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RESEARCH ARTICLE

Microbial safety of industrially reared *Hermetia illucens* larvae and frass: bacterial dynamics and prevalence of antibiotic resistance genes

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Abstract

The larvae of the black soldier fly (BSFL) can efficiently convert food waste into valuable biomass. They are also an alternative source of fat, protein, and chitin, but little is known about the total microbiota of the whole BSFL and its impact on the microbiological safety of food and feed. This study was conducted to determine bacterial microbiota dynamics of the whole BSFL and frass residues during the industrial rearing process, including the effects of thermal treatment, counts and identification of cultivable bacteria, and the presence of antibiotic resistance genes (ARGs). The second and the fourth instar larvae, frozen and dried fourth instar larvae, and frass samples were examined. The composition of the total bacterial microbiota in BSFL samples was similar and dominated by Proteobacteria while in frass Firmicutes prevailed. The lowest diversity was observed in the second instar larvae and the highest in the frass. The samples showed a relatively low bacterial community diversity. In agreement with the analysis of the total microbiota, the isolated cultivable bacterial strains were members of *Proteus*, *Providencia*, *Morganella*, *Staphylococcus*, *Klebsiella*, *Enterococcus*, and *Bacillus* genera. The counts of cultivable bacteria increased during the growth of larvae and were similar in the fourth instar larvae and frass residues. Dried larvae had the lowest number of viable counts and were dominated by spore-forming bacteria. The determined viable aerobic counts meet the criteria for edible insects. ARGs conferring resistance to aminoglycosides (*aac-aph*), β-lactams (*blaZ*), erythromycin (*ermA*), tetracycline (*tetM*, *tetW*), and vancomycin (*vanA*, *vanB*) were detected by PCR, with the highest diversity and detection rate in frass. The gene *tetM* was the most widespread and detected in all groups of the tested samples. The results of this work extend the scarce knowledge about the dynamics of microorganisms and ARGs in the industrial-scale food waste upcycling process by BSFL.

Keywords

black soldier fly – endospores – microbiota – tetracycline

1 Introduction

The global problem of food waste is increasingly being addressed through innovative solutions, such as the use of *Hermetia illucens*, also known as the black soldier fly. BSFL are efficient in upcycling food waste into valuable biomass, attracting sustainable companies to expand the use of BSFL on an industrial scale world-

wide (Rehman *et al.*, 2023). This process promotes the circular economy addressing issues like biological waste, sustainable protein production, and food scarcity.

Food waste has been recently shown to be a safe and efficient feed source for BSFL rearing (Naser El Deen *et al.*, 2023). However, the recycling of contaminated waste by insects poses a serious risk of the transmission of pathogenic microorganisms (Vandeweyer *et al.*, 2021). Therefore, proper monitoring of the recycling process must be assured.

Most of the BSFL research has been conducted under controlled laboratory conditions that do not directly translate into the dynamics observed in the large-scale experiments (Yang and Tomberlin, 2020). The majority of published data are focused on the gut microbiota of BSFL (Eke *et al.*, 2023), which is influenced by various factors, such as rearing substrate, temperature, developmental stage, feeding duration, and the density of the larvae, etc. (Bruno *et al.*, 2019; De Smet *et al.*, 2018; Li *et al.*, 2021; Querejeta *et al.*, 2023; Raimondi *et al.*, 2020; Schreven *et al.*, 2022; Varotto Boccazzi *et al.*, 2017). Some studies suggest that the microbiota is also strongly affected by the local microbiota in rearing facilities, as well as the geographical origin of *H. illucens* (Khamis *et al.*, 2020; Wynants *et al.*, 2019). One of the most challenging tasks is to ensure the safety of BSFL products when upcycling waste material which is not fully characterized and even less controlled.

Temperature treatments are a common practice to mitigate microbial contamination (Aleknavičius *et al.*, 2022). However, these are costly and ineffective against spore-forming bacteria such as *Bacillus* spp. Since BSFL contain a significant proportion of fats (about 20-41% of dry mass; Bessa *et al.*, 2020), high temperatures may be unfavourable in terms of lipid oxidation and generation of toxic polar compounds (Grootveld *et al.*, 2020). Efficient strategies to neutralise undesirable microorganisms are crucial for maximising the safety of waste upcycling.

BSFL frass residues, the end products of BSFL waste upcycling, are a valuable source of minerals, organic material, and bacteria (Lomonaco *et al.*, 2024). Although not directly used for food and feed, it is an efficient biofertilizer. The biosafety of frass must also be ensured to prevent the spread of harmful bacteria and ARGs. Recently, waste treatment by BSFL has gained attention as a useful tool to reduce ARGs in animal manure, by also lowering the abundance of antibiotic-resistant bacteria (Niu *et al.*, 2022; Zhao *et al.*, 2023a, b). BSFL efficiently degrade mycotoxins, pesticides, and tetracyclines, and are therefore suitable for the neutralisation of some antibiotics (Van Huis, 2020). The dynamics of the microbiota components in BSFL frass require further investigation.

Insects can participate in the ARGs transfer, conferring resistance to antibiotics commonly used in medicine, agriculture, and aquaculture (Rawat *et al.*, 2023). The widespread use of antibiotics exerts selective pressure that can lead to the development and persistence of antibiotic-resistant strains. To ensure the effective use of BSFL as food or feed, the risk of ARGs must be assessed. To date, only a few studies have assessed the prevalence of ARGs in BSFL and frass residues (Cai *et al.*, 2018; Cifuentes *et al.*, 2020; Liu *et al.*, 2020; Milanović *et al.*, 2021b). Tetracycline and erythromycin resistance genes are commonly found in most industrially reared insects, including *H. illucens* (Cai *et al.*, 2018; Cifuentes *et al.*, 2020; Liu *et al.*, 2020; Milanović *et al.*, 2021b).

In this study, the microbial safety of plant-based food waste-fed, industrially reared BSFL was evaluated. Three rearing stages were investigated: the second (2i) and the fourth instar larvae, and frass residues after BSFL harvesting (R). Three thermal treatments of the fourth instar larvae were assessed: freezing at –20 °C for 2 h (4i), for 24 h (F), and F drying at 105 °C for 2 h (D). Microbial safety in terms of total bacterial microbiota, viable counts on various growth media, isolation and identification of cultivable microorganisms, and the presence of ARGs were analysed. Of published works, in addition to the microbiota analysis of the whole larvae, only two inquired into industrial scale rearing (Gorrens *et al.*, 2022; Wynants *et al.*, 2019), only one that counted total aerobic counts also performed isolation and identification of cultivable bacteria (Gorrens *et al.*, 2021), only one assessed the impact of the post-harvesting treatment (Soomro *et al.*, 2021), and none have investigated the abundance of ARGs. Therefore, this work provides a novel comprehensive investigation considering multiple characteristics of BSFL safety that have not been previously analysed concomitantly.

2 Materials and methods

Origin and sampling of H. illucens *larvae and frass*

The *H. illucens* larvae and frass used in this study were produced and supplied by a local commercial producer (Insectum, Vilnius, Lithuania). The larvae were housed in metal containers (30 × 60 × 30 cm) with inward edges on top to prevent escape in the chamber at 26 °C and 50-60% relative humidity and constant filtered airflow (complete air exchange every 3 h). The sieving proce-

TABLE 1 Sample coding description

Coding	Sample description
2i	2nd instar larvae frozen at –20 °C for 2 h.
4i	4th instar larvae frozen at –20 °C for 2 h.
F	4th instar larvae frozen at –20 °C for 24 h.
D	4th instar larvae frozen at –20 °C for 24 h and dried at 105 °C for 2 h.
R	Frass residues after harvesting of 4th instar larvae.

dure was performed to separate the larvae and frass residues (Table 1). The larvae were fed on a wet (70% humidity) plant-based diet, namely homogenized food waste (6 parts of expired pasta, 4 parts of fruits, and vegetables, and 1 part of barley malt). Nutritional specification of the feed was about 43% carbohydrates, 7% protein, 2.5% fibre, and <1% fat. Larvae were washed under running tap water for 1 minute in a sieve with a 1 mm mesh size to remove the substrate as in Wynants *et al.* (2019). Before processing, the collected larvae (2i and 4i) were frozen at –20 °C for 2 h (for deactivation), whereas F samples were frozen at –20 °C for 24 h, as a part of industrial processing (Liceaga, 2021). To obtain dried larvae (D), the 4th instar larvae, after freezing for 24 h were dried in a conventional oven (MIWE Michael Wenz, Germany) at 105 °C for 2 h. The drying conditions were optimized in the rearing facility and the completion of the drying process was confirmed by the weight change (3.5-fold reduction). Samples for each BSFL stage were collected in triplicate, sampling 100 g of BSFL or frass from three separate containers of the same batch. One container yields 10 kg of the 4th instar larvae. Collected samples were processed immediately after harvesting.

