moënne-Loccoz Y, Duffy B, Défago G (2009) Effect of long-term vineyard monoculture on rhizosphere populations of pseudomonads carrying the antimicrobial biosynthetic genes *phlD* and/or *hcnAB*. FEMS Microbiol Ecol 68(1):25–36. https://doi.org/10.1111/j.1574-6941.2009.00649.x

Tagliavia M, Messina E, Manachini B, Cappello S, Quatrini P (2014) The gut microbiota of larvae of *Rhynchophorus ferrugineus* Oliver (Coleoptera: Curculionidae). BMC Microbiol 14:136. https://doi.org/10.1186/1471-2180-14-136

Taguchi Y, Oono Y (2004) Relational patterns of gene expression via non-metric multidimensional scaling analysis. Bioinformatics 21:730–740. https://doi.org/10.1093/bioinformatics/bti067

Teske A, Wawer C, Muyzer G, Ramsing NB (1996) Distribution of sulfate-reducing bacteria in a stratified fjord (Mariager Fjord, Denmark) as evaluated by most-probable-number counts and denaturing gradient gel electrophoresis of PCR-amplified ribosomal DNA fragments. Appl Environ Microbiol 62:1405–1415

Vogel H, Shukla SP, Engl T, Weiss B, Fischer R, Steiger S et al (2017) The digestive and defensive basis of carcass utilization by the burying beetle and its microbiota. Nat Commun 8:15186. https://doi.org/10.1038/ncomms15186

Vogel H, Adam A, Gutzeit H, Vilcinskas A (2018) Nutritional immunology: diversification and diet-dependent expression of antimicrobial peptides in the Black soldier fly *Hermetia illucens*. Dev Comp Immunol 78:141–148. https://doi.org/10.1016/j.dci.2017.09.008

Wang N, Yang X, Jiao S, Zhang J, Ye B, Gao S (2014) Sulfonamide-resistant bacteria and their resistance genes in soils fertilized with manures from Jiangsu Province, Southeastern China. PLoS ONE 9:e112626. https://doi.org/10.1371/journal.pone.0112626

Wang H, Li H, Gilbert JA, Li H, Wu L, Liu M, Wang L, Zhou Q, Yuan J, Zhang Z (2015) Housefly larva vermicomposting efficiently attenuates antibiotic resistance genes in swine manure, with concomitant bacterial population changes. Appl Environ Microbiol 81:7668–7679. https://doi.org/10.1128/AEM.01367-15

Wang H, Sangwan N, Li HY, Su JQ, Ouyang W, Zhang ZJ, Gilbert JA, Zhu YG, Ping F, Zhang HL (2016) The antibiotic resistome of swine manure is significantly altered by association with the *Musca domestica* larvae gut microbiota. ISME J 11:100–111. https://doi.org/10.1038/ismej.2016.103

Wynants E, Frooninckx L, Crauwels S, Verreth C, De Smet J, Sandrock C, Wohlfahrt J, Schelt J, Depraetere S, Lievens B, Miert S, Claes J, Van Campenhout L (2019) Assessing the microbiota of black soldier fly larvae (*Hermetia illucens*) reared on organic waste streams on four different locations at laboratory and large scale. Microb Ecol 77:913–930. https://doi.org/10.1007/s00248-018-1286-x

Xiao X, Mazza L, Yu Y, Cai M, Zheng L, Tomberlin J, Yu J, van Huis A, Yu Z, Fasulo S, Zhang J (2018) Efficient co-conversion process of chicken manure into protein feed and organic fertilizer by *Hermetia illucens* L. (Diptera: Stratiomyidae) larvae and functional bacteria. J Environ Manag 217:668–676. https://doi.org/10.1016/j.jenvman.2018.03.122

Yang Y, Yang J, Wu WM, Zhao J, Song Y, Gao L, Yang R, Jiang L (2015) Biodegradation and mineralization of polystyrene by plastic-eating mealworms: part 2. Role of gut microorganisms. Environ Sci Technol 49(20):12087–12093. https://doi.org/10.1021/acs.est.5b02663

Zheng L, Crippen TL, Singh B, Tarone AM, Dowd S, Yu Z, Wood TK, Tomberlin JK (2013) A survey of bacterial diversity from successive life stages of black soldier fly (Diptera: Stratiomyidae) by using 16S rDNA pyrosequencing. J Med Entomol 50:647–658. https://doi.org/10.1603/ME12199

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CHAPTER III

Isolation of *Hermetia illucens* larvae core gut microbiota by two different cultivation strategies

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Contributions:

YC designed the study; YC, performed the experiments and analysed the data; SG, YC wrote the paper which was proofed by all co-authors; AV and PK received the funding.

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ORIGINAL PAPER

Isolation of *Hermetia illucens* larvae core gut microbiota by two different cultivation strategies

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Abstract *Hermetia illucens* larvae (black soldier fly larvae, BSFL) convert efficiently organic waste to high quality biomass. To gain knowledge on the specific functions of gut microbes in this process it is a prerequisite to culture members of the core gut microbiota. Two different cultivation strategies were applied here for this purpose, a dilution-to-extinction cultivation and direct plating using six different media to culture aerobic heterotrophic bacteria. A total of 341 isolates were obtained by the dilution-to-extinction cultivation and 138 isolates by direct plating from guts of BSFL reared on chicken feed. Bacterial isolates were phylogenetically identified at the genus level by 16S rRNA gene sequencing (phylotyping) and differentiated at the strain level by genomic fingerprinting (genotyping). The main proportion of isolates was assigned to *Proteobacteria*, *Firmicutes* (*Bacilli*), and *Actinobacteria.* Predominant genera discussed in literature as member of a potential BSFL core gut microbiota, *Providencia, Proteus, Morganella, Enterococcus, Bacillus,* and members of the family *Enterobacteriaceae*, were isolated. A high intra-phylotype diversity was obtained by genomic fingerprinting which was especially enhanced by the dilution-to-extinction cultivation. This study showed that the application of different cultivation strategies including a dilution-to-extinction cultivation helps to culture a higher diversity of the BSFL gut microbiota and that genomic fingerprinting gives a better picture on the genetic diversity of cultured bacteria which cannot be covered by a 16S rRNA gene sequence based identification alone.

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Keywords Black soldier fly · Core gut microbiota · Cultivation approach · Dilution-to-extinction · Direct plating, genomic fingerprinting · *Hermetia illucens*

Introduction

Hermetia illucens larvae, known as black soldier fly larvae (BSFL), can efficiently convert a wide range of organic waste products in high quality biomass with high quality proteins and fats. For that reason larvae are increasingly used as animal feed and human food

source (Wang and Shelomi 2017) or for composting (da Silva and Hesselberg 2020). Larval gut microbiota play an important role in the digestion and nutrient provision and for the development, physiology, and the immune system of the larvae (Jeon et al. 2011; Engel and Moran 2013). An important role of the gut microbiota of BLSF during digestion of waste products of animal or human sources is the potential antibacterial activity during digestion which is especially important for the deactivation of pathogenic bacteria present in those substrates (Elhag et al. 2022; Erickson et al. 2004; Liu et al. 2008). The antimicrobial activity of BSFL gut microbes makes their cultivation especially attractive for the research of new antimicrobial compounds (Tegtmeier et al. 2021).

Different studies have already focused on the gut microbiota using mainly molecular cultivation independent methods to explore its community composition (Jeon et al. 2011; Zheng et al. 2013). Changes of the BSFL gut microbiota according to the employed substrate were reported in several publications (Bruno et al. 2019; Gorrens et al. 2021; Jeon et al. 2011; Klammsteiner et al. 2020; Shelomi et al. 2021; Wynants et al. 2019). Some of those studies reported also changes in the bacterial community in the residue of the employed substrates based on the larval digestion process (Bruno et al. 2019; Cifuentes et al. 2020; Wynants et al. 2019; Gold et al. 2020; Shelomi et al. 2021). Shelomi et al. (2021) showed that even through the waste microbiota strongly differed, many taxa were shared in the gut of the differently fed larvae, which indicates the presence of a BSFL specific gut core microbiota. A stable autochthonous bacterial community in the larval gut is one of the general conclusions across all molecular gut microbiota studies. However, there is still no clear consensus among scientists if BSFL have a defined bacterial core gut microbiota and which taxa belong to it (Gorrens et al. 2021). At least 20 genera have been frequently detected in the gut of BSFL reared under different conditions and analysed at different growth stages (Gorrens et al. 2021). As summarized by Gorrens et al. (2021), more than ten studies have detected *Dysgonomonas*, *Enterococcus*, *Morganella,* and *Providencia.* The genera *Klebsiella*, *Clostridium*, *Actinomyces*, *Bacillus*, *Pseudomonas,* and *Ignatzschineria* were all detected in more than three studies in gut samples of BSFL. The high detection frequency of these genera suggested their potential important role in the BSFL gut microbiota. At least some of those taxa could be considered as a core gut microbiota.

High throughput amplicon sequencing of bacterial 16S rRNA genes has been the most employed technique to explore the gut microbiota composition and all possible changes according to the rearing conditions of BSFL. This approach however does not reflect the overall bacterial diversity present below the genus level and cannot cover the genetic diversity present in an environment (Nichols 2007; De Smet et al. 2018). Only few studies are currently available which intended to culture bacteria of the BSFL gut microbiota (Jeon et al. 2011; Kim et al. 2014; Callegari et al. 2020; Shelomi et al. 2021; Gorrens et al. 2021; Tegtmeier et al. 2021). So far, only few different cultivation strategies were applied to culture specific functional members of a potential core gut microbiota. Some studies aimed to culture members of BSFL microbiota that have amylase, cellulase, pectinase, and esterase/lipase activities. For this purpose, Callegari et al. (2020) applied a pre-enrichment step with polymeric substrates as uric acid, filter paper, and carboxymethylcellulose. In contrast, Gorrens et al. (2021) reared BSFL in standard diets supplemented with polymeric compounds as lignin, pectin, keratin, cellulose, and hemicellulose and employed direct plating of serially diluted gut samples on plate count agar. Other studies applied non-selective direct plating approaches using complex media as Luria–Bertani or nutrient agar to isolate abundant heterotrophic gut associated bacteria from BSFL reared in different diets (Jeon et al. 2011; Shelomi et al. 2021). Tegtmeier et al. (2021) used an extended spectrum of cultivation conditions to study the diversity of physiological properties of endogenous BSFL bacteria. They used different buffers or broth media to prepare dilution series of BSFL homogenates which were plated on different selective and non-selective agar media. In contrast to other studies both, oxic and anoxic incubation conditions were applied. They isolated similar bacterial taxa as in the other studies but recovered an expanded range of bacterial diversity including some representatives of the less-abundant phylum *Actinobacteria* and potential new genera of anaerobic *Clostridiales.*

The aim of this study was to generate a culture collection of abundant aerobic heterotrophic bacteria representing members of the core gut microbiota from BSFL larvae as a starting point to investigate the

genetic potential of the BSFL core gut microbiota. To extend the diversity of cultured gut bacteria a dilution-to-extinction enrichment cultivation approach was applied beside a standard direct plating approach. The dilution-to-extinction enrichment cultivation was not yet applied to the cultivation of bacteria from the gut of BSFL larvae. This approach was originally introduced by Button et al. (1993) for the cultivation of typical small abundant but slow growing oligotrophic marine bacteria which are normally outcompeted by fast growing bacteria by the direct plating cultivation or which could not directly grow on an agar plate surface due to the higher oxygen tension there. For this approach, suspensions of environmental bacteria were directly serially diluted in an enrichment medium which based on filtered marine water which was supplemented with low amounts of complex substrates as peptone (Button et al. 1993). After several weeks of incubation, the enrichment cultures of the highest positive dilutions were streaked on agar plates containing the same media used for the enrichment. This approach was later described as a miniaturized dilution-to-extinction enrichment (Hoefman et al. 2012). We applied this approach to culture especially abundant, but slow growing bacteria from the gut microbiota or to culture gut symbionts that may have a problem to grow directly on an agar surface due to the high oxygen tension at the air agar surface interface. In the second approach, a standard direct plating approach, suspensions of the gut microbiota were serially diluted in sterile 0.9% (w/v) sodium chloride and plated directly on different agar media. A broad range of morphological different colonies was selected for the subsequent analyses. All obtained isolates (pure cultures) were identified by 16S rRNA gene sequencing (phylotyping) and differentiated at the strain level (intra-phylotype diversity) by genomic fingerprinting through BOX- and $(GTG)_5$-PCRs (genotyping).

Materials and methods

Sampling and gut dissection

Black solder fly larvae (BSFL) were supplied by the Bio.S Biogas company (Grimma, Germany). The larvae were reared for four weeks at 25 °C on commercial chicken feed.

Larvae were incubated at − 20 °C for 10 to 15 min to inactivate the larvae. Larvae were then surface-sterilized by incubation in 5% (v/v) sodium hypochlorite for 1 min and washed shortly in phosphate-buffered saline (PBS pH 7.0) before dissection of the guts. The dissection was carried out under the stereomicroscope on a sterile glass slide under oxic conditions. Each extracted gut was cleaned by washing in autoclaved pure water. The guts were collected in sterile 50 ml polypropylene tubes. Each of three sample replicates contained a pool of ten guts. Gut samples are shown in Supplementary Fig. S1. The guts were resuspended in 10 ml of 0.22 µm-filter-sterilized 0.2% (w/v) tetra-sodium-pyrophosphate (TSPP) and a mechanical treatment in a Stomacher ® 80 Biomaster (Seward Limited) was used two times for 1 min and 30 beats/s to detach bacterial cells from the gut material. The bacterial cell suspensions were used to culture core gut bacteria.

Cultivation

Each of the resuspended gut microbiota samples (three replicates, R1–R3) were used as inoculum for the dilution-to-extinction cultivation and for the direct plating approach. The cultivation approaches were started immediately after the gut microbiota was harvested. The procedure used for the direct plating approach started with a tenfold serial dilution of 1 ml of the cell suspensions in 0.9% (w/v) sodium chloride (NaCl). The dilution series was set up in glass tubes until 10^{-4}. Cell suspensions were always mixed by vortexing. An aliquot of each dilution (100 µl) was plated (from 10^0 to 10^{-4}) on brain heart infusion (BHI) agar (Difco), lysogeny broth (LB Lennox, ROTH) agar, trypticase soy (TS) (Difco) agar (Roth), Reasoner's 2A (R2A) agar (Oxoid), and Mueller–Hinton (MH) agar (Roth), respectively. All plates were incubated for 2 days under oxic conditions in the dark at 25 °C.

The dilution-to-extinction enrichment cultivation was performed in 96-well plates (Greiner BIO-ONE) using different media, half-concentrated R2A (½ R2A), R2A, LB, and TS broth.

The 96-well plate was pre-loaded with 180 µl sterile broth per well. In the first row the broth media were inoculated with 20 µl of the gut cell suspensions. Each of the three gut suspensions were used as a biological replicate once for each applied medium

as inoculum. The inoculated media in the first wells were serially diluted from A to H using a multichannel pipette. Cell-medium suspensions were mixed by pipetting up/down for 10 times and 20 µl of the suspensions were transferred to the next well, where mixing was repeated. The serial dilutions per sample contained eight dilution steps. Plates were closed with sterile plastic lids and incubated for 4 weeks at 25 °C in a closed container with a wet tissue to avoid the evaporation of the media. After this period of incubation, 10 µl were removed from the individual wells and were streaked on agar plates with the same media as used for the enrichment cultivation. The remaining samples in the 96-well plate were conserved at − 20 °C after the addition of glycerol in a final concertation of 20% (v/v). Agar plates were incubated for 2–3 days at 25 °C. Colonies with different morphologies were selected from the different agar plates. One to two inoculation loops full of fresh biomass of purified strains were suspended in 1.4 ml u-bottom push cap tubes (Micronic, Netherlands) with 250 or 500 µl Gibco newborn calf serum (NBCS, ThermoFisher Scientific) and stored at − 20 and − 80 °C for long-term preservation. In parallel one inoculation loop full of fresh biomass was resuspended in 100 or 500 µl molecular grade water (Roth) to obtain cell lysates using the freeze–thaw method (Schauss et al., 2015).

Genotyping

Genotypic differentiation (genotyping) of isolated bacteria was performed by genomic fingerprinting using BOX-PCR with primer BOX1AR (5′-CTA CGG CAA GGC GAC GCT GAC G-3′) (Versalovic et al. 1994). Analyses were performed according to Glaeser et al. (2013). For isolates which were identified as members of the genera *Enterococcus* and *Mammaliicoccus* (see below) BOX-PCR gave insufficient results, hence $(GTG)_5$-PCR was used according to Glaeser et al. (2016) with primer $(GTG)_5$ (5’-GTG GTG GTG GTG GTG-3’) (Versalovic et al. 1994). Genomic fingerprint patterns generated by agarose gel electrophoresis (Glaeser et al. 2013) were compared in BioNumerics version 8.0 (Applied Maths N.V.). A similarity matrix comparing the individual fingerprint patterns was calculated with the Pearson product–moment correlation (Pearson correlation). Cluster analysis was performed with the unweighted pair group method with arithmetic mean (UPGMA) with 1% position tolerance and 0.5% optimization. Isolates were assigned to one genotype if they shared identical genomic DNA fingerprint patterns.

Phylogenetic identification by partial 16S rRNA gene sequencing (phylotyping)

Representative isolates of each genotype were identified by partial 16S rRNA gene sequencing. The primer system 8F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1492R (5′-ACG GCT ACC TTG TTA CGA CTT-3′) (Lane 1991) was used to amplify the 16S rRNA gene as described previously (Aydogan et al. 2020). PCR products were sequenced with the Sanger method using primers 27F (5′-GAG TTT GAT CMT GGC TCA G-3′) and E786F (5′-GAT TAG ATA CCC TGG TAG-3′). PCR product purification and sequencing reactions were performed by LGC Genomics (Berlin, Germany).

DNA sequences were corrected manually using MEGA 7.0 (Kumar et al. 2016) based on the electropherograms. Ambiguous positions at the 5′ and 3′ ends of the sequences were removed. A first identification of the phylogenetic affiliation of the strains was done by BLAST analysis against the EzBioCloud database (Yoon et al. 2017) resulting in 16S rRNA gene sequence similarities of closely related type strains included in the database. Subsequently the sequences were added to the phylogenetic tree containing type strain sequences using the All-Species Living Tree Project (LTP) (Yarza et al. 2008), database releases 132 (June, 2018). Analysis was performed in ARB release 5.2 (Ludwig et al. 2004). The 16S rRNA gene sequences were first aligned with SINA v1.2.9 according to the SILVA seed alignment (http://www.arb-silva.de) (Pruesse et al. 2012) and imported into the LTP database tree using the parsimony quick add marked sequences tool implemented in ARB. The partial sequences were thereby placed into the database tree without changing the overall tree topology. The alignment of all sequences considered for phylogenetic analysis was checked manually considering the secondary structure of the 16S rRNA. A subtree containing the added partial 16S rRNA gene sequences and a selection of reference strains from the database tree was exported. For the definition of phylotypes additional phylogenetic analyses were performed in MEGA 7.0 using partial sequences

of the isolates and type strain reference sequences. Sequences were aligned with ClustalW implemented in MEGA 7.0. Analyses were performed separately for different bacterial phyla. Alignments were corrected manually. Pairwise sequence similarities were determined in MEGA 7.0 with the p-distance method without using evolutionary models. Phylogenetic trees for phylotype assignment were constructed with the Neighbour joining method using the Jukes Cantor Correction model. Trees were calculated considering 100 repetitions (bootstrap analysis). Isolates were assigned to phylotypes which were defined by the formation of monophyletic clusters within the phylogenetic tree which was supported by high bootstrap values. Sequences present within one cluster shared at least 99.0% partial 16S rRNA gene sequence similarities among each other.

Seriation analysis

Seriation analysis was performed in PAST4.04 (Hammer et al. 2001) to compare the occurrence patterns of phylotypes within the different replicates cultured by the two different cultivation strategies and in/on the different media (Franco et al. 2020). Analyses were based on an absence-presence (0/1) matrix using the algorithm described by Brower and Kile (1988).

Results

Overview of cultured bacteria (phylotyping)

Bacterial growth was obtained by both cultivation strategies in, or on all applied media for the investigated BSFL gut samples (three independent pools of guts of ten larvae). All wells of the serially diluted samples (up to 10^{-8}) in the dilution-to-extinction approach and all plated dilutions (up to 10^{-5}) showed bacterial growth. In total a collection of 479 isolates (341 from the dilution-to-extinction approach and 138 from direct plating) were cultured from the different agar media. The isolates represented the morphological diversity of abundant colony morphologies grown on the agar plates. Based on the 16S rRNA gene sequence analyses the isolates were assigned to ten different genera of *Proteobacteria* (*Stenotrophomonas, Pseudomonas, Alcaligenes, Proteus, Providencia, Morganella, Serratia, Klebsiella/Enterobacter,* and *Brucella*) and four different genera of *Firmicutes,* all of the order *Bacilli* (*Bacillus, Enterococcus, Mammaliicoccus,* and *Lysinibacillus*).

Isolates identified as *Enterobacteriaceae* (closely related to *Enterobacter/Klebsiella*) represented the largest proportion of isolates by both cultivation strategies (49% and 37% by the dilution-to-extinction and direct plating cultivation, respectively) (Fig. 1). The most abundant taxa cultured by the dilution-to-extinction approach were *Proteus* (13%), *Pseudomonas* (6%), *Providencia* (10%), and *Morganella* (8%) and by the direct plating approach *Morganella* (22%), *Proteus* (14%), *Mammaliicoccus* (9%), and *Bacillus* (7%). Other detected taxa occurred with a low relative abundance (0.3–4%).

Within the different genera, isolates were differentiated into up to six different phylotypes per genus which showed distinct clusters in the phylogenetic trees (Fig. 2, Supplementary Fig. S2a–c). A total of 18 different phylotypes were detected throughout this study. They all shared very high 16S rRNA gene sequence similarities (> 99%) to type strains of single or partially several related species. Due to the high 16S rRNA gene sequence similarity among members of the *Enterobacteriaceae* a phylotype differentiation was not possible for the isolates closely related to *Enterobacter/Klebsiella*. The strains were summarized as an *Enterobacteriaceae* clade. *Providencia* isolates represented two distinct phylotypes. Isolates of the first phylotype clustered with the type strain of *Providencia stuartii* and isolates of the second phylotype with several other *Providencia* species type strains with *Providencia rettgeri* as closest related one. All isolates identified as members of the genus *Proteus* belonged to one phylotype and clustered with the type strain of *Proteus mirabilis*. *Morganella* isolates formed one large cluster and were assigned to one phylotype. The isolates showed highest 16S rRNA gene sequence similarities to the type strains of *Morganella morganii* subsp. *morganii* and *Morganella morganii* subsp. *sorbia*. Isolates assigned to the genera *Serratia*, *Alcaligenes*, *Stenotrophomonas*, and *Mammaliicoccus* were always assigned to one phylotype and closest related to type strains of the species *Serratia marcescens, Stenotrophomonas maltophilia*, *Pseudomonas aeruginosa*, *Alcaligenes faecalis,* and *Mammaliicoccus sciuri* (formerly *Staphylococcus sciuri*).

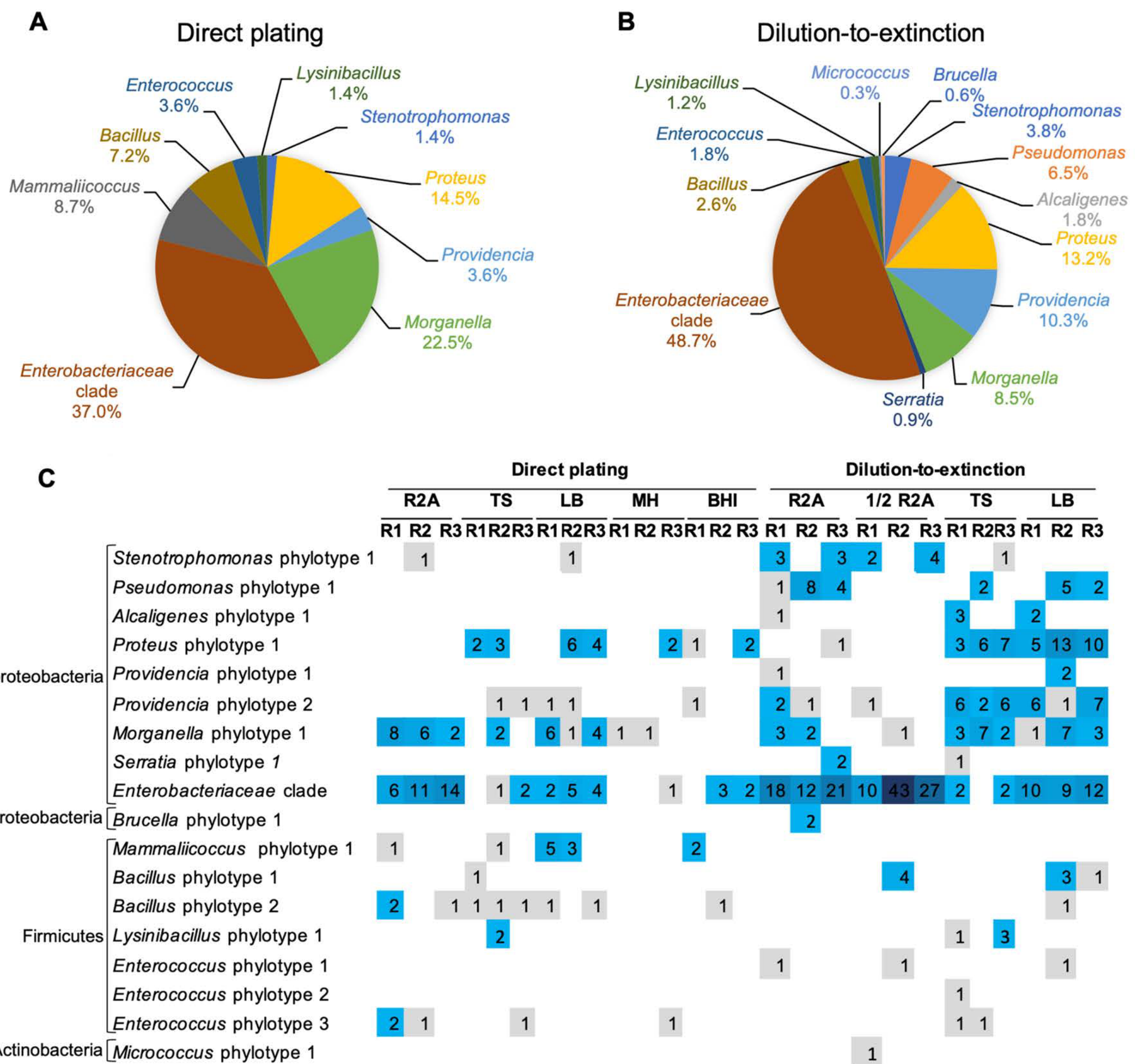


Fig. 1 Overview of the bacterial genera cultured from gut samples of black solder fly larvae (BSFL) by the use of two different cultivation methods, dilution-to-extinction enrichment cultivation and direct plating cultivation. **A**, **B** Genera distribution according to the detected phylotypes from all isolates cultured by direct plating (**A**) and dilution-to-extinction cultivation (**B**). Values were calculated based on a total of 138 isolates for the direct plating (**A**) and 341 isolates for the dilution-to-extinction cultivation (**B**). **C** Phylotype (defined based on 16S rRNA gene sequence data, Fig. 2) distribution according to the employed media (R2A, ½ R2A, TS, LB) and cultivation approaches. The numbers in **C** represent the numbers of studied isolates derived from individual colonies. R1-R3 represent studied replicates. Each replicate represented a pool of ten BSFL guts

Bacillus and *Enterococcus* isolates were assigned to two and three different phylotypes. These isolates were closely related to the type strains of *Bacillus cereus, Bacillus siamensis, Enterococcus faecalis, Enterococcus mediterraneensis,* and *Enterococcus durans/ Enterococcus avium,* respectively.