Viable counts

To estimate viable counts, 10 g of each sample (2i, 4i, F, D, and R) were aseptically homogenized with a manual grinder and diluted in a sterile 1× phosphate-buffered saline (PBS) solution (Reachem, India). All samples were diluted in 20 ml of 1× PBS, except for D 30 ml, and for R 40 ml were used because of a high absorption of the liquid. 10-fold dilutions of the samples were inoculated on the plates of selected growth media. Casein Soybean Digest (CASO) agar (Merck, Germany) was selected based on the preliminary observation, that this media produced the highest number of colony-forming units (CFUs) in comparison to other media used in this study. CASO agar plates were incubated at 37 °C for 24 hours before colony counting. Total mesophilic aerobes were counted in CASO and Plate Count Agar (PCA) (Liofilchem, Italy) plates. Aerobic bacterial spores were also counted on PCA plates but before plating homogenates were heated to 80 °C for 15 min followed by cooling in iced for 5 min. PCA plates were incubated at 30 °C for 48 h. The data were expressed as the mean of log CFU per gram ± standard error of independent triplicate biological experiments. A two-way analysis of variance (ANOVA, $P < 0.05$) and Tukey's honestly significant difference test ($P < 0.05$) was used for statistical analysis. Calculations were performed using Microsoft Office Excel 2019.

DNA extraction

10 g of each sample were homogenized with 1× PBS, like for the viable counts experiment. Samples were centrifuged at 800 × *g* for 10 min. Supernatant collected and centrifuged at 16000 × *g* for 2 min. The supernatant was discarded. DNA extraction was performed using about 200 mg of collected sediment per sample, using the Genomic DNA Purification Kit (Thermo Fisher Scientific Baltics, Vilnius, Lithuania), according to manufacturer instructions. The quality and quantity of the extracted DNA were evaluated spectrophotometrically at 260, 280, and 234 nm, using NanoPhotometer P330 (Implen GmbH, Munich, Germany).

DNA sequencing

The next-generation sequencing was performed by ZymoBiomics Targeted Sequencing Service (Zymo Research, Irvine, CA, USA). Bacterial diversity was analysed based on the PCR amplification of V3 and V4 regions of the 16S rRNA genes using 341F (5′-CCTACGGGNGGCWGCAG-3′) and 785R (5′-GACTACH VGGGTATCTAATCC-3′) primers (Klindworth *et al.*, 2013). The final library was sequenced on Illumina MiSeq™ with a v3 reagent kit (600 cycles). The sequencing was performed with 10% PhiX spike-in.

Processing and analysis of the sequencing data

Raw sequencing data, demultiplexed by ZymoBiomics, were obtained in FASTQ format and subsequently processed using QIIME2 (v2020.06) (Bolyen *et al.*, 2019). Initially, Cutadapt (v2.8) was employed to excise amplicon primers (Martin, 2011). The DADA2 plugin facilitated the denoising, filtering, and trimming of reads (Callahan *et al.*, 2016). Taxonomic classification of amplicon sequence variants (ASVs) was performed using the RDP database, with a classifier trained on the specific region (Klindworth *et al.*, 2013). A *de novo* tree was constructed using the multiple sequence align-

ment program (MAFFT) within QIIME2 and used for subsequent diversity analyses incorporating phylogenetic distances (Price *et al.*, 2010). Alpha diversity metrics, including Shannon's Diversity, Faith's Phylogenetic Diversity (PD), and Pielou's Evenness, were calculated within QIIME2 using rarefied counts (subsampled to the smallest read count across samples). For statistical comparison of alpha diversity metrics across groups, p-values were computed using the Qiime2 alpha-group-significance method. Box plots for alpha diversity were created using Microsoft Office Excel 2019 for visualization. Beta diversity was assessed using several metrics: unweighted UniFrac distances, which measure phylogenetic distances between taxa; and weighted UniFrac distances, which consider both phylogenetic distances and relative abundance (Lozupone and Knight, 2005). Statistical significance of beta diversity differences among groups was assessed using the build-in QIIME2 beta-group-significance method, which applies PERMANOVA test (Anderson, 2001). Principal coordinates analysis (PCoA) plots were generated using ClustVis to visualize beta diversity (Metsalu and Vilo, 2015). The heatmap was created using SRplot (Tang *et al.*, 2023).

Isolation and identification of cultivable bacteria

Randomly selected colonies from viable counts experiment, with a preference to morphological differences, were subjected to further analysis. DNA was extracted by alkaline lysis by placing the cells from a plate into 25 µl aqueous 0.02 M NaOH (Lachema, Czech Republic) solution and heated at 95 °C for 10 min. 2 µl of lysed cells were added to the PCR mixture. For identification of bacteria, primers W001 (5′-AGAGTTTGATCMTGGCTC-3′) and W002 (5′-GNTACCTTGTTACGACTT-3′) were used to amplify V3–V4 region of 16S rRNA genes.

The reaction mixture was composed of 5 µl DreamTaq buffer, 1 µl of 2 mM dNTP mix, 1 µl of each primer (10 µmol/L), 2.5 units of DreamTaq DNA polymerase, 4 µl of lysed cells, and sterile distilled water to the final volume of 50 µl. The following PCR conditions were employed for bacterial DNA amplification: 95 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 45 °C for 30 s, and 72 °C for 2 min. The final extension was carried out at 72 °C for 10 min for both PCR reaction types. The size of all PCR products was analysed by 1% agarose gel electrophoresis compared to GeneRuler DNA Ladder Mix. All chemicals for PCR reactions were manufactured by Thermo Fisher Scientific Baltics, Vilnius, Lithuania, while the primers were obtained from Metabion, Germany.

Obtained DNA fragments were subjected to restriction fragment length polymorphism (RFLP) analysis. *BsuRI* (*HaeIII*) (10 U/µl) and *HhaI* (10 U/µl) restriction enzymes with corresponding reaction buffers Buffer R (10×) and Tango Buffer (10×) were purchased from Thermo Fisher Scientific Baltics, Vilnius, Lithuania. PCR products were cut using restriction endonucleases separately. The reaction mixture per sample consisted of 0.5 µl of restriction enzyme, 1 µl reaction buffer and 3.5 µl distilled water. 5 µl of the PCR product was added to each reaction mixture and incubated at 37 °C for 1 hour. Reaction products were analysed by agarose gel electrophoresis.

Based on the diversity of colony morphology and RFLP analysis data, diverse PCR products were purified using the GeneJet PCR purification kit (Thermo Fisher Scientific Baltics, Vilnius, Lithuania) and sequenced using W001 and/or W002 primers at BaseClear (Leiden, the Netherlands). Sequencing data was analysed using Chromas (version 2.6.6) software. The generated sequences were compared with those found in the FASTA network service of the EMBL-EBI database (accessed on 6 April 2023). Sequences with identity scores higher than 97% were assigned to the described genus and/or species.

Detection of antibiotic resistance genes

The total extracted DNA from 2i, 4i, F, and R was tested for the presence of ARGs. The presence of 13 ARGs was examined, namely, genes granting resistance to aminoglycosides (*aac-aph*), β-lactams (*blaZ*, *mecA*), erythromycin (*ermA*, *ermB*, *ermC*), tetracycline (*tetK*, *tetM*, *tetO*, *tetS*, *tetW*) and vancomycin (*vanA*, *vanB*). ARGs were detected by PCR assay, using primers and PCR conditions as described in (Milanović *et al.*, 2021b). The following modifications for the reaction mixture were made: 2.5 µl DreamTaq buffer, 0.5 µl of 2 mM dNTP mix, 0.5 µl of each ARG-specific primer (10 µmol/L), 1.25 units of DreamTaq DNA polymerase, 2 µl of extracted total gDNA, and sterile distilled water to the final volume of 25 µl. The size of PCR products was analysed by 1% (w/v) agarose gel electrophoresis, comparing the size with molecular weight marker GeneRuler DNA Ladder Mix. Nuclease-free water was used as a negative control in PCR reactions. All PCR reactives used were from Thermo Fisher Scientific Baltics, Vilnius, Lithuania, while the primers were synthesised at Metabion, Germany.

TABLE 2 Bacterial 16S rDNA metagenomic analysis data summary[1]

Sample	Total obtained reads	High-quality reads	ASVs	Shannon diversity	Faith pd	Pielou evenness
2i1	29385	19923	33	2.6	1.8	0.5
2i2	31448	21643	32	2.7	1.6	0.5
2i3	42352	28962	28	2.8	1.5	0.6
4i1	40855	27703	37	3.6	5.8	0.7
4i2	43486	27076	31	3.4	1.3	0.7
4i3	43657	28611	33	3.6	1.2	0.7
F1	40911	25554	33	3.8	1.1	0.7
F2	44590	28873	36	3.6	1.2	0.7
F3	37998	23262	28	3.5	1.1	0.7
R1	45858	29269	38	2.9	1.8	0.6
R2	47697	29278	47	3.3	2.5	0.6
R3	43211	26133	48	3.6	2.5	0.6

1 2i = 2nd instar, 4i = 4th instar larvae, F = 24 h-frozen 4th instar larvae, R = frass residues.