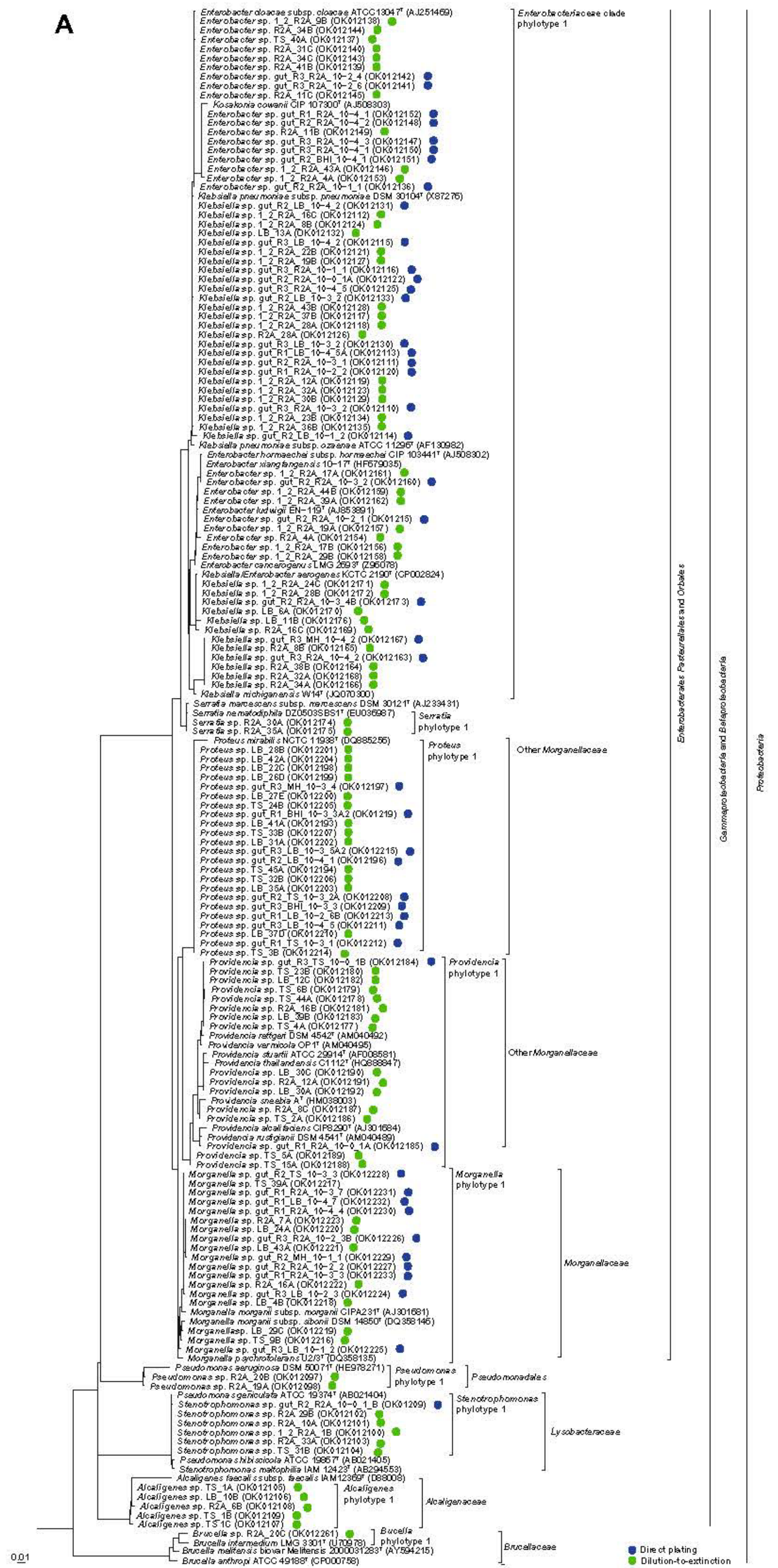


Fig. 2 Placement of the 16S rRNA gene sequenced isolates into the type strain tree provided in the LTP database releases 132 (June, 2018) without changing the tree topology. **A** Proteobacteria. **B** Other phyla. Analysis was performed in ARB. Circles behind the isolate numbers indicate the applied cultivation method. Acc. numbers of the 16S rRNA gene sequences are given in parenthesis. Blue and green dots represent the isolates obtained from direct plating and dilution-to-extinction approaches, respectively. (Color figure online)

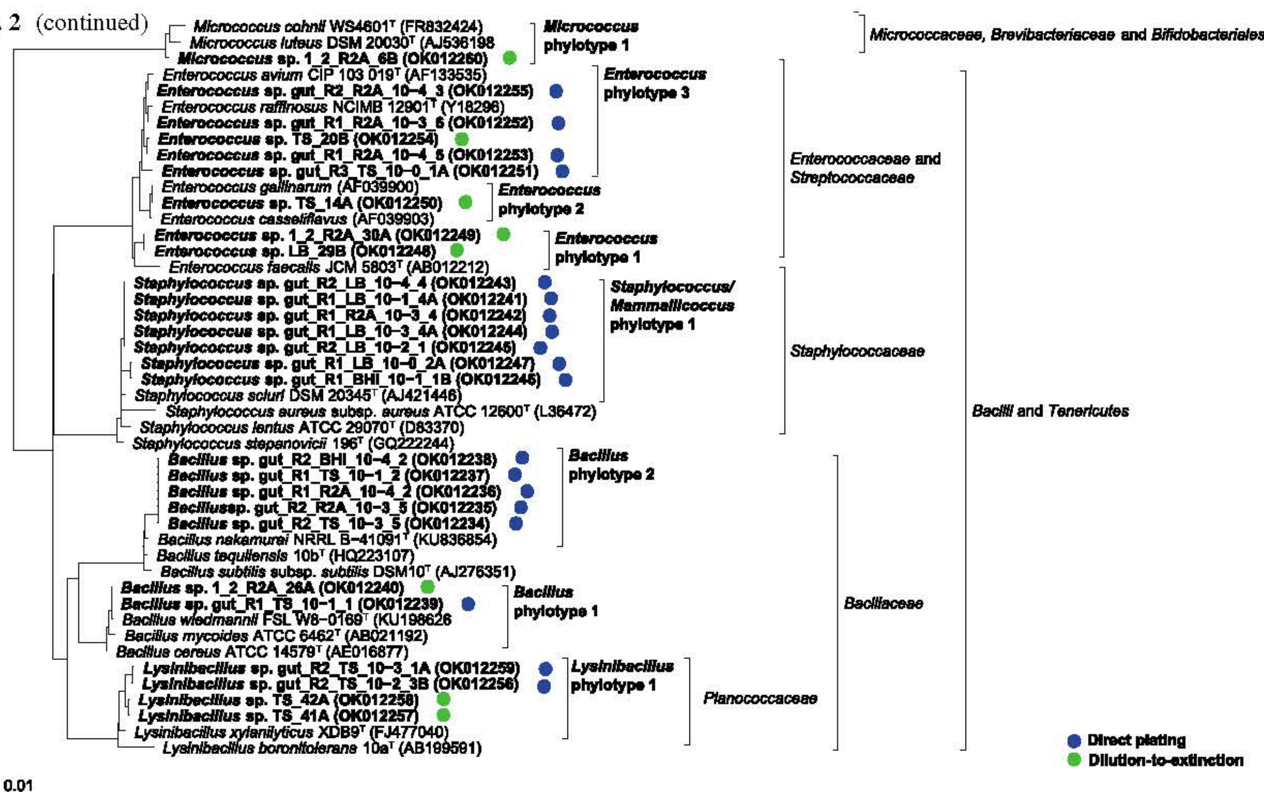


Fig. 2 (continued)

Phylotype distribution

Seriation analysis showed that six different phylotypes including phylotypes of the genera *Pseudomonas, Alcaligenes, Providencia, Serratia, Brucella, Micrococcus,* and *Enterococcus* occurred exclusively among the isolates cultured by the dilution-to-extinction cultivation while only the *Mammaliicoccus* phylotype was only cultured by the direct plating approach (Fig. 2).

Genotyping

Genomic fingerprinting, BOX and $(GTG)_5$-PCR based patterns, enabled the comparison of the isolated bacteria at a higher genetic resolution (strain level). The analysis showed that the genetic diversity of the cultured bacteria was higher than obtained by the phylotype based differentiation. Genomic fingerprinting indicated a high intra-phylotype based genetic diversity (Fig. 3).

The highest genotype diversity was determined for isolates assigned to the *Enterobacteriaceae* clade and the genera *Providencia, Enterococcus,* and *Morganella* (Fig. 4). Only isolates assigned to the genera *Mammaliicoccus*, *Alcaligenes,* and *Serratia* showed for all isolates identical genomic fingerprint patterns within a phylotype, indicating genetically identity or clonality. Some genotypes were isolated by both cultivation strategies and from different media, while others were only cultured from individual cultivation strategies and individual media.

Specific observations

A high abundance of swarming bacteria, overgrowing the colonies of other bacteria, were obtained by the direct plating approach on TS, MH, and LB agar, but not on R2A and half concentrated R2A agar. On TS agar some bacterial colonies inhibited the growth of the swarming bacterium in the area around the colonies (Supplementary Fig. S3). The swarming bacteria were identified as *Proteus* spp. Genomic fingerprinting indicated that different *Proteus* spp. seemed to be present in the BSF gut (Fig. 4). The *Proteus*-inhibiting bacteria were identified as *Bacillus* spp. closely related to the

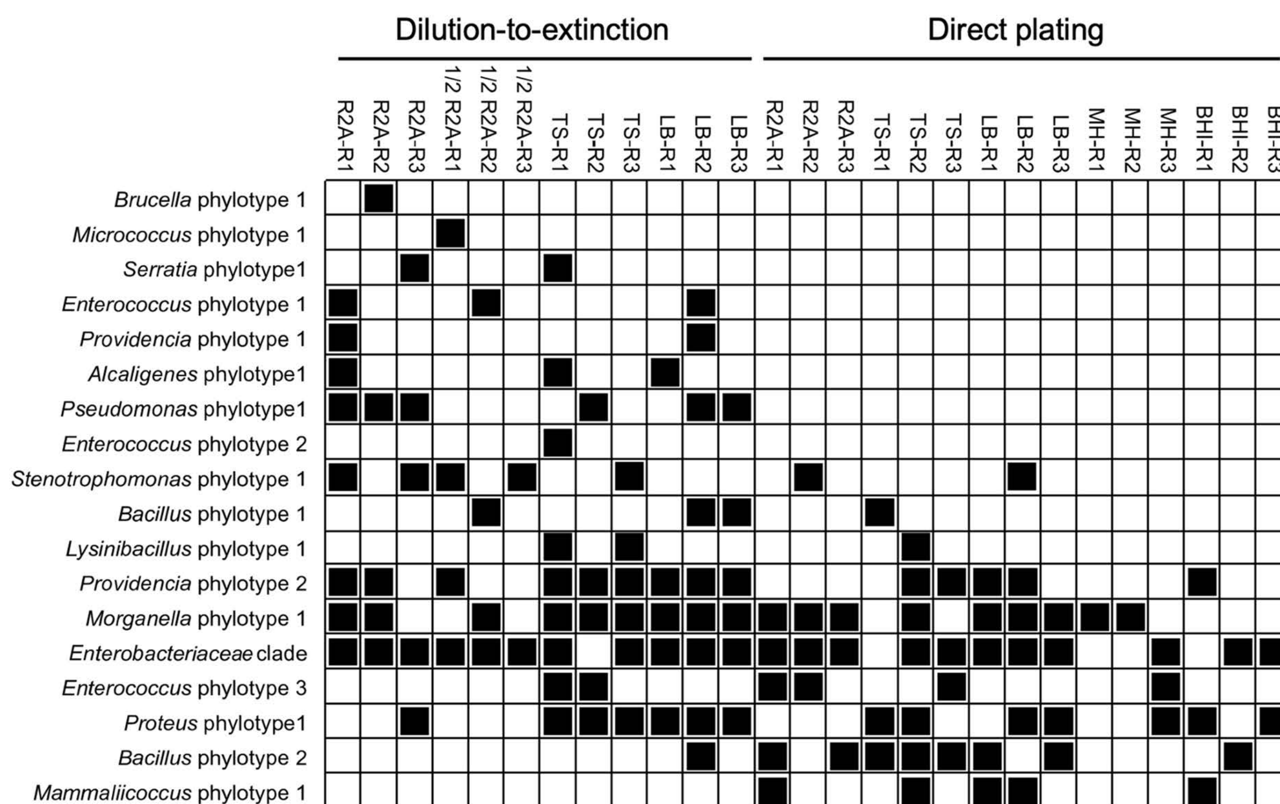


Fig. 3 Seriation analysis based on an absence-presence (0/1) matrix illustrating the presence of each phylotype/clade in all different media through miniaturized dilution-to-extinction and direct plating approach

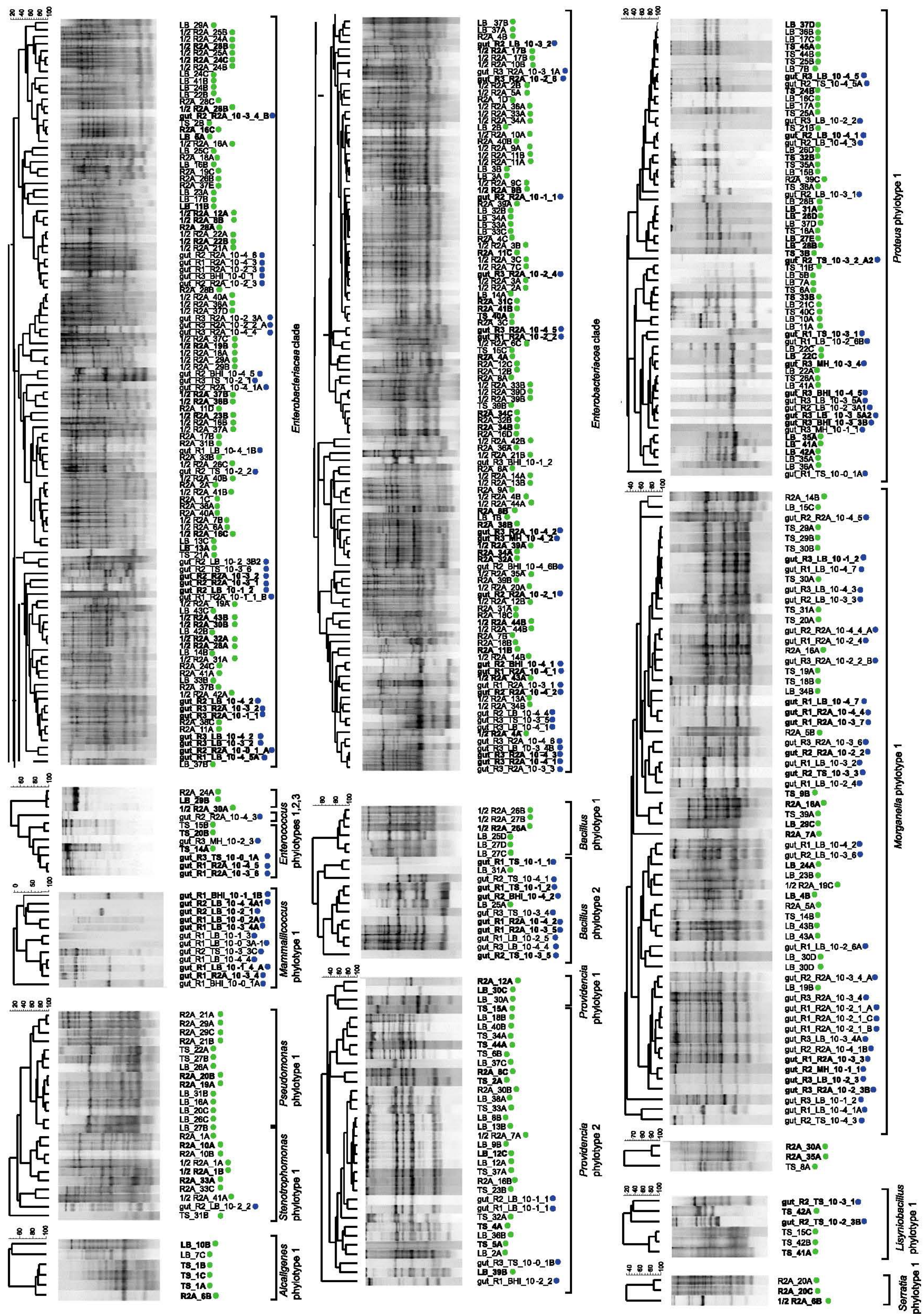
type strains of different *Bacillus* species including *Bacillus siamensis, Bacillus amyloliquefaciens,* and *Bacillus velezensis*. The 16S rRNA gene sequence similarities to those type strains were 99.89–100.0%.