3 Results and discussion

Diversity and richness of black soldier fly and frass residues microbiota

The bacterial community of BSFL was investigated by high-throughput sequencing of the 16S rRNA genes. Four stages of BSFL industrial processing were analysed with three replicates each (Table 2).

In total, 12 individual samples were analysed. Total reads, high-quality reads and ASVs per sample were similar between the groups. The R group, which was the only group consisting of residues but not the larvae itself, had the highest numbers of high-quality reads and ASVs in relation to the larval samples. The average number of ASVs was 35 ± 6.47 per sample, whereas in the R group it was 44 ± 5.51 ASVs, indicating greater microbial richness in R.

Total microbiota analysis showed the differences among the different BSFL industrial-rearing stages. Alpha diversity analysis revealed that 2i had the lowest, while 4i and F had the highest diversity scores, and the differences were statistically significant (Supplementary Figure S1). It is consistent with an observation in a recent study, that bacterial richness and diversity increase during *H. illucens* development from larval to pupal stage (Querejeta *et al.*, 2023). The microbiota of R was statistically significantly more diverse in terms of phylogenetic analysis (Faith PD) and had the greatest bacterial richness.

Beta diversity analysis was performed by pseudo-F statistics of weighted and unweighted sample groups using permutational multivariate analysis of variance (PERMANOVA). In contrast to alpha diversity, BSFL samples did not show statistically significant differences in beta diversity ($P > 0.05$) (Table 3).

TABLE 3 Beta diversity analysis of BSFL samples based on weighted and unweighted UniFrac distance metrics[1]

	Unweighted pseudo-F	UniFrac *P*-value	Weighted pseudo-F	UniFrac *P*-value
2i vs 4i	1.64	0.091	18.28	0.092
2i vs F	4.56	0.102	41.12	0.097
2i vs R	2.59	0.098	391.56	0.102
4i vs F	1.07	0.205	0.55	0.59
4i vs R	1.69	0.202	122.79	0.095
F vs R	6.82	0.108	156.98	0.103

1 2i = 2nd instar, 4i = 4th instar larvae, F = 24 h-frozen 4th instar larvae, R = frass residues.

Consistent with sample preparation, the most similar communities reside in 4i and F, which are represented by the lower pseudo-F values and the higher p-values of both unweighted and weighted metrics (Table 3). This confirms the minimal impact of prolonged freezing on the bacterial community diversity of the fourth instar larvae. R group is the most diverse since comparisons with each other group, given the highest values of weighted pseudo-F metric (Table 3), however, the difference is still not statistically significant. This suggests that any apparent differences could arise due to random variation rather than true underlying differences in metagenomics data.

Bacterial communities between 2i, 4i, F and R were different, and the differences were evaluated based on PCoA. Both unweighted and weighted UniFrac distances

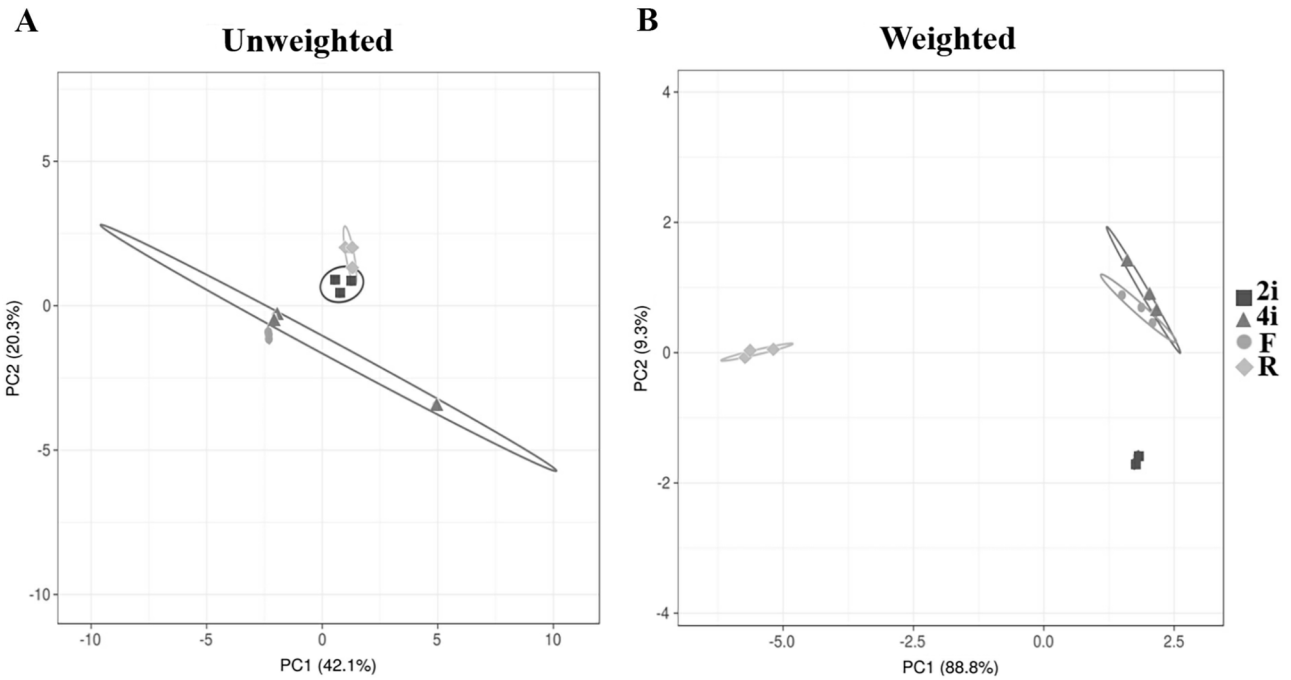

FIGURE 1 Comparison of bacterial communities between different stages of black soldier fly industrial growth and processing by principal coordinate analysis (PCoA). Plots are displaying unweighted (A) and weighted (B) Euclidian distance among samples. 2i = second instar, 4i = fourth instar larvae, F = 24 h-frozen fourth instar larvae, R = frass residues.

showed a close relation between the F and 4i groups (Figure 1). The distance between communities of 2i and R samples, and 4i and F samples is far more evident in the weighted test.

Bacterial communities in different black soldier fly processing stages

The taxonomic composition of both growth stages of BSFL was similar, where Proteobacteria dominated, while R was mainly inhabited by Firmicutes (Figure 2). At the phylum level, domination of Proteobacteria was also observed in BSFL fed with plant-based Gainesville diet (Raimondi *et al.*, 2020), while in another study with the same diet, Bacteroidetes prevailed (Zheng *et al.*, 2013). The proportion of Firmicutes increased about 3.6-3.8 times when progressing from the second to the fourth stage of BSFL and to the frass (Supplementary Table S1). Actinobacteria were mainly found in the fourth instar BSFL.

Almost all Proteobacteria were members of Enterobacteriaceae family (Figure 2, Supplementary Table S1). The representation of Enterococcaceae family (Firmicutes) increased from stage 2i to stage R, starting from 2.73% in 2i, followed by 10.09% and 13.85% in 4i and F, with the peak of 20.76% in R (Supplementary Table S1). Other families were less abundant. R stand out with the high (52.52%) ratio of Staphylococcaceae (of which 52.31% belong to *Staphylococcus* sp.) and a significant 18.76% of Lactobacillaceae (with 4.01% of *Lactobacillus* sp. bacteria).

At the genus level, members of Enterobacteriaceae, namely *Proteus* sp. and *Providencia* sp. were present at the highest ratios (about 19-54%) in 2i, 4i, and F, whereas more than 20% of total bacteria in 4i and F were *Morganella* sp. (Figure 2, Supplementary Table S1). *Klebsiella* sp. from the same family was less abundant (below 2%) but found in all samples, like *Enterococcus* sp. (Supplementary Table S1). *Ignatzschineria* sp. was also present in all samples with a minimum of 0.01% in 2i and a maximum of 0.98% in F. Some genera of bacteria were detected only in 2i and R, like *Bacillus* sp. (0.64% and 0.26%, respectively) and *Lactobacillus* sp. (0.24% and 4.01%, respectively), while *Corynebacterium* sp. was only found in R (0.51%) and *Pseudomonas* sp. only in 2i (0.73%), both with trace amounts of 0.01% in 2i and R samples, respectively. The relatively low abundance of *Bacillus* may be related to the limited protein content in plant-based substrate, since *Bacillus* spp. were associated with protein digestion (Yu *et al.*, 2022).

Differences between 4i and F revealed that prolonged freezing favours the detection of bacteria. Although the average ratios of the detected bacteria were similar, prolonged freezing increased the detection of Actinobacteria phylum (from 10.52% to 13.20%); Enterococcaceae family (from 10.09% to 13.85%) of which *Enterococcus* sp. (from 0.25% to 0.72%); *Klebsiella* sp.

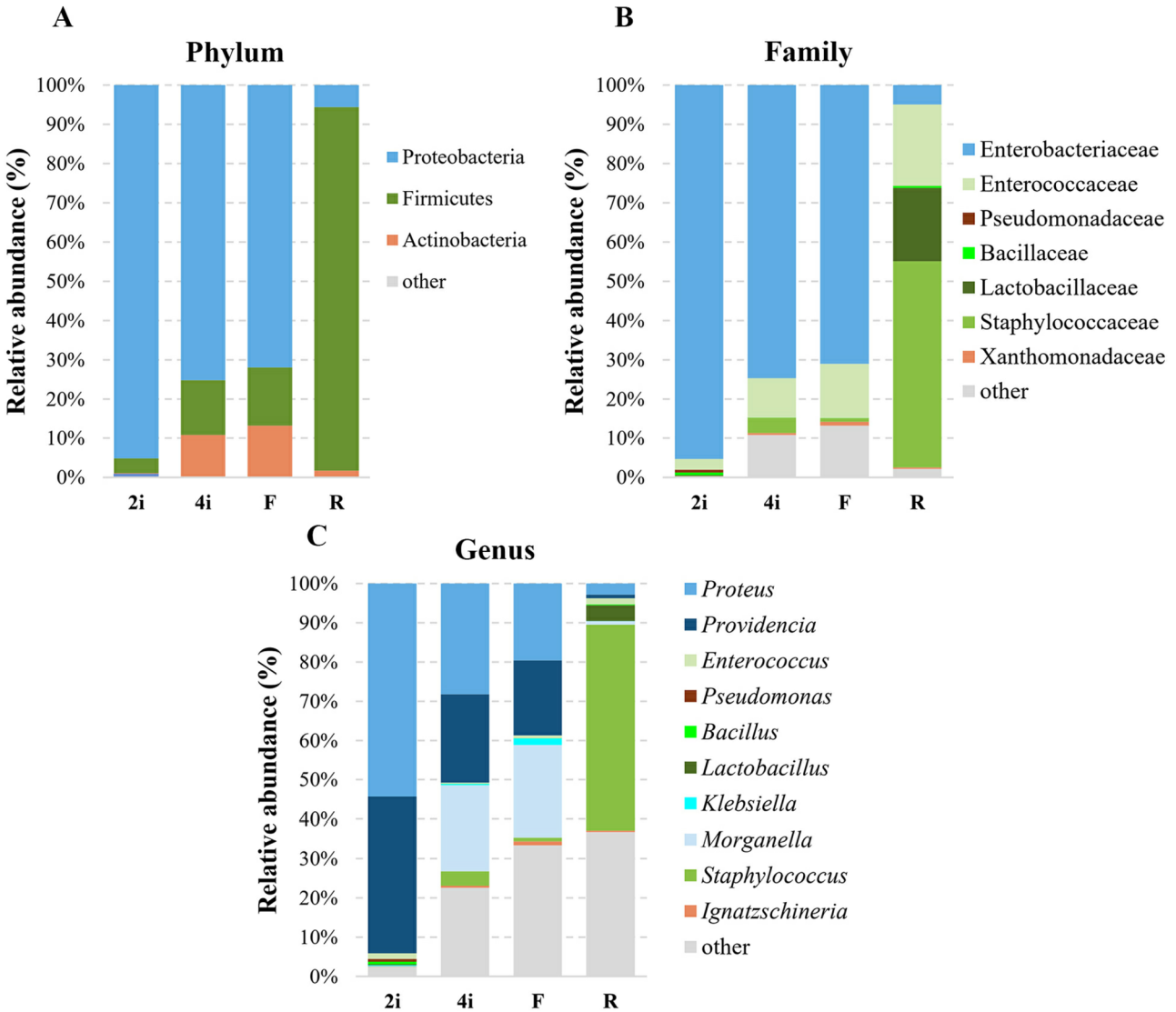

FIGURE 2 Taxonomic composition of bacterial communities at black soldier fly rearing and processing stages. Relative abundance of ASVs at phylum, family, and genus levels are represented. 2i = 2nd instar, 4i = 4th instar larvae, F = 24 h-frozen 4th instar larvae, R = frass residues.

(from 0.32% to 1.79%); *Morganella* sp. (from 21.88% to 23.47%) of Enterobacteriaceae family; and Xanthomonadaceae family member *Ignatzschineria* sp. (from 0.52% to 0.98%) (Supplementary Table S1). On average, there were three families, and five genera less represented in the longer frozen samples, namely Flavobacteriaceae of which *Imtechella* sp. (0.26% vs 0.00%); Staphylococcaceae (3.87 vs 0.94) of which *Jeotgalicoccus* sp. (0.26% vs 0.01%), *Staphylococcus* sp. (3.61% vs 0.94%); Enterobacteriaceae (74.71% vs 71.03%) of which *Proteus* sp. (28.20% vs 19.62%), *Providencia* sp. (3.61% vs 0.94%). However, these differences were not observed uniformly regarding individual samples. Most of the discrepancy between the abundance ratios was minor (below 4%), except for *Proteus* sp. which was by 8.58% less represented in F than 4i. Relative abundance of *Proteus* genera bacteria was also shown to decrease in prepupae, when BSFL were reared at lower temperatures (Raimondi *et al.*, 2020).

Distribution of the most common ASVs in bacterial communities of each BSFL processing stage is represented by the heatmap (Figure 3).

Staphylococcus (ASV5, ASV10, ASV11, ASV23, ASV20, and ASV32), Lactobacillaceae (ASV9), *Lactobacillus* (ASV17) and Corynebacterium (ASV41) are commonly found in R (Figure 3). Enterococcaceae (ASV5 and ASV12) and *Enterococcus* (ASV28) were present not only in R but also in 4i and F. *Morganella* (ASV2, ASV22, ASV16, and ASV31) and *Ignatzschineria* (ASV25) were hallmarks of the fourth instar larvae. Most *Proteus* and *Providencia* ASVs were found in 2i exclusively, with several exceptions (*Providencia* ASV3, ASV24, ASV14; *Pro-*