Discussion

The parallel application of two different strategies for the cultivation of aerobic heterotrophic bacteria from BSFL guts enabled the identification of a high diversity of bacteria assigned to genera discussed as members of a potential BSFL core gut microbiota (Gorrens et al. 2021). The application of the two cultivation strategies enhanced thereby the genetic diversity of the cultured bacteria with more different phylotypes and genotypes.

In our previous study we investigated the gut microbiota of BSFL fed with the same substrate by a culture-independent 16S rRNA gene amplicon sequencing based approach (Cifuentes et al. 2020). In that study nine genera were proposed as members of a potential gut core microbiota, *Providencia*, *Enterobacter*, *Bacillus*, *Enterococcus*, *Klebsiella, Morganella*, *Dysgonomonas*, *Lachnospiraceae,* and *Actinomyces.* This was in accordance with other studies that investigated the BSFL gut microbiota (Gorrens et al. 2021).

Here we were able to culture representatives of six of those genera, *Providencia*, *Enterobacter*, *Bacillus*, *Enterococcus*, *Klebsiella*, and *Morganella*. Representatives of the other three genera, *Dysgonomonas*, *Lachnospiraceae,* and *Actinomyces*, could not be isolated. One reason may be that only aerobic cultivation conditions were applied. Members of respective genera may require anaerobic cultivation conditions (Pramono et al. 2015; Feingold and Meislich 2018). Similar observations were made in the study of Tegtmeier et al. (2021). *Dysgonomonas* were not mentioned at all in their study. However, a successful isolation of *Dysgonomonas* under anaerobic cultivation conditions was for example reported for cockroaches (Vera-Ponce de León et al.


Enterobacteriaceae clade
Enterococcus phylotypes 1,2,3
Mammaliicoccus phylotype 1
Pseudomonas phylotype 1
Stenotrophomonas phylotype 1
Alcaligenes phylotype 1
Bacillus phylotype 1
Bacillus phylotype 2
Providencia phylotype 1
Providencia phylotype 2
Proteus phylotype 1
Morganella phylotype 1
Lysinibacillus phylotype 1
Serratia phylotype 1

◂**Fig. 4** Genotyping of all identified phylotypes isolated from BSFL gut microbiota, based on fingerprint pattern obtained by genomic fingerprinting using primers targeting BOX and $(GTG)_5$ repetitive elements. $(GTG)_5$-PCR was employed exclusively with *Mammaliicoccus* and *Enterococcus* phylotypes. Cluster analysis was performed in BioNumerics (Applied Maths) using UPGMA clustering, based on a dissimilarity matrices generated by the Pearson correlation. Bold isolates were identified through 16S rRNA gene sequencing. Blue and green dots represent the isolates cultured by direct plating and dilution-to-extinction cultivation. (Color figure online)

2020). Tegtmeier et al. (2021) pointed out that they were also not able to culture *Lachnospiraceae* and *Actinomyces* however respective taxa were detected in the culture independent community analysis of the gut microbiota. None of the other studies that cultures BSFL gut bacteria cultured those genera.

Bacteria assigned to six more genera that were not defined as potential core gut microbiota but detected through 16S rRNA gene amplicon sequencing of the BSFL gut microbiota of larvae from the same provider and fed with the same diet (Cifuentes et al. 2020) were also isolated here. The isolates were identified as members of the genera *Proteus*, *Alcaligenes*, *Mammaliicoccus*, *Micrococcus*, *Stenotrophomonas*, and *Pseudomonas*.

We performed a comparison to other studies that isolated BSFL gut microbes (Table 1). Based on the information given in the different publications bacteria assigned to 37 genera were cultured from BSFL guts. Members of two genera (*Proteus* and *Enterococcus*) were isolated in five studies including ours. Four genera (*Providencia, Morganella, Klebsiella*, and *Bacillus*), were cultured in four different studies including ours. Seven genera were isolated in three different studies among those five which were detected also in our study (*Enterobacter, Alcaligenes, Stenotrophomonas, Mammaliicoccus, Brucella*). The number of different detected genera ranged from just three by Jeon et al. (2011) and Shelomi et al. (2021) to up to 27 genera in the study of Tegtmeier et al. (2021). The higher number of taxa cultured by Tegtmeier et al. (2021) compared to our study (15 different detected genera) may be linked to the inclusion of anaerobic cultivation conditions by Tegtmeier et al. (2021).

Members of *Enterobacteriaceae* (*Klebsiella*/*Enterobacter*), *Proteus*, *Providencia*, *Morganella,* and *Enterococcus* were represented by several isolates in our study. Representatives of those genera were isolated in different studies from BSFL larvae reared under different rearing conditions and using different cultivation strategies (Callegari et al. 2020; Gorrens et al. 2021; Tegtmeier et al. 2021). Only some hints are available for the function of those bacteria in the BSFL gut. Feeding experiments with different substrates and in parallel performed gut microbiota studies indicated their role in the degradation of polymeric substances (Jiang et al. 2019; Gold et al. 2020; Mazza et al. 2020; Gorrens et al. 2021; Schreven et al. 2021). The use of different cultivation or enrichment media indicated the presence of specific enzymatic activities in respective taxa (Callegari et al. 2020; Gorrens et al. 2021; Tegtmeier et al. 2021).

The addition of BSFL isolates to the feed of BSFL demonstrated that members of the BSFL gut microbiota, as bacteria of the genus *Proteus* enhance the BSFL biomass production (Mazza et al. 2020; Sontowski and van Dam 2020). BFSL based vermicomposting studies indicated the important role of *Enterococcus* and *Providencia* strains in the BSFL gut due to their complex carbohydrate-degrading enzymes and nitrogen, hydrogen, and sulphur metabolism (Jiang et al. 2019; Gold et al. 2020; Gorrens et al. 2021). Further studies indicated the potential capability of *Providencia* to digest xylan (Sontowski and van Dam 2020) and an important role for enterococci during the starvation process in BSFL (Yang et al. 2021). Despite the fact that *Morganella* was reported as pathogen by some *Diptera* spp. (Sontowski and van Dam 2020), members of the genus are frequently found in high abundance in the gut microbiota of healthy appearing BSFL (Table 1).

Different studies indicated that *Providencia* and *Proteus* are involved in the hydrolysis of urea (Gold et al. 2020; Klammsteiner et al. 2020). *Klebsiella* and *Enterobacter* showed a strong correlation to nitrogen fixation in *Diptera* species which significantly contributed to the nitrogen uptake of the larvae (Behar et al. 2005). Specific pectinolytic activities have been recently attributed to *Klebsiella* spp. isolated from the BSFL gut (Callegari et al. 2020; Gorrens et al. 2021). Knowledge on the function of *Dysgonomonas* spp. is only available from isolates cultured from other insects. Those studies indicated an important role of *Dysgonomonas* in the degradation of starch, pectin and cellulose due to the abundance of carbohydrate-active enzyme (CAZyme)-coding genes (Vera-Ponce de León et al. 2020).

Table 1 Comparison of detected genera mentioned in studies which cultured gut bacteria from BSFL

Genera	This study	Jeon et al. (2011)	Callegari et al. (2020)	Shelomi et al. (2021)	Gorrens et al. (2021)	Tegtmeier et al. (2021)	No. of studies
Proteus	x	x	x		x	x	5
Enterococcus	x		x	x	x	x	5
Providencia	x		x		x	x	4
Morganella	x		x		x	x	4
Klebsiella	x		x		x	x	4
Bacillus	x	x	x			x	4
Enterobacter	x		x		x		3
Alcaligenes	x		x			x	3
Stenotrophomonas	x		x		x		3
Mammaliicoccus	x		x			x	3
Brucella	x		x			x	3
Escherichia			x		x	x	3
Citrobacter			x	x		x	3
Pseudomonas	x		x				2
Micrococcus	x		x				2
Lysinibacillus	x					x	2
Acinetobacter			x	x			2
Vagococcus			x			x	2
Bordetella			x			x	2
Leucobacter			x			x	2
Myroides			x			x	2
Paenicaligenes		x				x	2
Sphingobacterium					x	x	2
Serratia	x						1
Pantoea			x				1
Koukoulia						x	1
Corynebacterium						x	1
Rhodococcus						x	1
Dietzia						x	1
Kocuria						x	1
Brevibacterium						x	1
Microbacterium						x	1
Clostridium						x	1
Neglecta						x	1
Chryseobacterium					x		1
Rummeliibacillus					x		1
Glutamicibacter					x		1
Genera/study	15	3	21	3	12	27	

Compared with the other three large collections of the BSFL gut isolates (Callegari et al. 2020; Gorrens et al. 2021; Tegtmeier et al. 2021), Callegari et al. (2020) and Tegtmeier et al. (2021), have the most shared phylotypes with our study. The shared phylotypes correspond to genera with low abundance in our study as *Mammaliicoccus*, *Alcaligenes*, *Stenotrophomonas*, *Brucella,* and *Bacillus.* Members of the genus *Bacillus* and *Stenotrophomonas* were present in high abundance in the study of Callegari et al. (2020).

Their study indicated that *Bacillus* spp. are involved in the breakdown of cellulose and starch while *Stenotrophomonas* spp. are involved in the digestion of casein due to pectinase and lipase activities.

Some of the genera that were cultured exclusively by the dilution-to-extinction approach in our study, like *Micrococcus* and *Pseudomonas,* were also cultured by Callegari et al. (2020) and detected previously by our culture-independent 16S rRNA gene amplicon sequencing approach (Cifuentes et al. 2020). The fact that in three studies that cultured bacteria from the gut of BSFL, only Callegari et al. (2020) and the present study have successfully isolated *Pseudomonas* indicates that enrichment methods may be required for a successful cultivation of gut associated *Pseudomonas*. In general, *Pseudomonas* strains can be easily cultured on different complex media (Palleroni 2015). However, here and in another study of other insect guts (unpublished data) we obtained specific subgroups of *Pseudomonas* which were only culturable by the application of the dilution-to-extinction enrichment cultivation in liquid medium and extended time of incubation. The reason for this selective cultivability of some *Pseudomonas* was not further studied yet.

The bacterial collection from Tegtmeier et al. (2021) was obtained from BSFL of the same provider as the BSFLs studied here. This may be one reason why a high proportion of identical taxa were isolated in both studies. Among those members of the genus *Lysinibacillus*. Members of this genus were recently correlated to an increase of the BSFL weight if they were added as food supplement (Mazza et al. 2020; Schreven et al. 2021).

Serratia spp. were cultured exclusively in our study from BSFL guts (Table 1). Strains of this genus were in our study exclusively cultured by the dilution-to-extinction cultivation. *Serratia* spp. were also cultured from gut samples of *Diptera* spp. (Jang and Nishijima 1990; Zurek et al. 2000). They are discussed to be on the one hand insect pathogens (Grimont and Grimont 1978), but also present in healthy insects indicating a non-pathogenic interaction with those (Jang and Nishijima 1990; Zurek et al. 2000). *Serratia* spp. in contrast are one of the most frequently isolated genera from cockroaches (Guzman and Vilcinskas 2020). Studies summarized by Guzman and Vilcinskas (2020) also indicated that they are normal members of the gut microbiota of cockroaches because *Serratia* were isolated from healthy, sick, and dead cockroaches, respectively.

Only a part of the studies that cultured BSFL gut microbes provided 16S rRNA gene sequence data of the identified bacteria which made a phylogenetic diversity based study problematic.

Beside our study only Tegtmeier et al. (2021) looked in some extend also to a higher genetic resolution including genomic fingerprinting. Phylotyping (16S rRNA gene sequence based analysis) and genotyping (genomic fingerprinting using e.g. BOX-PCR) as performed here showed a higher genetic diversity of BSFL gut bacteria than obtained by 16S rRNA gene amplicon based studies. Amplicon data should, due to the short sequence fragments obtained by Illumina sequencing, just be used for a genus based assignment of the gut microbiota.

The comparison between the available 16S rRNA gene sequences of bacteria cultured by Callegari et al. (2020) and Tegtmeier et al. (2021), showed that for some genera different phylotypes were isolated in the different studies (Supplementary Fig. S4). According to the phylogenetic tree (Supplementary Fig. S4), more than one phylotype of *Providencia* was identified in our and other studies. The detection of different *Providencia* spp. was also shown for the gut of *Diptera* spp. (Kuzina et al. 2001; Toth et al. 2006). The reason for the diversity of gut associated *Providencia* species is not yet known.

The detected members of the *Enterobacteriaceae* clade represents a high number of possible species, assigned to the genera *Klebsiella* and *Enterobacter*. A distinction of members of this clade requires at least a multilocus sequence analysis (MLSA) or a core genome based phylogenetic analysis (Glaeser and Kämpfer 2015; Adeolu et al. 2016). Comparison of *Enterobacteriaceae* clade isolates of this study and those obtained by Callegari et al. (2020) and Tegtmeier et al. (2021) indicated a high diversity of different *Enterobacteriaceae* clade bacteria present in the guts of BSFL already by the comparison of the 16S rRNA gene sequences.

Species and strain specific variations were found in the efficacy to support BSFL growth and substrate utilization efficiency (Yu et al. 2011; Mazza et al. 2020). Our study showed that for several genera different phylotypes (potentially different species) and genetically different strains of the same phylotype (different genotypes) could be isolated from BSFL gut samples

dependent on the applied cultivation strategies. Due to the limitation of the applied genomic fingerprinting PCR techniques to intra-lab comparison it is not possible to compare isolates from different studies to check if genetically identical strains were present in the guts of BSFL in different BSFL studies.

Several of the detected bacteria were closely related to bacterial pathogens. Cultured representatives are required for species assignment which is a requirement for risk group assessment. Further on isolates can be screened for pathogenicity factors or used for infection experiments to understand if BSFL associated strains indeed represent pathogens or just phylogenetically related to pathogenic strains.

As Gold et al. (2020) pointed out, little is known about specific functions and evolutionary adaptation of the BSFL gut microbes. In vitro studies are suggested to get more knowledge on the activities of those bacteria. Cultivation of gut and residue microbes is a pre-requisite for such studies. Large strain collections of gut microbes are required to study species and strain specific traits in the rearing process (Gold et al. 2020). Currently there are no genome sequence based studies of BSFL gut microbes available, which would explain either the diversity or the common shared genes of BSFL gut associated taxa, in general or of specific genera. It may be possible that gut associated strains share specific gene sets which can explain their adaptation to BSFL guts. That phenomenon was shown for *Aeromonas* strains, which occur as one of the two dominating gut symbionts in the gut of medicinal leeches (Marden et al. 2016; Ott et al. 2016).