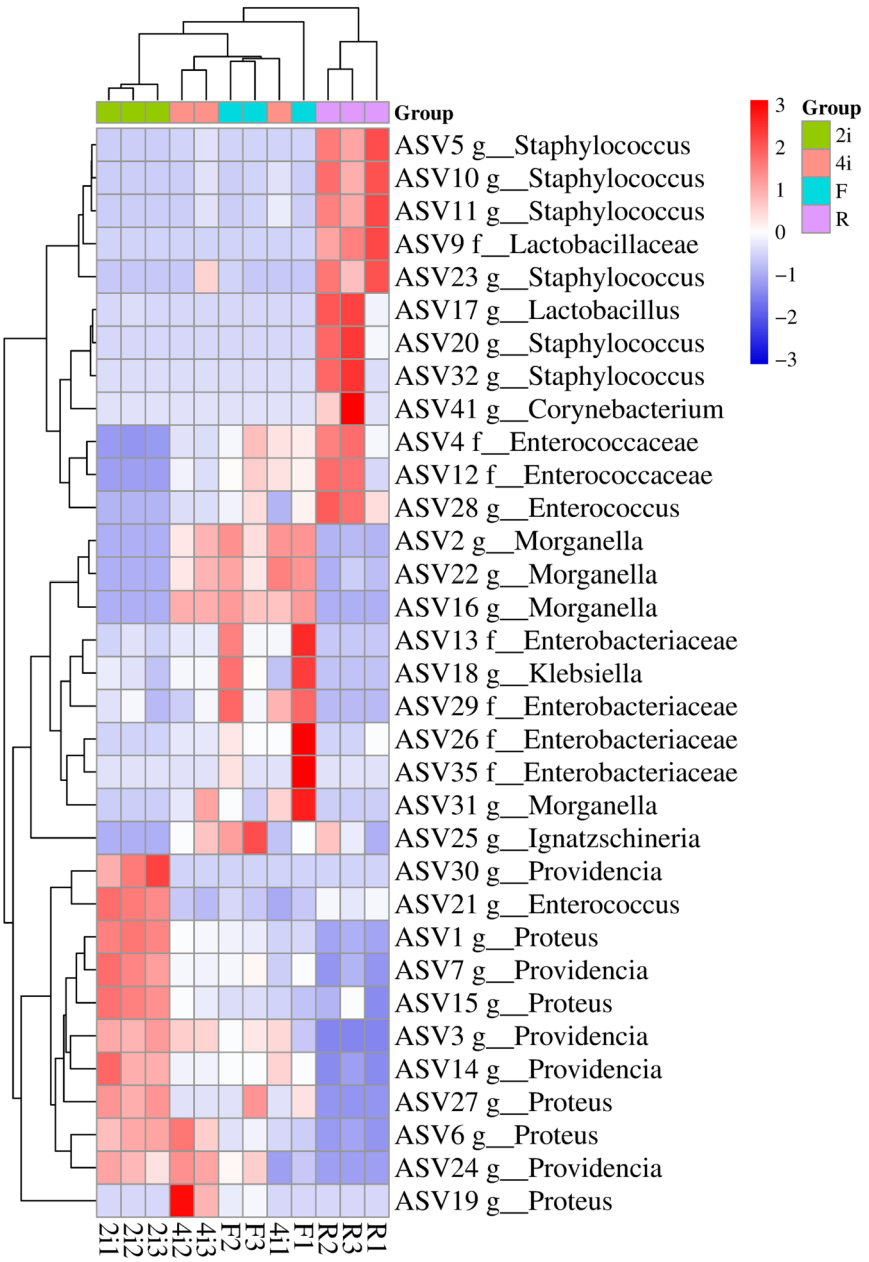

FIGURE 3 Heatmap of the most abundant unique bacterial ASVs (>1% relative abundance) in black soldier fly industrial growth and processing stages. 2i = 2nd instar, 4i = 4th instar larvae, F = 24 h-frozen 4th instar larvae, R = frass residues.

teus ASV27, ASV6) that were also detected in the fourth instar larvae. Analysis of ASVs showed prominent clustering of 2i and R, whereas 4i and F remained very similar.

The composition of BSFL microbiota resembled more to the one that was observed upon feeding with nutrient-restricted diets, regarding the large proportion of Gammaproteobacteria and a lot smaller of Bacilli class bacteria (Supplementary Table S1), whereas in a full nutrient diet, more than 90% of bacteria were Bacilli (Marasco *et al.*, 2022). The bacterial community of frass is considered to be largely dependent on the substrate (Schreven *et al.*, 2022), however, the relative abundance of Firmicutes, Proteobacteria, and Actinobacteria were very similar to the other study where the plant waste and kitchen waste was used for BSFL rearing (Deng *et al.*, 2022). We were unable to analyse the total microbiota of D due to unsuccessful attempts to extract sufficient high-quality gDNA suitable for subsequent PCR reactions, likely because of the high lipids and chitin content. Generally low bacterial diversity was observed in our study.

Viable counts and cultivable bacteria diversity

The assessment of microorganisms is essential to ensure the safety of food consumption. To evaluate the number

TABLE 4 Viable counts during the processing of *H. illucens* larvae. Numbers (in log CFU/g) are expressed as means ± standard error

Sample[1]	Total mesophilic aerobes		Aerobic bacterial spores (PCA)
	CASO	PCA	
2i	5.1 ± 0.1^{c}	5.1 ± 0.0^{c}	2.7 ± 0.3^{a}
4i	6.1 ± 0.1^{de}	6.2 ± 0.2^{de}	2.9 ± 0.2^{a}
F	6.7 ± 0.1^{e}	6.6 ± 0.1^{e}	2.8 ± 0.0^{a}
D	4.3 ± 0.1^{b}	4.3 ± 0.2^{b}	4.1 ± 0.0^{b}
R	6.4 ± 0.1^{e}	5.5 ± 0.1^{cd}	2.8 ± 0.2^{a}

1 Samples of 2nd instar (2i) and 4th instar (4i) larvae; 24 h-frozen (F) and heat-dried (D) 4th instar larvae; and frass residues (R) are represented. Means marked with different letters are significantly different ($P < 0.05$).

of living microorganisms in different stages of industrial growth and processing of BSFL and frass, the viable counts assay was performed by plating the samples on CASO, PCA, and YPD plates. CFUs were observed on all plates. YPD growth medium, supplemented with chloramphenicol, was chosen for yeast isolation. However, microscopical inspection of CFUs on YPD plates and PCR assays revealed only the presence of bacteria but not yeasts (data not shown). Since no yeast colonies were observed, the YPD medium was excluded from further investigation. The numbers of viable counts are presented in Table 4.

CASO medium appeared to be the most favourable growth medium for the cultivation of bacteria in BSFL samples, although the viable counts were similar (except for R) on PCA medium as well. The highest counts of total mesophilic aerobes were detected in F, 4i, and R on CASO medium, and 4i and F on PCA, ranging from 6.1 to 6.7 log CFU/g (Table 4). There was no statistically significant difference in viable counts between 4i and F on any media, consistent with the minimal processing difference. The total bacteria count in 2i was smaller than in 4i and F, indicating an increase in bacterial counts during the growth of the larvae. Only in R, statistically significantly higher counts were detected in CASO than in the PCA medium (6.4 and 5.5 log CFU/g, respectively).

The highest viable counts were found in the F (6.7 ± 0.1 log CFU/g), while the lowest were in D (4.3 ± 0.1 log CFU/g). Freezing followed by roasting at 105 °C for 2 hours resulted in up to 2.4 logs reduction of viable counts (Table 4). D produced the lowest (from 3.9 to 4.3 log CFU/g) and statistically equal counts on all the tested media, showing that most CFUs come from spores. However, D has about 10 times statistically significantly higher ($P < 0.05$) spore counts, than other samples. Counts of endospores are considered to remain similar throughout the life cycle of *H. illucens* (Van Looveren *et al.*, 2024). It may be related to the higher number of individual larvae in the D sample, since freshly harvested BSFL can contain about 75% of humidity, the majority of which is lost with drying (Shelomi, 2020). Drying at 105 °C has been shown as an efficient strategy to preserve harvested edible insects, however spore-forming bacteria remain a potential risk (Klunder *et al.*, 2012). Thus, thermal treatment can be an effective strategy to reduce microbial contamination of the BSFL, however potential dangers must be evaluated to ensure a safe post-harvesting.

Viable counts were 1-4 logs less, or in some cases similar alike in other works, where waste was used as a substrate (Osimani *et al.*, 2021; Raimondi *et al.*, 2020; Wynants *et al.*, 2019). Differences may be related to the distinct sample processing. BSFL frass containing even higher numbers of CFUs (up to 10^9 CFU/g) had not impaired soil hygiene, since frass bacteria were outcompeted by soil microorganisms (Klammsteiner *et al.*, 2020). Regulation of insects as food or feed are distinct worldwide (Lee *et al.*, 2022). *H. illucens* is currently not accepted as an edible insect for humans in EU. BSFL are allowed as a feed only in aquaculture, while substrates for BSFL are limited agro-industrial vegetable waste is permitted, according to the regulations in EU (Lee *et al.*, 2022). However, there are currently no strict regulations for the microbiological safety of any edible insects, since it is a complex task (Grabowski *et al.*, 2022). Only Netherlands have specified contamination limits for total aerobic counts below 5.7 log CFU/g and some bacteria but not for aerobic spore-forming bacteria (Kox, 2019). EU regulations for ground meat permits counts below 6.7 log CFU/g. According to the local Lithuanian hygiene standard HN 26:2006, the maximum permitted level of total microorganisms processed meat products is up to 4 log CFU/g and in smoked fish products up to 5 log CFU/g. The limit of 4 log CFU/g is exceeded in most of the samples, except for spore forming bacteria, where only D were slightly above the threshold with 4.1 log CFU/g (Table 4). Freezing and drying of BSFL almost achieves the microbiological criteria for certain meat and fish foodstuffs in Lithuania. This additional treatment at high temperature can reduce the microbial counts to the strictest permissible limit.

In total, 172 cultivable bacteria isolates were obtained in this study. RFLP analysis of the isolated strains showed 28 different profiles (Supplementary Table S2),

TABLE 5 Cultivable bacteria isolated from *H. illucens* larvae and frass

Phylum	Genus/species	Sample[1]
Actinobacteria	*Rothia terrae*	4i
Firmicutes	*Bacillus amyloliquefaciens*	D
	Bacillus cereus	2i, D
	Bacillus licheniformis	2i, 4i, D, R
	Bacillus licheniformis/aerius	R
	Bacillus sp.[2]	2i, 4i, D, R
	Bacillus sp.[3]	F, R
	Bacillus thuringiensis/cereus	2i
	Enterococcus avium	4i
	Enterococcus faecalis	4i, F
	Oceanobacillus caeni	4i, D
	Sporosarcina newyorkensis	4i
	Staphylococcus pasteuri	4i
	Staphylococcus simulans	F, R
Proteobacteria	*Klebsiella pneumoniae/variicola*	4i
	Morganella morganii	4i, F
	Proteus mirabilis/vulgaris	4i
	Proteus sp.[4]	4i
	Providencia rettgeri	4i
	Providencia stuartii	4i

1 Abundance of cultivable bacteria in the samples of 2nd instar (2i) and 4th instar (4i) larvae; 24 h-frozen (F) and heat-dried (D) 4th instar larvae; and frass residues (R) is represented.
2 *B. amyloliquefaciens*/*B. subtilis* subsp. *stercoris*/*B. velezensis*/*B. tequilensis*/*B. subtilis*.
3 *B. amyloliquefaciens*/*B. velezensis*/*B. siamensis*.
4 *P. mirabilis*/*P. cibarius*/*P. vulgaris*/*P. terrae* subsp. *cibarius*.

whereas partial 16S rDNA sequencing led to the identification of 7 bacteria at the genus and 13 strains at the species level (Table 5).

In agreement with the total microbiota analysis, isolated cultivable bacterial strains were members of *Proteus*, *Providencia*, *Morganella*, *Staphylococcus*, *Klebsiella*, *Enterococcus*, and *Bacillus* genera. 4i had the greatest number of different bacterial species, where all 10 genera were represented. Four genera, namely *Bacillus*, *Enterococcus*, *Staphylococcus*, and *Morganella* were also found in F. *Bacillus* sp. was isolated from 2i. R hosted *Staphylococcus* and *Bacillus* bacteria, while D was limited to cultivable spore-forming *Bacillus* and *Oceanobacillus* strains. Thus, the highest diversity of cultivable bacteria was isolated from the 4i. *Enterococcus*, *Klebsiella*, *Morganella*, *Providencia*, and *Scrofimicrobium* genera are members of the BSFL gut core microbiota, as described by a recent meta-analysis, while *Proteus* was also very abundant (IJdema *et al.*, 2022). Meta-analysis was mostly focused on the microbiota of the insect gut since only two studies (Gorrens *et al.*, 2022; Wynants *et al.*, 2019) were included in the analysis. However, the whole BSFL microbiota showed significantly higher bacterial diversity than only in the gut (IJdema *et al.*, 2022). The most represented genera in BSFL samples seem to be members of the core microbiota.

Prolonged freezing at –20 °C did not result in any statistically significant differences between 4i and F samples. Freezing and/or storage of insects or other samples is a common practice in research, mainly to preserve the original state of the research object prior to the further analysis. Freezing at –20 °C is a common practice for insect killing, however, blanching or other methods are more effective in the reduction of microbiological contamination (Larouche *et al.*, 2019). BSFL killing by freezing at –20°C reduced the viable counts of *Pseudomonas* spp., when compared to other methods (Larouche *et al.*, 2019). We have only detected *Pseudomonas* spp. in 2i samples with less than 1% of relative abundance (Supplementary Table S1). Microorganisms show different tolerance for freezing, thereby, some are preserved while others are slowly diminishing (Archer, 2004). Effects of prolonged freezing are slow, thus the difference between

2 and 24 hours was probably too small to detect significant differences in this work.

An exclusive prevalence of certain bacteria genera in the BSFL rearing stage was observed. A significant ratio *Morganella morganii* (approximately 20%) was exclusively present in the fourth instar BSFL (Figure 2, Supplementary Table S1), consistent with the data of previous studies that it is associated with later stages of larval development (Querejeta *et al.*, 2023; Shelomi, 2020). *M. morganii* was linked to the mortality of the fruit fly (Salas *et al.*, 2017), phenol production (Marshall *et al.*, 2016), urea hydrolysis (Gold *et al.*, 2020), and inhibition of pathogenic bacteria *Pseudomonas aeruginosa* (Tegtmeier *et al.*, 2021). *Staphylococcus* sp. was the most abundant genera unique to frass. It could originate from the food waste substrate, but in a previous study, BSFL rearing resulted in the complete elimination of these bacteria from the substrate (Gold *et al.*, 2020). *Staphylococcus* increased the lipolytic activities of BSFL (Meng *et al.*, 2023).

Identification of bacteria in D samples revealed that the major risk (or benefit) in dried larvae are spore-forming bacteria of *Bacillus* sp. *Bacillus* spp. were commonly detected bacteria not only in D but in all samples.

Bacteria species isolated in this work may be valuable BSFL microbial community members. *Bacillus* sp., *Providencia* sp., *Klebsiella* sp., *Proteus* sp., certain strains of *Paenibacillus* sp., *Enterococcus faecalis* were tested as probiotics for BSF rearing, and showed beneficial effects, with *Proteus* sp. being the most elusive by showing inconsistent results, as described in (Gorrens *et al.*, 2023). *Klebsiella pneumoniae* strains, isolated from BSFL gut, were non-toxic and shown to contribute to BSFL tolerance to sulfonamides and cadmium (Shi *et al.*, 2022). A recent review provides a summary of the functional roles of the insect-associated bacterial strains, where the members of *Bacillus* sp. stand out, for the digestion of carbohydrates, protein, and lipids (Carpentier *et al.*, 2024). BSFL-associated *Providencia* sp., *Morganella* sp., *Klebsiella* sp., and *Bacillus* sp. strains showed inhibition of some pathogenic bacteria (Tegtmeier *et al.*, 2021). *Rothia terrae* – the only isolated actinobacteria – was reported to have some enzymatic activities that can provide benefits in the food industry (Angelidis *et al.*, 2015; Chou *et al.*, 2008). Different results have been observed of the impact on the microbiota of the animals consuming BSFL (Salahuddin *et al.*, 2024), and while clear reasons for these differences are unknown, BSFL microbial composition may also impact the nutritional outcomes.

Although *B. licheniformis* and *B. cereus*, *Enterococcus* sp., *M. morganii*, and *K. pneumoniae* are associated with foodborne diseases, the most common causes of infection are poor food storage conditions or inadequate hygiene (Davis and Price, 2016; Fiore *et al.*, 2019; Gu *et al.*, 2019; Liu *et al.*, 2016; Tewari and Abdullah, 2015) Almost all cultivable bacteria identified from BSFL could be opportunistic pathogens, but their adverse effects are most commonly determined in immunocompromised people or in hospital-acquired infections, where uncontrolled growth of certain bacteria is observed. Investigation of the properties of individual isolated strains is required to better understand their role in BSFL rearing and product safety.

TABLE 6 Antibiotic resistance genes in total gDNA of BSFL samples. The number indicates the presence of corresponding ARG out of the three tested replicates of each sample, confirmed by PCR assay

Gene	Sample[1]			
	2i	4i	F	R
aac-aph	–	–	–	1
blaZ	–	–	1	–
mecA	–	–	–	–
ermA	–	–	–	3
ermB	–	–	–	–
ermC	–	–	–	–
tetK	–	–	–	–
tetM	1	2	1	3
tetO	–	–	–	–
tetS	–	–	–	–
tetW	–	–	–	2
vanA	–	–	–	1
vanB	–	–	1	3

1 2i = 2nd instar, 4i = 4th instar larvae, F = 24 h-frozen 4th instar larvae, R = frass residues.

Antibiotic resistance genes

Total DNA extracted from 2i, 4i, F, and R were screened for the presence of common genes conferring resistance to aminoglycosides (*aac-aph*), β-lactams (*blaZ*, *mecA*), erythromycin (*erm-* genes), tetracycline (*tet-* genes) and vancomycin (*van-* genes). The presence of ARGs was confirmed by PCR assay (Table 6).

Analysis of ARGs confirmed that genes conferring resistance to tetracyclines (namely *tetM*) were found in all BSFL industrial processing stages tested (Table 6). There was almost no difference between 2i and 4i, where only the *tetM* gene was detected in part of the tested replicates. More ARGs were detected in F than 4i samples but it is rather a coincidence, since the genes

were detected in only one replicate out of three. Most of the ARGs were not detected in all replicates, suggesting random distribution and/or relatively small amounts (manufacturer states that DreamTaq DNA polymerase sensitivity for human gDNA is 30 pg) of these genes. R contained the highest variety of ARGs (*aac-aph*, *ermA*, *tetM*, *tetW*, *vanA* and *vanB*), that correlates with the highest diversity of bacteria. Furthermore, *ermA*, *tetM*, and *vanB* were found in all frass samples, making R the most ARG-rich fraction of BSFL products (Table 6).

Even though the use of antibiotics for non-medical applications is prohibited by EU regulations (1831/2003/EC), the spread of ARGs in the food chain poses a serious risk. Our results are similar to those of another study, in which tetracycline-resistance genes were the most commonly detected in BSFL samples, and the highest diversity of ARGs was also found in frass (Milanović *et al.*, 2021b), whereas other works also reported the presence of *tetM* genes in BSFL samples (Cai *et al.*, 2018; Cifuentes *et al.*, 2020; Liu *et al.*, 2020). To date, tetracyclines are the most widely used antibiotics (Granados-Chinchilla and Rodríguez, 2017). Resistance to antibiotics of the tetracycline family is commonly detected in samples from farms, soil, and water, with *tetM* being the most widely transferred between different bacteria due to its association with mobile genetic elements (Roberts and Schwarz, 2016).