Conclusion

This study confirmed the findings of previous studies that few taxa are repetitively detected by the analysis of the gut microbiota of BSFL which seemed to be members of a core gut microbiota. The strain level based resolution performed in our study by genomic fingerprinting of isolated gut bacteria indicated, that the diversity of the gut microbiota within the abundant taxa is much higher than indicated by the culture independent 16S rRNA gene amplicon sequencing studies. Our data enlarge the knowledge of the intra-species diversity for BFSL gut bacteria, as it has been reported before for other insect gut microbiota (Ellegaard and Engel 2016).

The application of different media and different cultivation strategies has extended the genetic diversity for different taxa. Especially the dilution-to-extinction cultivation, originally developed for the cultivation of abundant slow growing marine oligotrophs (Button et al. 1993), extended the diversity of cultured bacteria for BSFL gut microbes. If there are specific strains within the abundant taxa that show different growth behaviours and were therefore just cultured by the dilution-to-extinction approach must be further studied.

Strain collections are the first step to move on to explore specific functions and the evolutionary adaptation of the cultured symbionts of the insect gut. Comparative genomics of cultured BSFL gut bacteria with representatives of the same taxa from other insect guts or free-living or even pathogenic strains must be the next step forward to determine genomics traits linked to functional traits as metabolic capacities and evolutionary adaptation to the specific ecological niches as the larval gut.

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Author contributions SG, YC designed the study; YC, performed the experiments and analysed the data; SG, YC wrote the paper which was proofed by all co-authors; AV and PK received the funding.

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Data availability The Genbank/EBML/DDBJ of the 16S rRNA gene sequences generated from isolates are available under the accession numbers OK012097 to OK012261.

Code availability Not applicable.

Declarations

Conflict of interest There are no conflict of interests.

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References

Adeolu M et al (2016) Genome-based phylogeny and taxonomy of the "*Enterobacteriales*": proposal for *Enterobacterales* ord. nov. divided into the families *Enterobacteriaceae*, *Erwiniaceae* fam. nov., *Pectobacteriaceae* fam. nov., *Yersiniaceae* fam. nov., *Hafniaceae* fam. nov., *Morganellaceae* fam. nov., and *Budviciaceae* fam. nov. Int J Syst Evol Microbiol 66(12):5575–5599. https://doi.org/10.1099/ijsem.0.001485

Aydogan EL et al (2020) Global warming shifts the composition of the abundant bacterial phyllosphere microbiota as indicated by a cultivation-dependent and -independent study of the grassland phyllosphere of a long-term warming field experiment. FEMS Microbiol Ecol 96(8):1–19. https://doi.org/10.1093/femsec/fiaa087

Behar A, Yuval B, Jurkevitch E (2005) Enterobacteria-mediated nitrogen fixation in natural populations of the fruit fly *Ceratitis capitata*. Mol Ecol 14(9):2637–2643

Brower JC, Kile KM (1988) Sedation of an original data matrix as applied to paleoecology. Lethaia 21(1):79–93. https://doi.org/10.1111/j.1502-3931.1988.tb01756.x

Bruno D, Bonelli M, De Filippis F, Di Lelio I, Tettamanti G, Casartelli M, Ercolini D, Caccia S (2019) The intestinal microbiota of *Hermetia illucens* larvae is affected by diet and shows a diverse composition in the different midgut regions. Appl Environ Microbiol 85:e01864-18. https://doi.org/10.1128/AEM.01864-18

Button DK et al (1993) Viability and isolation of marine bacteria by dilution culture: theory, procedures, and initial results. Appl Environ Microbiol 59(3):881–891

Callegari M et al (2020) Hydrolytic profile of the culturable gut bacterial community associated with *Hermetia illucens*. Front Microbiol 11:1965. https://doi.org/10.3389/fmicb.2020.01965

Cifuentes Y et al (2020) The gut and feed residue microbiota changing during the rearing of *Hermetia illucens* larvae. Antonie Van Leeuwenhoek, Int J Gen Mol Microbiol 113(9):1323–1344. https://doi.org/10.1007/s10482-020-01443-0

da Silva GDP, Hesselberg T (2020) A review of the use of black soldier fly larvae, *Hermetia illucens* (Diptera: Stratiomyidae), to compost organic waste in tropical regions. Neotrop Entomol 49(2):151–162. https://doi.org/10.1007/s13744-019-00719-z

De Smet J et al (2018) Microbial community dynamics during rearing of black soldier fly larvae (*Hermetia illucens*) and impact on exploitation potential. Appl Environ Microbiol 84(9):1–17

Elhag O et al (2022) Inhibition of zoonotic pathogens naturally found in pig manure by black soldier fly larvae and their intestine bacteria. Insects 13(1):66. https://doi.org/10.3390/insects13010066

Ellegaard KM, Engel P (2016) Beyond 16S rRNA community profiling: intra-species diversity in the gut microbiota. Front Microbiol 7:1475. https://doi.org/10.3389/fmicb.2016.01475

Engel P, Moran NA (2013) The gut microbiota of insects—diversity in structure and function. FEMS Microbiol Rev 37(5):699–735. https://doi.org/10.1111/1574-6976.12025

Erickson M, Sheppard C, Liao J, Doyle M (2004) Reduction of *Escherichia coli* O157:H7 and Salmonella enterica Serovar Enteritidis in Chicken Manure by Larvae of the Black Soldier Fly. J Food Prot 67:685–690. https://doi.org/10.4315/0362-028X-67.4.685

Feingold AR, Meislich D (2018) 195—Anaerobic gram-positive nonsporulating *Bacilli* (including actinomyces). In: Long SS, Prober CG, Fischer MBT-P (eds) Principles and practice of pediatric infectious diseases. Elsevier, Amsterdam, pp 1019-1022.e1

Franco A et al (2020) High diversity of *Vibrio* spp. associated with different ecological niches in a marine aquaria system and description of *Vibrio aquimaris* sp. nov. Syst Appl Microbiol 43(5):126123. https://doi.org/10.1016/j.syapm.2020.126123

Glaeser SP, Kämpfer P (2015) Multilocus sequence analysis (MLSA) in prokaryotic taxonomy. Syst Appl Microbiol 38(4):237–245. https://doi.org/10.1016/j.syapm.2015.03.007

Glaeser SP et al (2013) *Niabella hirudinis* and *Niabella drilacis* sp. nov., isolated from the medicinal leech *Hirudo verbana*. Int J Syst Evol Microbiol 63(Pt 9):3487–3493. https://doi.org/10.1099/ijs.0.050823-0

Glaeser SP et al (2016) Cultivation of vancomycin-resistant enterococci and methicillin-resistant staphylococci from input and output samples of German biogas plants. FEMS Microbiol Ecol. https://doi.org/10.1093/femsec/fiw010

Gold M et al (2020) Identification of bacteria in two food waste black soldier fly larvae rearing residues. Front Microbiol. https://doi.org/10.3389/fmicb.2020.582867

Gorrens E et al (2021) Isolation and Identification of dominant bacteria from black soldier fly larvae (*Hermetia illucens*) envisaging practical applications. Front Microbiol. https://doi.org/10.3389/fmicb.2021.665546

Grimont PA, Grimont F (1978) The genus *Serratia*. Ann Rev Microbiol 32:221–248. https://doi.org/10.1146/annurev.mi.32.100178.001253

Guzman J, Vilcinskas A (2020) Bacteria associated with cockroaches: health risk or biotechnological opportunity? Appl Microbiol Biotechnol. https://doi.org/10.1007/s00253-020-10973-6

Hammer O, Harper D, Ryan P (2001) PAST: paleontological statistics software package for education and data analysis. Palaeontol Electron 4:1–9

Hoefman S et al (2012) Miniaturized extinction culturing is the preferred strategy for rapid isolation of fast-growing methane-oxidizing bacteria. Microb Biotechnol 5(3):368–378. https://doi.org/10.1111/j.1751-7915.2011.00314.x

Jang EB, Nishijima KA (1990) Identification and attractancy of bacteria associated with *Dacus dorsalis* (Diptera: Tephritidae). Environ Entomol 19(6):1726–1731

Jeon H et al (2011) The intestinal bacterial community in the food waste-reducing larvae of *Hermetia illucens*. Curr Microbiol 62(5):1390–1399. https://doi.org/10.1007/s00284-011-9874-8

Jiang C et al (2019) Black soldier fly larvae (*Hermetia illucens*) strengthen the metabolic function of food waste biodegradation by gut microbiome. Microb Biotechnol. https://doi.org/10.1111/1751-7915.13393

Kim E et al (2014) Identification and physiological characters of intestinal bacteria of the black soldier fly, *Hermetia illucens*. Korean J Appl Entomol 53(1):15–26. https://doi.org/10.5656/ksae.2013.09.0.049

Klammsteiner T et al (2020) The core gut microbiome of black soldier fly (*Hermetia illucens*) larvae raised on low-bioburden diets. Front Microbiol. https://doi.org/10.3389/fmicb.2020.00993

Kumar S, Stecher G, Tamura K (2016) MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol 33(7):1870–1874

Kuzina LV et al (2001) Isolation and identification of bacteria associated with adult laboratory Mexican fruit flies, *Anastrepha ludens* (Diptera: Tephritidae). Curr Microbiol 42(4):290–294

Lane DJ (1991) 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M (eds) Nucleic acid techniques in bacterial systematics. Wiley, Hoboken, pp 115–175

Liu Q, Tomberlin JK, Brady JA, Sanford MR, Yu Z (2008) Black soldier fly (Diptera: Stratiomyidae) larvae reduce *Escherichia coli* in dairy manure. Environ Entomol 37(6):1525–1530. https://doi.org/10.1603/0046-225X-37.6.1525

Ludwig W et al (2004) ARB: a software environment for sequence data. Nucleic Acids Res 32(4):1363–1371

Marden JN et al (2016) Host matters: medicinal leech digestive-tract symbionts and their pathogenic potential. Front Microbiol 7:1569. https://doi.org/10.3389/fmicb.2016.01569

Mazza L et al (2020) Management of chicken manure using black soldier fly (Diptera: Stratiomyidae) larvae assisted by companion bacteria. Waste Manage 102:312–318

Nichols D (2007) Cultivation gives context to the microbial ecologist. FEMS Microbiol Ecol 60(3):351–357. https://doi.org/10.1111/j.1574-6941.2007.00332.x

Ott BM et al (2016) A tale of transmission: *Aeromonas veronii* activity within leech-exuded mucus. Appl Environ Microbiol 82(9):2644–2655. https://doi.org/10.1128/AEM.00185-16

Palleroni NJ (2015) *Pseudomonas*. In: Whitman WB, Rainey F, Kämpfer P, Trujillo M, Chun J, DeVos P, Hedlund B, Dedysh S (eds) Bergey's manual of systematics of archaea and bacteria, vol 1. Wiley, Hoboken

Pramono AK et al (2015) *Dysgonomonas termitidis* sp. nov., isolated from the gut of the subterranean termite *Reticulitermes speratus*. Int J Syst Evol Microbiol 65(2):681–685. https://doi.org/10.1099/ijs.0.070391-0

Pruesse E, Peplies J, Glöckner FO (2012) SINA: accurate high-throughput multiple sequence alignment of ribosomal RNA genes. Bioinformatics 28(14):1823–1829. https://doi.org/10.1093/bioinformatics/bts252

Schauss T et al (2015) Improved detection of Extended Spectrum Beta-Lactamase (ESBL)-producing *Escherichia coli* in input and output samples of German biogas plants by a selective pre-enrichment procedure. PLoS ONE 10(3):e0119791. https://doi.org/10.1371/journal.pone.0119791

Schreven JJ et al (2021) Relative contributions of egg-associated and substrate-associated microorganisms to black soldier fly larval performance and microbiota. FEMS Microbiol Ecol 97(5):fiab054

Shelomi M et al (2021) Microbes associated with black soldier fly (Diptera: Stratiomiidae) degradation of food waste. Environ Entomol 49(2):405–411. https://doi.org/10.1093/EE/NVZ164

Sontowski R, van Dam NM (2020) Functional variation in dipteran gut bacterial communities in relation to their diet, life cycle stage and habitat. Insects 11(8):543

Tegtmeier D et al (2021) Culture-independent and culture-dependent characterization of the black soldier fly gut microbiome reveals a large proportion of culturable bacteria with potential for industrial applications. Microorganisms 9(8):1642

Toth EM et al (2006) Bacteria isolated from the different developmental stages and larval organs of the obligate parasitic fly, *Wohlfahrtia magnifica* (Diptera: Sarcophagidae). Microb Ecol 51(1):13–21

Vera-Ponce de León A et al (2020) Cultivable, host-specific bacteroidetes symbionts exhibit diverse polysaccharolytic strategies. Appl Environ Microbiol 86(8):e00091-e120

Versalovic J et al (1994) Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. Methods Mol Cell Biol 5(1):25–40

Wang Y-S, Shelomi M (2017) Review of black soldier fly (*Hermetia illucens*) as animal feed and human food. Foods 6(10):91. https://doi.org/10.3390/foods6100091

Wynants E, Frooninckx L, Crauwels S, Verreth C, De Smet J, Sandrock C, Wohlfahrt J, Van Schelt J, Depraetere S, Lievens B, Van Miert S, Claes J, Van Campenhout L (2019) Assessing the Microbiota of Black Soldier Fly Larvae (*Hermetia illucens*) reared on organic waste streams on four different locations at laboratory and large scale. Microbial Ecol 77(4):913–930. https://doi.org/10.1007/s00248-018-1286-x

Yang F, Tomberlin JK, Jordan HR (2021) Starvation alters gut microbiome in black soldier fly (Diptera: Stratiomyidae) larvae. Front Microbiol 12:160

Yarza P et al (2008) The all-species living tree project: a 16S rRNA-based phylogenetic tree of all sequenced type strains. Syst Appl Microbiol 31(4):241–250

Yoon S-H et al (2017) Introducing EzBioCloud: a taxonomically united database of 16S rRNA gene sequences and whole-genome assemblies. Int J Syst Evol Microbiol 67(5):1613–1617. https://doi.org/10.1099/ijsem.0.001755

Yu G et al (2011) Inoculating poultry manure with companion bacteria influences growth and development of black soldier fly (Diptera: Stratiomyidae) larvae. Environ Entomol 40(1):30–35

Zheng L et al (2013) Bacteria mediate oviposition by the black soldier fly, *Hermetia illucens* (L.), (Diptera:

Stratiomyidae). Sci Rep 3(1):2563. https://doi.org/10.1038/srep02563

Zurek L, Schal C, Watson DW (2000) Diversity and contribution of the intestinal bacterial community to the development of *Musca domestica* (Diptera: Muscidae) larvae. J Med Entomol 37(6):924–928

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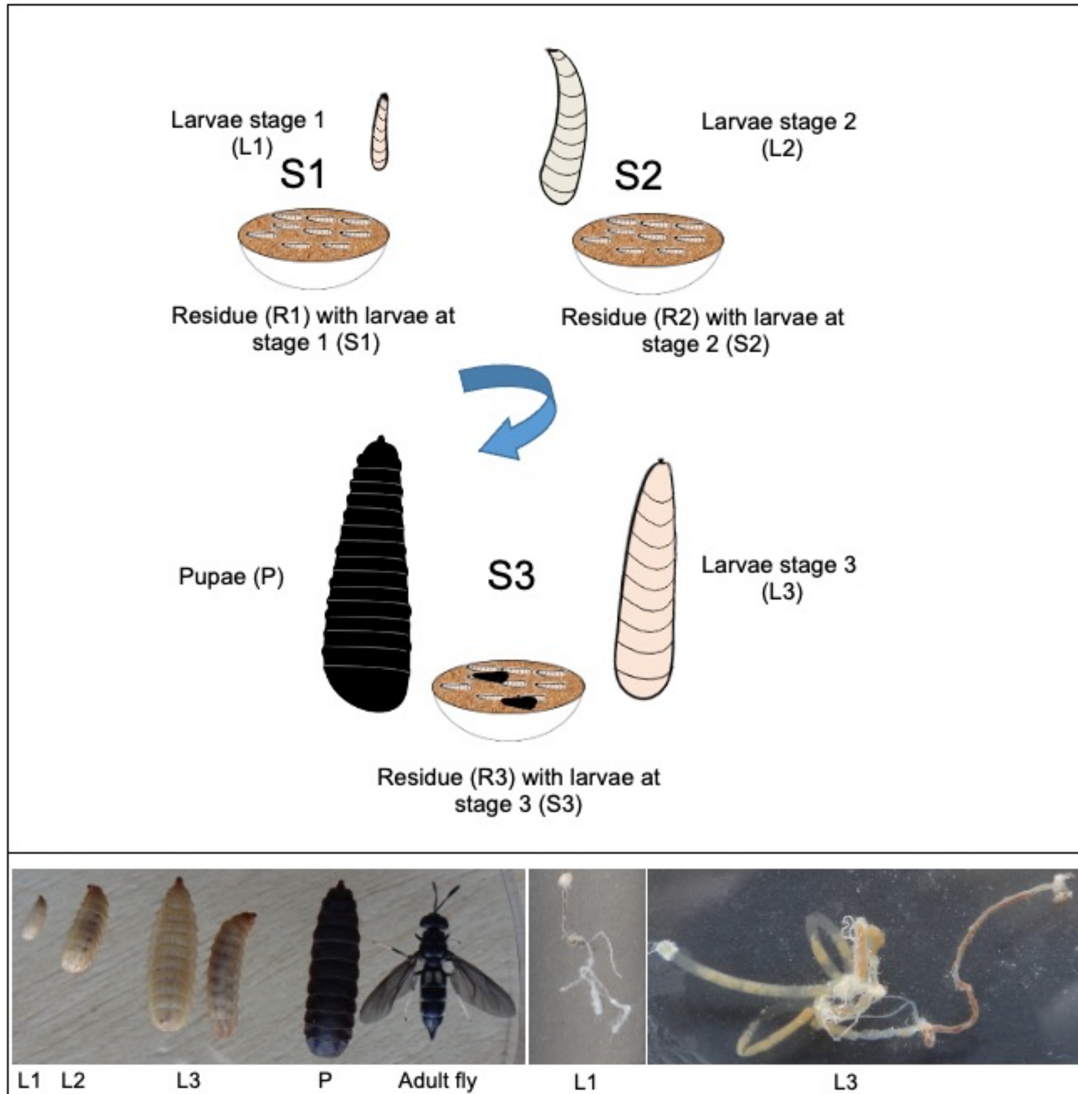
Annex

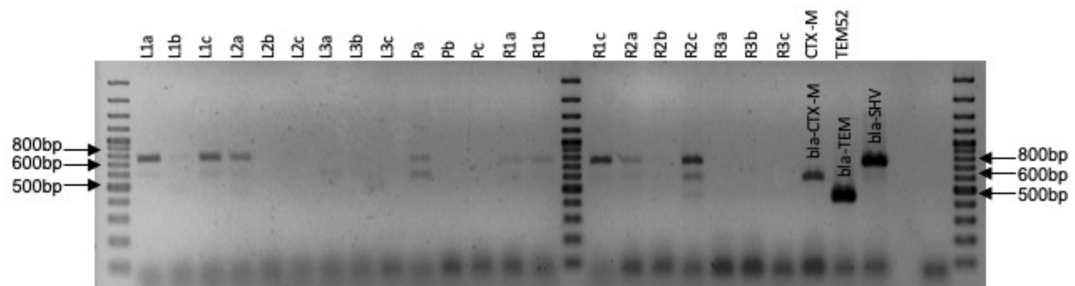
Chapter I, Supplementary material



Supplementary Fig 1 Scheme of the experimental design

Larvae (L) and pupae (P) of *Hermetia illucens* were harvested at three different growth stages (S1 to S3). The growth substrate was added at the beginning of the feeding experiment and processed during the rearing process without further addition of new substrate. The residue of the substrate (R) was harvested in parallel to larvae/pupae (R1 - R3).

Figure A.1



Supplementary Fig 2 Ethidium bromide stained agarose gel depicting PCR products of bla_{TEM}, bla_{CTX-M}, and bla_{SHV} in fed residue (R) and gut (L, P9) samples taken from three different rearing stages (1-3). a-c: biological replicates

Figure A.2

Supplementary Table 1. Data of applied quantitative PCR (qPCR) assays.

Target gene	Sulfonamide resistance gene *sul2*	SHV β-lactamases gene *bla*$_{SHV}$	tetracycline resistance protein TetM coding gene *tetM*	Alternative penicillin binding protein coding gene *mecA*	Quaternary ammonium compound (QAC) resistance genes coding for a transmembrane protein (*qacE/qacEΔ1*).	*Bacteria* 16S rRNA gene
Primer system used for qPCR	sul2F/sul2R	blaSHV-F/blaSHV-R	tet(M)F/tet(M)R	MECUP1/MECUP2	qacEallF/qacEallR/ qacEallP	UnivF/UnivR
Primer sequence and references used for qPCR	*sul2*-F TCGTCAACATAACC TCGGACAG *sul2*-R GTTGCGTTTGATAC CGGCAC (Byrne-Bailey et al. 2009)	*bla*SHV-F ATGCGTTATATTCG CCTGTG *bla*SHV-R TGCTTTGTTATTCG GGCCAA) (Paterson et al. 2003)	tet(M)-F ACAGAAAGCTTATT ATATAAC tet(M)-R TGGCGTGTCTATGA TGTTCAC (Kobayashi et al. 2007)	MECUP1 GGGATCATAGCGTC ATTATTC MECUP2 AACGATTGTGACAC GATAGCC (Poulsen et al. 2003).	qacEallF CGCATTTTATTTTCT TTCTCTGGTT qacEallR CCCGACCAGACTGC ATAAGC qacEallP FAM- TGAAATCCATCCCT GTCGG TGT-TAMRA (Jechalke et al. 2014)	Universal-F GTGSTGCAYGGYTG TCGTCA Universal-R ACGTCRTCCMCACC TTCCTC (Maeda et al. 2003).
Length qPCR products [bp]	478	747	170	526	68	147
Initial denaturation	98°C, 2 min	98°C, 2min	98°C, 2min	98°C, 2min	50°C, 95°C, 10min	98°C, 2min
Denaturation	98°C, 5 sec	98°C, 5 sec	98°C, 5 sec	98°C, 5 sec	95°C, 15 sec	98°C, 5 sec
Primer annealing (Acquisition 1)[1]	60°C, 5 sec	60°C, 5 sec	51°C, 5 sec	55°C, 5 sec	60°C, 1 min	60°C, 5 sec

Extra step for primer dimer melting (Acquisition 2)[1]	79.9°C, 5 sec	--	74°C, 5 sec	--	--	--
Acquisition used for analysis	79.9°C	60°C	74°C	60°C		60°C
Number of cycles	45	45	45	45	40	45
Melting curve[2]	65-95°C, 0.5°C/sec	65-95°C, 0.5°C/sec	65-95°C, 0.5°C/sec	65-95°C, 0.5°C/sec	--	65-95°C, 0.5°C/sec
Template for qPCR standards	*E. coli* ESBL37-B15-13-1E	*E. coli* DSM 22311	Cloned PCR product from environmental sample	*Staphylococcus aureus* MRSA ST398	*E. coli* ESBL37-B15-13-1E	*Luteolibater cuticulihirudinis* E100^T
Standard-DNA fragment	qPCR primers were applied and products cloned with the pJet1.2 system and reamplified from plasmids using pjet1.2-F CGACTCACTATAGG GAGAGCGGC pjet1.2-R AAGAACATCGATTT TCCATGGCAG	blaSHV S1-F TGGTTATGCGTTAT ATTCGCC blaSHV S2-R GGTTAGCGTTGCCA GTGCT (Pai et al.1999)	qPCR primers were applied and products cloned with the pJet1.2 system and reamplified from plasmids using pjet1.2-F CGACTCACTATAGG GAGAGCGGC pjet1.2-R AAGAACATCGATTT TCCATGGCAG	qPCR primers were applied and products cloned with the pJet1.2 system and reamplified from plasmids using pjet1.2-F CGACTCACTATAGG GAGAGCGGC pjet1.2-R AAGAACATCGATTT TCCATGGCAG	qacEΔ1F ATCGCAATAGTTGG CGAAGT sul1bR GCAAGGCGGAAAC CCGCGCC (Sandvang *et al.* 1998)	EUB9-F- (GAGTTTGATCMTG GCTCAG) EUB1492-R- (ACGGYTACCTTGTT ACGACTT) (Weisburg *et al.* 1991)
Employed standard range in qPCR	$1.0x10^3$- $1.0x10^7$	$1.0x10^3$- $1.0x10^7$	$1.0x10^2$- $1.0x10^8$	$1.0x10^5$- $1.0x10^8$	$1.0x10^2$- $1.0x10^8$	$1.0x10^3$- $1.0x10^7$
Efficiency (%)	100.9%	109.1%	99.1%	97.2%	96.8%	86.3%

R2	0.985	0.996	0.999	0.993	0.987	0.999
Slope	-3.301	-3.122	-3.3444	-3.391	-3.401	-3.701
y-int	34.581	34.684	35.492	40.769	41.606	37.381

[1]Aquisition was performed at an increased temperature (Acquisition 2) if primer dimers were formed by the applied primer system to melt primer dimers before acquisition. The temperature was evaluated by melt-curve analysis. At the respective temperature primer dimers were totally melt while PCR products of the target genes did not start to melt.

[2]Positive or negative results were confirmed with the melting curves of each sample accompanied with agarose gel analyses of the PCR product.

References

Byrne-Bailey KG, Gaze WH, Kay P, Boxall ABA, Hawkey PM, Wellington EMH (2009) Prevalence of sulfonamide resistance genes in bacterial isolates from manured agricultural soils and pig slurry in the United Kingdom. Antimicrob Agents Chemother 53:696–702. https://doi.org/10.1128/AAC.00652-07

Jechalke S, Schreiter S, Wolters B, Dealtry S, Heuer H, Smalla K (2014) Widespread dissemination of class 1 integron components in soils and related ecosystems as revealed by cultivation-independent analysis. Front Microbiol 4:420

Kobayashi T, Suehiro F, Cach Tuyen B, Suzuki S (2007) Distribution and diversity of tetracycline resistance genes encoding ribosomal protection proteins in Mekong River sediments in Vietnam. FEMS Microbiol Ecol 59:729–737. https://doi.org/10.1111/j.1574-6941.2006.00244.x

Maeda H, Fujimoto C, Haruki Y, Maeda T, Kokeguchi S, Petelin M, Arai H, Tanimoto I, Nishimura F, Takashiba S (2003) Quantitative real-time PCR using TaqMan and SYBR green for *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, TetQ gene and total bacteria. FEMS Immunol Med Microbiol 39:81–86. https://doi.org/10.1016/S0928-8244(03)00224-4

Pai H, Lyu S, Lee JH, Kim J, Kwon Y, Kim JW, Choe KW (1999) Survey of extended-spectrum beta-lactamases in clinical isolates of *Escherichia coli* and *Klebsiella pneumoniae*: prevalence of TEM-52 in Korea. J Clin Microbiol 37:1758–1763

Paterson DL, Hujer KM, Hujer AM, Yeiser B, Bonomo MD, Rice LB, Bonomo RA (2003) Extended-spectrum beta-lactamases in *Klebsiella pneumoniae* bloodstream isolates from seven countries: dominance and widespread prevalence of SHV- and CTX-M-type beta-lactamases. Antimicrob Agents Chemother 47:3554–3560. https://doi.org/10.1128/AAC.47.11.3554-3560.2003

Poulsen AB, Skov R, Pallesen LV (2003) Detection of methicillin resistance in coagulase-negative staphylococci and in staphylococci directly from simulated blood cultures using the EVIGENE MRSA Detection Kit. J Antimicrob Chemother 51:419–421. https://doi.org
/10.1093/jac/dkg084

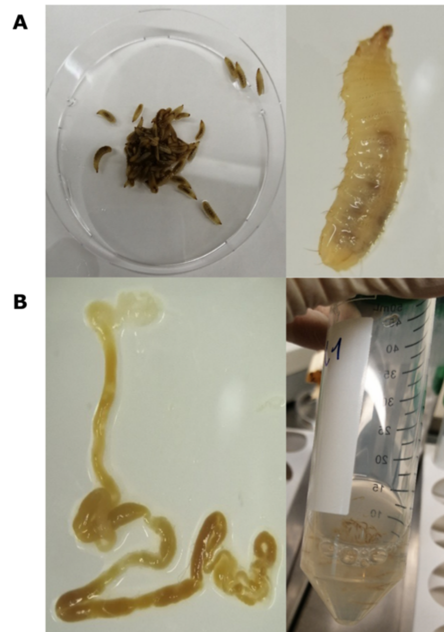
Sandvang D, Aarestrup FM, Jensen LB (1998) Characterisation of integrons and antibiotic resistance genes in Danish multiresistant *Salmonella enterica* Typhimurium DT104. FEMS Microbiol Lett 160:37–41. https://doi.org/10.1111/j.1574-6968.1998.tb12887.x

Weisburg WG, Barns SM, Pelletier DA, Lane DJ (1991) 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol Res 173:697–703. https://doi.org/10.1128/jb.173.2.697-703.1991

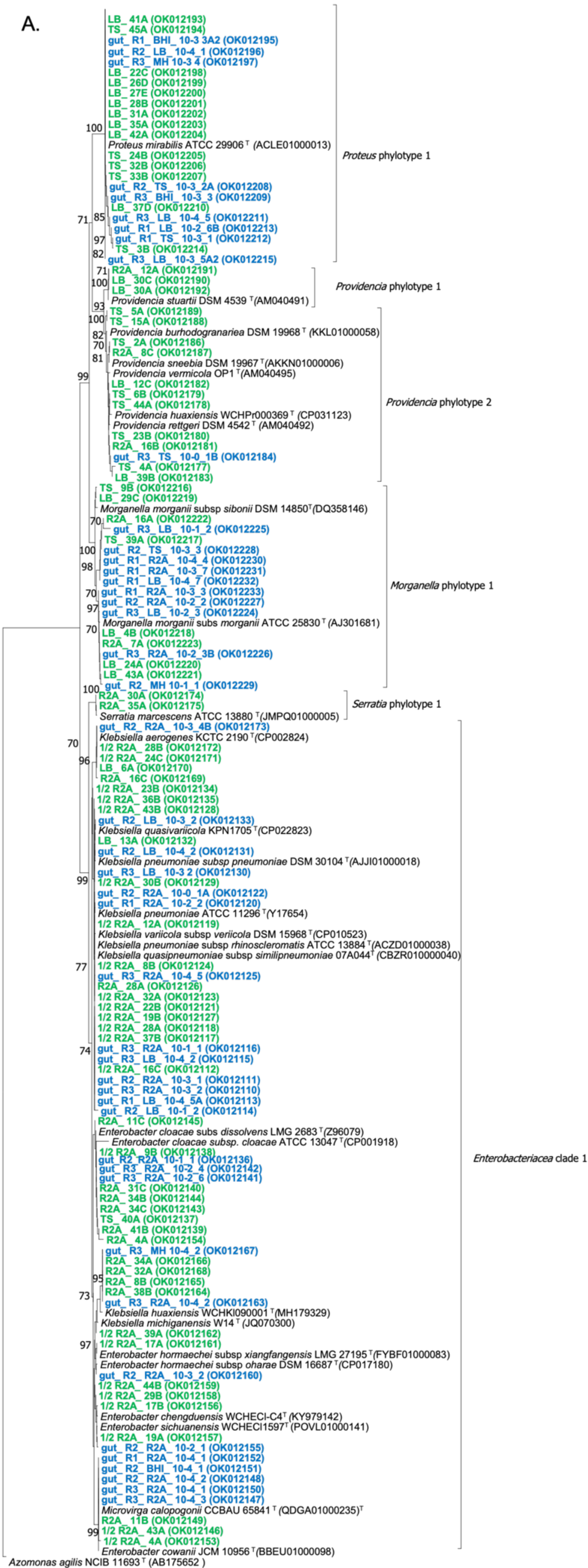
Annex

Chapter II, Supplementary material

Supplementary Figures



Supplementary Fig S1. A. BSFL before dissection. **B.** Dissected guts and resuspended guts in sterile 50 ml polypropylene tubes.