The ARGs found in this work are related to the BSFL-associated bacteria. Tetracycline resistance genes are found in diverse bacterial genera, while other ARGs detected in this work have been detected only in specific bacterial genera. *M. morganii* contributes to the spread of ARGs, even though none of the tested ARGs were reported in this species (Liu *et al.*, 2016). The *ermA* gene is commonly detected in staphylococci (Lina *et al.*, 1999), whereas *aac-aph* is more widespread among different genera and are found in enterococci, staphylococci, and streptococci (Lalitha Aishwarya *et al.*, 2020). Vancomycin resistance genes are associated with the health threat of clinical isolates of enterococci (Moosavian *et al.*, 2018) and staphylococci (Bakeer *et al.*, 2024). These genera were also detected in this work, however, methicillin resistance gene (*mecA*), which is a hallmark of methicillin-resistant staphylococci, was not found, suggesting that samples studied here might not be a serious risk of multi-drug-resistant bacteria. *Providencia*, *Enterococcus*, *Morganella*, and *Klebsiella* were reported not only to be tetracycline-resistant but also to be involved in the degradation of antibiotics (Pei *et al.*, 2023; Ruan *et al.*, 2024). The relationship between the composition of BSFL bacterial microbiota and the prevalence of ARGs is not straightforward (Cai *et al.*, 2018; Liu *et al.*, 2020). The transfer of ARGs from ARGs-containing BSFL to BSFL-fed fish has already been demonstrated (Milanović *et al.*, 2021a). Thus, it is important to control the spread of ARGs with BSFL products.

4 Conclusions

This work is a multifaceted investigation of microbial safety in the industrial BSFL rearing on plant-based food waste. We investigated for the first time the composition of the whole BSFL microbiota, viable counts, cultivable bacteria isolation and identification, and abundance of ARGs by investigating two BSFL life stages, two BSFL thermal treatments, and frass in one study. Industrial BSFL rearing on plant-based food waste resulted in low bacterial diversity. Frass residues stood out with the most diverse bacterial community and as the most ARGs-rich product in the BSFL rearing process. Resistance to tetracycline was the most detected throughout the stages of BSFL rearing. Thermal treatment of BSFL (freezing at –20 °C and subsequent drying at 105 °C for 2 hours) was sufficient to reach about 4 log CFU/g viable counts to meet the highest available EU standards for microbiological contamination of insects as food. Isolated cultivable bacteria can provide beneficial and pathogenic traits both to BSFL and consumers. In future research, based on the results of this study, we can better understand the roles of BSFL-associated bacteria by investigating the properties of the isolated strains and comparing the effects of post-harvesting treatments on the microbial safety of BSFL. This work makes a valuable contribution to the development of guidelines for BSFL as a more widely used food and feed source by providing comprehensive data on microbiological safety, including ARGs, in the industrial BSFL-rearing process.

Supplementary material

Supplementary material is available online at:
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Conflict of interest

Author Stanislavas Tracevičius is employed by the company Insectum. The remaining authors have no conflict of interest to declare.

Data availability

All sequences obtained in this work are available in the Sequence Read Archive (SRA) of the National Center for Biotechnology Information (NCBI), under accession number PRJNA1163095.

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References

Aleknavičius, D., Lukša, J., Strazdaitė-Žielienė, Ž. and Servienė, E., 2022. The bacterial microbiota of edible insects *Acheta domesticus* and *Gryllus assimilis* revealed by high content analysis. Foods 11: 1073.

Anderson, M.J., 2001. A new method for non-parametric multivariate analysis of variance. Austral Ecology 26: 32-46.

Angelidis, A.S., Kalamaki, M.S. and Georgiadou, S.S., 2015. Identification of non-*Listeria* spp. bacterial isolates yielding a β-d-glucosidase-positive phenotype on Agar *Listeria* according to Ottaviani and Agosti (ALOA). International Journal of Food Microbiology 193: 114-129.

Archer, D.L., 2004. Freezing: an underutilized food safety technology? International Journal of Food Microbiology 90: 127-138.

Bakeer, W., Gaafar, M., El-Gendy, A.O., El Badry, M.A., Khalil, M.G., Mansour, A.T., Alharbi, N.K., Selim, H.M.R.M. and Bendary, M.M., 2024. Proven anti-virulence therapies in combating methicillin- and vancomycin-resistant *Staphylococcus aureus* infections. Frontiers in Cellular and Infection Microbiology 14: 1403219.

Bessa, L.W., Pieterse, E., Marais, J. and Hoffman, L.C., 2020. Why for feed and not for human consumption? The black soldier fly larvae. Comprehensive Reviews in Food Science and Food Safety 19: 2747-2763.

Bolyen, E., Rideout, J.R., Dillon, M.R., Bokulich, N.A., Abnet, C.C., Al-Ghalith, G.A., Alexander, H., Alm, E.J., Arumugam, M., Asnicar, F., Bai, Y., Bisanz, J.E., Bittinger, K., Brejnrod, A., Brislawn, C.J., Brown, C.T., Callahan, B.J., Caraballo-Rodríguez, A.M., Chase, J., Cope, E.K., Da Silva, R., Diener, C., Dorrestein, P.C., Douglas, G.M., Durall, D.M., Duvallet, C., Edwardson, C.F., Ernst, M., Estaki, M., Fouquier, J., Gauglitz, J.M., Gibbons, S.M., Gibson, D.L., Gonzalez, A., Gorlick, K., Guo, J., Hillmann, B., Holmes, S., Holste, H., Huttenhower, C., Huttley, G.A., Janssen, S., Jarmusch, A.K., Jiang, L., Kaehler, B.D., Kang, K.B., Keefe, C.R., Keim, P., Kelley, S.T., Knights, D., Koester, I., Kosciolek, T., Kreps, J., Langille, M.G.I., Lee, J., Ley, R., Liu, Y.-X., Loftfield, E., Lozupone, C., Maher, M., Marotz, C., Martin, B.D., McDonald, D., McIver, L.J., Melnik, A.V., Metcalf, J.L., Morgan, S.C., Morton, J.T., Naimey, A.T., Navas-Molina, J.A., Nothias, L.F., Orchanian, S.B., Pearson, T., Peoples, S.L., Petras, D., Preuss, M.L., Pruesse, E., Rasmussen, L.B., Rivers, A., Robeson, M.S., Rosenthal, P., Segata, N., Shaffer, M., Shiffer, A., Sinha, R., Song, S.J., Spear, J.R., Swafford, A.D., Thompson, L.R., Torres, P.J., Trinh, P., Tripathi, A., Turnbaugh, P.J., Ul-Hasan, S., Van Der Hooft, J.J.J., Vargas, F., Vázquez-Baeza, Y., Vogtmann, E., Von Hippel, M., Walters, W., Wan, Y., Wang, M., Warren, J., Weber, K.C., Williamson, C.H.D., Willis, A.D., Xu, Z.Z., Zaneveld, J.R., Zhang, Y., Zhu, Q., Knight, R. and Caporaso, J.G., 2019. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nature Biotechnology 37: 852-857.

Bruno, D., Bonelli, M., De Filippis, F., Di Lelio, I., Tettamanti, G., Casartelli, M., Ercolini, D. and 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.

Cai, M., Ma, S., Hu, R., Tomberlin, J.K., Thomashow, L.S., Zheng, L., Li, W., Yu, Z. and Zhang, J., 2018. Rapidly mitigating antibiotic resistant risks in chicken manure by *Hermetia illucens* bioconversion with intestinal microflora. Environmental Microbiology 20: 4051-4062.

Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A. and Holmes, S.P., 2016. DADA2: High-resolution sample inference from *Illumina amplicon* data. Nature Methods 13: 581-583.

Carpentier, J., Abenaim, L., Luttenschlager, H., Dessauvages, K., Liu, Y., Samoah, P., Francis, F. and Caparros Megido, R., 2024. Microorganism contribution to mass-reared edible insects: opportunities and challenges. Insects 15: 611.

Chou, Y.-J., Chou, J.-H., Lin, K.-Y., Lin, M.-C., Wei, Y.-H., Arun, A.B., Young, C.-C. and Chen, W.-M., 2008. *Rothia terrae* sp. nov. isolated from soil in Taiwan. International Journal of Systematic and Evolutionary Microbiology 58: 84-88.

Cifuentes, Y., Glaeser, S.P., Mvie, J., Bartz, J.-O., Müller, A., Gutzeit, H.O., Vilcinskas, A. and Kämpfer, P., 2020. The gut and feed residue microbiota changing during the rearing

of *Hermetia illucens* larvae. Antonie Van Leeuwenhoek 113: 1323-1344.