A.
LB_ 41A (OK012193)
TS_ 45A (OK012194)
gut_ R1_ BHI_ 10-3 3A2 (OK012195)
gut_ R2_ LB_ 10-4_1 (OK012196)
gut_ R3_ MH 10-3 4 (OK012197)
LB_ 22C (OK012198)
LB_ 26D (OK012199)
LB_ 27E (OK012200)
LB_ 28B (OK012201)
LB_ 31A (OK012202)
LB_ 35A (OK012203)
LB_ 42A (OK012204)
Proteus mirabilis ATCC 29906 T (ACLE01000013)
TS_ 24B (OK012205)
TS_ 32B (OK012206)
TS_ 33B (OK012207)
gut_ R2_ TS_ 10-3_2A (OK012208)
gut_ R3_ BHI_ 10-3_3 (OK012209)
LB_ 37D (OK012210)
gut_ R3_ LB_ 10-4_5 (OK012211)
gut_ R1_ LB_ 10-2_6B (OK012213)
gut_ R1_ TS_ 10-3_1 (OK012212)
TS_ 3B (OK012214)
gut_ R3_ LB_ 10-3_5A2 (OK012215)
Proteus phylotype 1
R2A_ 12A (OK012191)
LB_ 30C (OK012190)
LB_ 30A (OK012192)
Providencia stuartii DSM 4539 T (AM040491)
Providencia phylotype 1
TS_ 5A (OK012189)
TS_ 15A (OK012188)
Providencia burhodogranariea DSM 19968 T (KKL01000058)
TS_ 2A (OK012186)
R2A_ 8C (OK012187)
Providencia sneebia DSM 19967 T (AKKN01000006)
Providencia vermicola OP1 T (AM040495)
LB_ 12C (OK012182)
TS_ 6B (OK012179)
TS_ 44A (OK012178)
Providencia huaxiensis WCHPr000369 T (CP031123)
Providencia rettgeri DSM 4542 T (AM040492)
TS_ 23B (OK012180)
R2A_ 16B (OK012181)
gut_ R3_ TS_ 10-0_1B (OK012184)
TS_ 4A (OK012177)
LB_ 39B (OK012183)
Providencia phylotype 2
TS_ 9B (OK012216)
LB_ 29C (OK012219)
Morganella morganii subsp sibonii DSM 14850T (DQ358146)
R2A_ 16A (OK012222)
gut_ R3_ LB_ 10-1_2 (OK012225)
TS_ 39A (OK012217)
gut_ R2_ TS_ 10-3_3 (OK012228)
gut_ R1_ R2A_ 10-4_4 (OK012230)
gut_ R1_ R2A_ 10-3_7 (OK012231)
gut_ R1_ LB_ 10-4_7 (OK012232)
gut_ R1_ R2A_ 10-3_3 (OK012233)
gut_ R2_ R2A_ 10-2_2 (OK012227)
gut_ R3_ LB_ 10-2_3 (OK012224)
Morganella morganii subs morganii ATCC 25830 T (AJ301681)
LB_ 4B (OK012218)
R2A_ 7A (OK012223)
gut_ R3_ R2A_ 10-2_3B (OK012226)
LB_ 24A (OK012220)
LB_ 43A (OK012221)
gut_ R2_ MH 10-1_1 (OK012229)
Morganella phylotype 1
R2A_ 30A (OK012174)
R2A_ 35A (OK012175)
Serratia marcescens ATCC 13880 T (JMPQ01000005)
Serratia phylotype 1
gut_ R2_ R2A_ 10-3_4B (OK012173)
Klebsiella aerogenes KCTC 2190 T (CP002824)
1/2 R2A_ 28B (OK012172)
1/2 R2A_ 24C (OK012171)
LB_ 6A (OK012170)
R2A_ 16C (OK012169)
1/2 R2A_ 23B (OK012134)
1/2 R2A_ 36B (OK012135)
1/2 R2A_ 43B (OK012128)
gut_ R2_ LB_ 10-3_2 (OK012133)
Klebsiella quasivariicola KPN1705 T (CP022823)
LB_ 13A (OK012132)
gut_ R2_ LB_ 10-4_2 (OK012131)
Klebsiella pneumoniae subsp pneumoniae DSM 30104 T (AJJI01000018)
gut_ R3_ LB_ 10-3 2 (OK012130)
1/2 R2A_ 30B (OK012129)
gut_ R2_ R2A_ 10-0_1A (OK012122)
gut_ R1_ R2A_ 10-2_2 (OK012120)
Klebsiella pneumoniae ATCC 11296 T (Y17654)
1/2 R2A_ 12A (OK012119)
Klebsiella variicola subsp veriicola DSM 15968 T (CP010523)
Klebsiella pneumoniae subsp rhinoscleromatis ATCC 13884 T (ACZD01000038)
Klebsiella quasipneumoniae subsp similipneumoniae 07A044 T (CBZR010000040)
1/2 R2A_ 8B (OK012124)
gut_ R3_ R2A_ 10-4_5 (OK012125)
R2A_ 28A (OK012126)
1/2 R2A_ 32A (OK012123)
1/2 R2A_ 22B (OK012121)
1/2 R2A_ 19B (OK012127)
1/2 R2A_ 28A (OK012118)
1/2 R2A_ 37B (OK012117)
gut_ R3_ R2A_ 10-1_1 (OK012116)
gut_ R3_ LB_ 10-4_2 (OK012115)
1/2 R2A_ 16C (OK012112)
gut_ R2_ R2A_ 10-3_1 (OK012111)
gut_ R3_ R2A_ 10-3_2 (OK012110)
gut_ R1_ LB_ 10-4_5A (OK012113)
gut_ R2_ LB_ 10-1_2 (OK012114)
R2A_ 11C (OK012145)
Enterobacter cloacae subs dissolvens LMG 2683 T (Z96079)
Enterobacter cloacae subsp. cloacae ATCC 13047 T (CP001918)
1/2 R2A_ 9B (OK012138)
gut_R2_R2A_10-1_1 (OK012136)
gut_ R3_ R2A_ 10-2_4 (OK012142)
gut_ R3_ R2A_ 10-2_6 (OK012141)
R2A_ 31C (OK012140)
R2A_ 34B (OK012144)
R2A_ 34C (OK012143)
TS_ 40A (OK012137)
R2A_ 41B (OK012139)
R2A_ 4A (OK012154)
gut_ R3_ MH 10-4_2 (OK012167)
R2A_ 34A (OK012166)
R2A_ 32A (OK012168)
R2A_ 8B (OK012165)
R2A_ 38B (OK012164)
gut_ R3_ R2A_ 10-4_2 (OK012163)
Klebsiella huaxiensis WCHKI090001 T (MH179329)
Klebsiella michiganensis W14 T (JQ070300)
1/2 R2A_ 39A (OK012162)
1/2 R2A_ 17A (OK012161)
Enterobacter hormaechei subsp xiangfangensis LMG 27195 T (FYBF01000083)
Enterobacter hormaechei subsp oharae DSM 16687 T (CP017180)
gut_ R2_ R2A_ 10-3_2 (OK012160)
1/2 R2A_ 44B (OK012159)
1/2 R2A_ 29B (OK012158)
1/2 R2A_ 17B (OK012156)
Enterobacter chengduensis WCHECI-C4T (KY979142)
Enterobacter sichuanensis WCHECI1597T (POVL01000141)
1/2 R2A_ 19A (OK012157)
gut_ R2_ R2A_ 10-2_1 (OK012155)
gut_ R1_ R2A_ 10-4_1 (OK012152)
gut_ R2_ BHI_ 10-4_1 (OK012151)
gut_ R2_ R2A_ 10-4_2 (OK012148)
gut_ R3_ R2A_ 10-4_1 (OK012150)
gut_ R3_ R2A_ 10-4_3 (OK012147)
Microvirga calopogonii CCBAU 65841 T (QDGA01000235)T
R2A_ 11B (OK012149)
1/2 R2A_ 43A (OK012146)
1/2 R2A_ 4A (OK012153)
Enterobacter cowanii JCM 10956 T (BBEU01000098)
Enterobacteriacea clade 1
Azomonas agilis NCIB 11693 T (AB175652)
0.02
100
71
85
97
82
71
100
93
100
82
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81
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97
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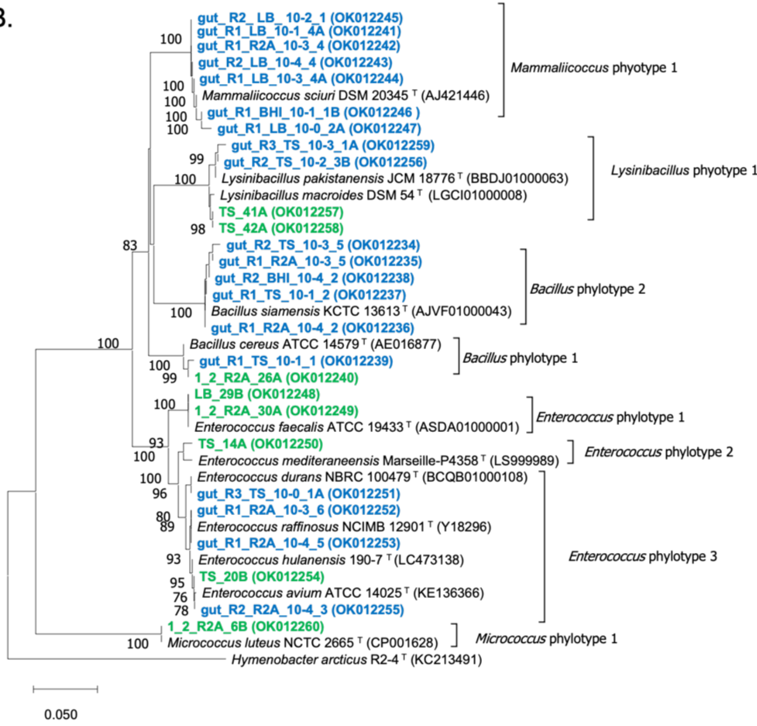

B.
gut_R2_LB_10-2_1 (OK012245)
gut_R1_LB_10-1_4A (OK012241)
gut_R1_R2A_10-3_4 (OK012242)
gut_R2_LB_10-4_4 (OK012243)
gut_R1_LB_10-3_4A (OK012244)
Mammaliicoccus sciuri DSM 20345 T (AJ421446)
gut_R1_BHI_10-1_1B (OK012246)
gut_R1_LB_10-0_2A (OK012247)
Mammaliicoccus phyotype 1
gut_R3_TS_10-3_1A (OK012259)
gut_R2_TS_10-2_3B (OK012256)
Lysinibacillus pakistanensis JCM 18776 T (BBDJ01000063)
Lysinibacillus macroides DSM 54 T (LGCI01000008)
TS_41A (OK012257)
TS_42A (OK012258)
Lysinibacillus phyotype 1
gut_R2_TS_10-3_5 (OK012234)
gut_R1_R2A_10-3_5 (OK012235)
gut_R2_BHI_10-4_2 (OK012238)
gut_R1_TS_10-1_2 (OK012237)
Bacillus siamensis KCTC 13613 T (AJVF01000043)
gut_R1_R2A_10-4_2 (OK012236)
Bacillus phylotype 2
Bacillus cereus ATCC 14579 T (AE016877)
gut_R1_TS_10-1_1 (OK012239)
1_2_R2A_26A (OK012240)
Bacillus phylotype 1
LB_29B (OK012248)
1_2_R2A_30A (OK012249)
Enterococcus faecalis ATCC 19433 T (ASDA01000001)
Enterococcus phylotype 1
TS_14A (OK012250)
Enterococcus mediteraneensis Marseille-P4358 T (LS999989)
Enterococcus phylotype 2
Enterococcus durans NBRC 100479 T (BCQB01000108)
gut_R3_TS_10-0_1A (OK012251)
gut_R1_R2A_10-3_6 (OK012252)
Enterococcus raffinosus NCIMB 12901 T (Y18296)
gut_R1_R2A_10-4_5 (OK012253)
Enterococcus hulanensis 190-7 T (LC473138)
TS_20B (OK012254)
Enterococcus avium ATCC 14025 T (KE136366)
gut_R2_R2A_10-4_3 (OK012255)
Enterococcus phylotype 3
1_2_R2A_6B (OK012260)
Micrococcus luteus NCTC 2665 T (CP001628)
Micrococcus phylotype 1
Hymenobacter arcticus R2-4 T (KC213491)
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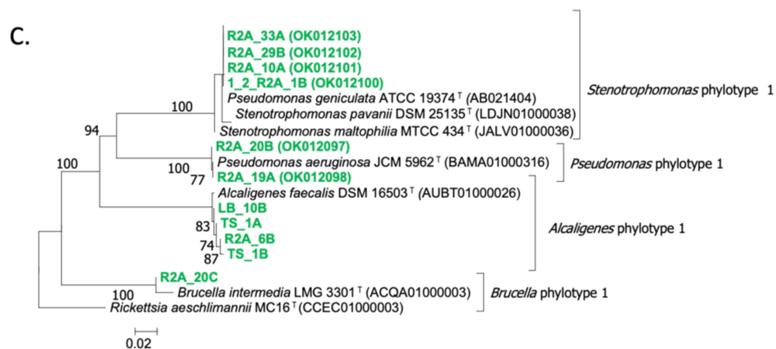
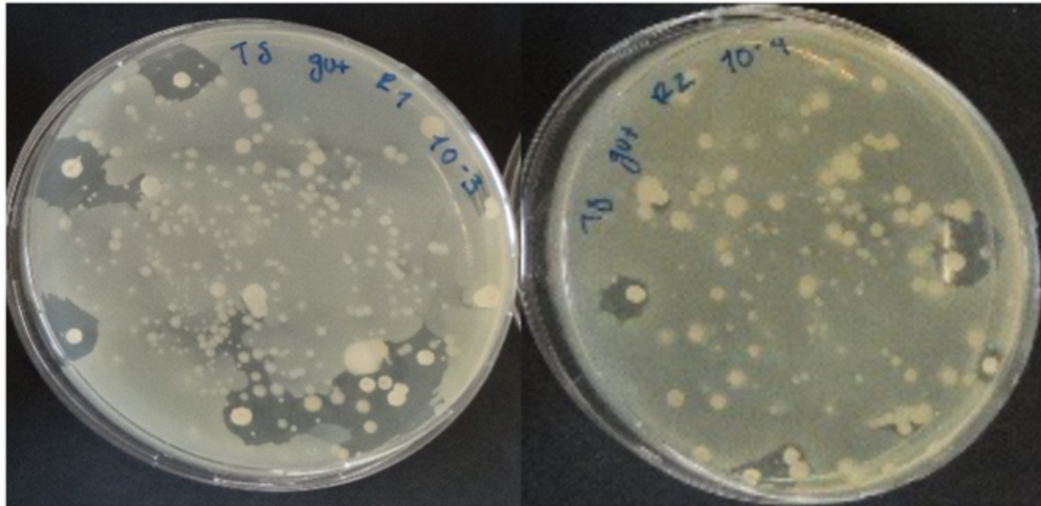
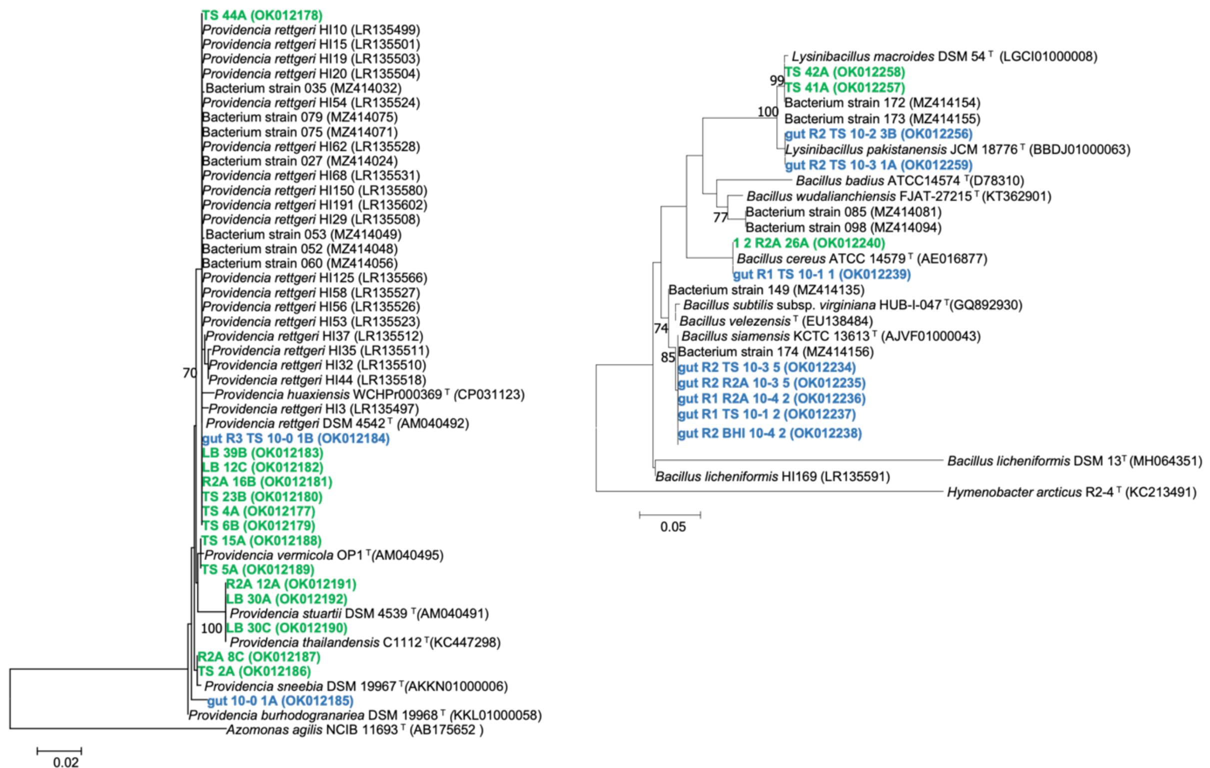


Supplementary Fig S2. Neighbour joining trees showing the phylotype assignment of the bacterial isolates cultured from BSFL gut samples based on partial 16S rRNA gene sequences. Trees were calculated for the *Enterobacteriaceae* clade (**A**), *Firmicutes* and *Actinobacteria* (**B**), and *Alpha*- and other *Gammaproteobacteria* (**C**). Analysis was performed in MEGA7 using the Jukes-Cantor distance correction as evolutionary model and 100 replications for bootstrap analysis. Bootstrap values (>70 %) are given at the branch nodes. All isolated culture by the direct plating are in blue bold and those from the dilution-to-extinction in green bold. Accession numbers of isolates and type strains are given in brackets.



Supplementary Fig S3. Bacterial growth on TS agar after direct plating of serially diluted cell suspensions derived from the BSFL gut samples. An inhibition of swarming bacteria (identified as *Proteus* spp.) was obtained as clear inhibition zones around some colonies identifies as *Bacillus* spp.

R2A 11C (OK012145)
TS 40A (OK012137)
R2A 41B (OK012139)
R2A 31C (OK012140)
gut R3 R2A 10-2 6 (OK012141)
gut R3 R2A 10-2 4 (OK012142)
R2A 34C (OK012143)
R2A 34B (OK012144)
Enterobacter ludwigii EN-119T (JTLO01000001)
Enterobacter cloacae subsp. dissolvens ATCC 23373T (AJ417485)
1 2 R2A 29B (OK012158)
Enterobacter cloacae ATCC 13047T T (AJ417484)
R2A 4A (OK012154)
1 2 R2A 17B (OK012156)
1 2 R2A 19A (OK012157)
gut R2 R2A 10-2 1 (OK012155)
gut R1 R2A 10-4 1 (OK012152)
gut R2 BHI 10-4 1 (OK012151)
gut R3 R2A 10-4 1 (OK012150)
R2A 11B (OK012149)
88
gut R2 R2A 10-4 2 (OK012148)
gut R3 R2A 10-4 3 (OK012147)
1 2 R2A 43A (OK012146)
1 2 R2A 4A (OK012153)
Enterobacter cowanii CIP 107300T (AJ508303)
gut R2 R2A 10-1 1 (OK012136)
1 2 R2A 44B (OK012159)
Klebsiella michiganensis W14 T (JQ070300)
1 2 R2A 17A (OK012161)
Enterobacter hormaechei subsp. hoffmannii DSM 14563 T(CP017186)
Enterobacter hormaechei subsp oharae DSM 16687 T(CP017180)
Enterobacter hormaechei subsp xiangfangensis LMG 27195 T(FYBF01000083)
gut R2 R2A 10-3 2 (OK012160)
1 2 R2A 39A (OK012162)
gut R3 MH 10-4 2 (OK012167)
R2A 34A (OK012166)
R2A 8B (OK012165)
R2A 38B (OK012164)
gut R3 R2A 10-4 2 (OK012163)
Bacterium strain 122 (MZ414118)
Bacterium strain 121 (MZ414117)
Bacterium strain 111 (MZ414107)
Klebsiella oxytoca ATCC 13182 T (FJ971867)
Klebsiella michiganensis HI207 (LR135612)
Klebsiella michiganensis HI201 (LR135607)
Klebsiella michiganensis HI198 (LR135605)
Klebsiella michiganensis HI197 (LR135604)
Klebsiella michiganensis HI183 (LR135598)
Klebsiella michiganensis HI176 (LR135594)
Klebsiella michiganensis HI168 (LR135590)
R2A 32A (OK012168)
Klebsiella michiganensis HI154 (LR135582)
Klebsiella michiganensis HI161 (LR135586)
Klebsiella michiganensis HI160 (LR135585)
Enterobacter huaxiensis 090008 T(MK049964)
Enterobacter chengduensis WCHECl-C4 T(KY979142)
Klebsiella quasipneumoniae subsp. similipneumoniae 07A044T T(HG933295)
gut R2 R2A 10-3 4B (OK012173)
1 2 R2A 24C (OK012171)
LB 6A (OK012170)
R2A 16C (OK012169)
1 2 R2A 23B (OK012134)
Bacterium strain 067 (MZ414063)
Bacterium strain 057 (MZ414053)
Bacterium strain 054 (MZ414050)
Klebsiella aerogenes KCTC 2190 T(CP002824)
78
Enterobacter aerogenes HI131 (LR135572)
Enterobacter aerogenes HI110 (LR135558)
Enterobacter aerogenes HI109 (LR135557)
Enterobacter aerogenes HI108 (LR135556)
Enterobacter aerogenes HI107 (LR135555)
Enterobacter aerogenes HI105 (LR135554)
Enterobacter aerogenes HI104 (LR135553)
1 2 R2A 28B (OK012172)
Enterobacter aerogenes HI72 (LR135534)
LB 13A (OK012132)
gut R2 LB 10-4 2 (OK012131)
gut R3 LB 10-3 2 (OK012130)
1 2 R2A 30B (OK012129)
1 2 R2A 43B (OK012128)
1 2 R2A 19B (OK012127)
R2A 28A (OK012126)
gut R3 R2A 10-4 5 (OK012125)
1 2 R2A 8B (OK012124)
1 2 R2A 32A (OK012123)
gut R2 R2A 10-0 1A (OK012122)
1 2 R2A 22B (OK012121)
gut R1 R2A 10-2 2 (OK012120)
1 2 R2A 12A (OK012119)
1 2 R2A 28A (OK012118)
1 2 R2A 37B (OK012117)
gut R3 R2A 10-1 1 (OK012116)
gut R3 LB 10-4 2 (OK012115)
gut R2 LB 10-1 2 (OK012114)
gut R1 LB 10-4 5A (OK012113)
1 2 R2A 16C (OK012112)
gut R2 R2A 10-3 1 (OK012111)
gut R3 R2A 10-3 2 (OK012110)
Bacterium strain 139 (MZ414130)
Bacterium strain 064 (MZ414060)
Bacterium strain 064 (MZ414060)
Bacterium strain 063 (MZ414059)
Klebsiella variicola subsp veriicola DSM 15968 T(CP010523)
1 2 R2A 36B (OK012135)
Klebsiella variicola DSM 15968 T(KP783170)
Klebsiella quasipneumoniae subsp similipneumoniae 07A044T (CBZR010000040)
Klebsiella pneumoniae subsp rhinoscleromatis ATCC 13884 T(ACZD01000038)
Klebsiella pneumoniae subsp. ozaenae ATCC 11296 T (AF130982)
Klebsiella quasipneumoniae HI165 (LR135588)
Klebsiella granulomatis HI151 (LR135581)
gut R2 LB 10-3 2 (OK012133)
Azomonas agilis NCIB 11693T (AB175652)
0.02



Supplementary Fig S4. Neighbour joining trees showing the phylogenetic placement of the bacterial isolates from Callegari et al. (2020), Tegtmeier et al. (2021), and this study from BSFL gut microbiota. Trees were calculated based on partial 16S rRNA gene sequences in MEGA7 using the Jukes-Cantor distance correction as evolutionary model and 100 replications for bootstrap analysis. Bootstrap values (> 70 %) are given at the branch nodes. All isolates from this study are in blue bold for direct plating and green bold for dilution-to-extinction. Accession numbers are given in brackets.

Abbreviations

AMP: Antimicrobial Peptide
ANOVA: Analysis of Variance
ANOSIM: Analysis of Similarities
ARG: Antibiotic Resistance Gene
BHI: Brain Heart Infusion
BLAST: Basic Local Alignment Search Tool
BSFL: Black Soldier Fly Larvae
BOX-PCR: BOX-Polymerase Chain Reaction
Ct value: Cycle threshold
DNA: Deoxyribonucleic acid
ESBL: Extended Spectrum Beta-Lactamase
g: gram
µg: microgram
LB: Lysogeny Broth
MC: Monte Carlo
MH: Mueller-Hinton
mL: milliliter
NMDS: Non-Metric Multidimensional Scaling
OTU: Operational Taxonomic Unit
PCR: Polymerase Chain Reaction
PERMANOVA: Permutational Multivariate Analysis of Variance
PERMDISP: Permutational analysis of multivariate dispersions
QAC: Quaternary Ammonium Compound
qPCR: Quantitative Polymerase Chain Reaction
R2A: Reasoner's 2A Agar
½ R2A: Half-concentrated Reasoner's 2A Broth
16S rRNA: 16S Ribosomal RNA
S1, S2, S3: These abbreviations refer to the three larval development stages examined in the study
SIMPER: Similarity Percentages
SRA: Sequence Read Archive

TS: Tryptic Soy

TSPP: Tetra-Sodium Pyrophosphate

UPGMA: Unweighted Pair Group Method with Arithmetic Mean

List of publications

Publication I

Cifuentes, Yina, Stefanie P. Glaeser, Jacques Mvie, Jens-Ole Bartz, Ariane Müller, Herwig O. Gutzeit, Andreas Vilcinskas, and Peter Kämpfer. "The gut and feed residue microbiota changing during the rearing of Hermetia illucens larvae." *Antonie Van Leeuwenhoek* 113 (2020): 1323-1344.

Publication II

Cifuentes, Yina, Andreas Vilcinskas, Peter Kämpfer, and Stefanie P. Glaeser. "Isolation of Hermetia illucens larvae core gut microbiota by two different cultivation strategies." *Antonie Van Leeuwenhoek* 115, no. 6 (2022): 821-837.

Publication III

Krause, Hans-Martin, Joe G. Ono-Raphel, Edward Karanja, Felix Matheri, Martina Lori, Yina Cifuentes, Stefanie P. Glaeser et al. "Organic and conventional farming systems shape soil bacterial community composition in tropical arable farming." Applied Soil Ecology 191 (2023): 105054.

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