Davis, G.S. and Price, L.B., 2016. Recent research examining links among *Klebsiella pneumoniae* from food, food animals, and human extraintestinal infections. Current Environmental Health Reports 3: 128-135.

De Smet, J., Wynants, E., Cos, P. and Van Campenhout, L., 2018. Microbial community dynamics during rearing of black soldier fly larvae (*Hermetia illucens*) and impact on exploitation potential. Applied and Environmental Microbiology 84: e02722-17.

Deng, B., Zhu, J., Wang, G., Xu, C., Zhang, X., Wang, P. and Yuan, Q., 2022. Effects of three major nutrient contents, compost thickness and treatment time on larval weight, process performance and residue component in black soldier fly larvae (*Hermetia illucens*) composting. Journal of Environmental Management 307: 114610.

Eke, M., Tougeron, K., Hamidovic, A., Tinkeu, L.S.N., Hance, T. and 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: 40.

Fiore, E., Van Tyne, D. and Gilmore, M.S., 2019. Pathogenicity of Enterococci. Microbiology Spectrum 7: 7.4.9.

Gold, M., Von Allmen, F., Zurbrügg, C., Zhang, J. and Mathys, A., 2020. Identification of bacteria in two food waste black soldier fly larvae rearing residues. Frontiers in Microbiology 11: 582867.

Gorrens, E., Lecocq, A. and De Smet, J., 2023. The use of probiotics during rearing of *Hermetia illucens*: potential, caveats, and knowledge gaps. Microorganisms 11: 245.

Gorrens, E., Smet, J.D., Vandeweyer, D., Bossaert, S., Crauwels, S., Lievens, B. and Campenhout, L.V., 2022. The bacterial communities of black soldier fly larvae (*Hermetia illucens*) during consecutive, industrial rearing cycles. Journal of Insects as Food and Feed 8: 1061-1076. https://doi.org/10.3920/JIFF2021.0150

Gorrens, E., Van Moll, L., Frooninckx, L., De Smet, J. and Van Campenhout, L., 2021. Isolation and identification of dominant bacteria from black soldier fly larvae (*Hermetia illucens*) envisaging practical applications. Frontiers in Microbiology 12: 665546.

Grabowski, N.Th., Abdulmawjood, A., Acheuk, F., Barragán Fonseca, K., Chhay, T., Costa Neto, E.M., Ferri, M., Franco Olivas, J., González Aguilar, D.G., Keo, S., Lertpatarakomol, R., Miech, P., Piofczyk, T., Proscia, F., Mitchaothai, J., Guerfali, M.M., Sayed, W., Tchibozo, S. and Plötz, M., 2022. Insects—a source of safe and sustainable fFood?—"Jein" (yes and no). Frontiers in Sustainable Food Systems 5: 701797.

Granados-Chinchilla, F. and Rodríguez, C., 2017. Tetracyclines in food and feeding stuffs: from regulation to analytical methods, bacterial resistance, and environmental and health implications. Journal of Analytical Methods in Chemistry 2017: 1315497.

Grootveld, M., Percival, B.C., Leenders, J. and Wilson, P.B., 2020. Potential adverse public health effects afforded by the ingestion of dietary lipid oxidation product toxins: significance of fried food sources. Nutrients 12: 974.

Gu, H.-J., Sun, Q.-L., Luo, J.-C., Zhang, J. and Sun, L., 2019. A first study of the virulence potential of a *Bacillus subtilis* isolate from deep-sea hydrothermal vent. Frontiers in Cellular and Infection Microbiology 9: 183.

Ijdema, F., De Smet, J., Crauwels, S., Lievens, B. and Van Campenhout, L., 2022. Meta-analysis of larvae of the black soldier fly (*Hermetia illucens*) microbiota based on 16S rRNA gene amplicon sequencing. FEMS Microbiology Ecology 98: fiac094.

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., Ekesi, S. and Tanga, C.M., 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.

Klammsteiner, T., Turan, V., Fernández-Delgado Juárez, M., Oberegger, S. and Insam, H., 2020. Suitability of black soldier fly frass as soil amendment and implication for organic waste hygienization. Agronomy 10: 1578.

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M. and Glöckner, F.O., 2013. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Research 41: e1.

Klunder, H.C., Wolkers-Rooijackers, J., Korpela, J.M. and Nout, M.J.R., 2012. Microbiological aspects of processing and storage of edible insects. Food Control 26: 628-631.

Kox, L., 2019. Advice on animal and public health risks of insects reared on former foodstuffs as raw material for animal feed. https://doi.org/10.13140/RG.2.2.22058.16327

Lalitha Aishwarya, K.V., Venkataramana Geetha, P., Eswaran, S., Mariappan, S. and Sekar, U., 2020. Spectrum of aminoglycoside modifying enzymes in gram-negative bacteria causing human infections. Journal of Laboratory Physicians 12: 27-31.

Larouche, J., Deschamps, M.-H., Saucier, L., Lebeuf, Y., Doyen, A. and Vandenberg, G.W., 2019. Effects of killing methods on lipid oxidation, colour and microbial load of black soldier fly (*Hermetia illucens*) larvae. Animals 9: 182.

Lee, J.-H., Kim, T.-K., Cha, J.Y., Jang, H.W., Yong, H.I. and Choi, Y.-S., 2022. How to develop strategies to use insects as ani-

mal feed: digestibility, functionality, safety, and regulation. Journal of Animal Science and Technology 64: 409-431.

Li, X., Zhou, S., Zhang, J., Zhou, Z. and Xiong, Q., 2021. Directional changes in the intestinal bacterial community in black soldier fly (*Hermetia illucens*) larvae. Animals 11: 3475.

Liceaga, A.M., 2021. Processing insects for use in the food and feed industry. Current Opinion in Insect Science 48: 32-36.

Lina, G., Quaglia, A., Reverdy, M.-E., Leclercq, R., Vandenesch, F. and Etienne, J., 1999. Distribution of genes encoding resistance to Macrolides, Lincosamides, and Streptogramins among Staphylococci. Antimicrobial Agents and Chemotherapy 43: 1062-1066.

Liu, C., Yao, H., Chapman, S.J., Su, J. and 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.

Liu, H., Zhu, J., Hu, Q. and Rao, X., 2016. *Morganella morganii*, a non-negligent opportunistic pathogen. International Journal of Infectious Diseases 50: 10-17.

Lomonaco, G., Franco, A., De Smet, J., Scieuzo, C., Salvia, R. and Falabella, P., 2024. Larval frass of *Hermetia illucens* as organic fertilizer: composition and beneficial effects on different crops. Insects 15: 293.

Lozupone, C. and Knight, R., 2005. UniFrac: a new phylogenetic method for comparing microbial communities. Applied and Environmental Microbiology 71: 8228-8235.

Marasco, R., Fusi, M., Callegari, M., Jucker, C., Mapelli, F., Borin, S., Savoldelli, S., Daffonchio, D. and Crotti, E., 2022. Destabilization of the bacterial interactome identifies nutrient restriction-induced dysbiosis in insect guts. Microbiology Spectrum 10: e0158021.

Marshall, D.G., Jackson, T.A., Unelius, C.R., Wee, S.L., Young, S.D., Townsend, R.J. and Suckling, D.M., 2016. *Morganella morganii* bacteria produces phenol as the sex pheromone of the New Zealand grass grub from tyrosine in the colleterial gland. Die Naturwissenschaften 103: 59.

Martin, M., 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet.journal 17: 10.

Meng, Y., Zhang, X., Zhang, Z., Li, J., Zheng, P., Li, J., Xu, J., Xian, J. and Lu, Y., 2023. Effects of microorganisms on growth performance, body composition, digestive enzyme activity, intestinal bacteria flora and antimicrobial peptide (AMP) content of black soldier fly larvae (*Hermetia illucens*). Animals 13: 2722.

Metsalu, T. and Vilo, J., 2015. ClustVis: a web tool for visualizing clustering of multivariate data using Principal Component Analysis and heatmap. Nucleic Acids Research 43: W566-W570.

Milanović, V., Cardinali, F., Aquilanti, L., Maoloni, A., Garofalo, C., Zarantoniello, M., Olivotto, I., Riolo, P., Ruschioni, S., Isidoro, N., Cattalani, M., Cardinaletti, G., Clementi, F. and Osimani, A., 2021a. 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., Olivotto, I., Ceccobelli, S., Tavoletti, S., Clementi, F. and Osimani, A., 2021b. Occurrence of antibiotic resistance genes in *Hermetia illucens* larvae fed coffee silverskin enriched with *Schizochytrium limacinum* or *Isochrysis galbana* microalgae. Genes 12: 213.

Moosavian, M., Ghadri, H. and Samli, Z., 2018. Molecular detection of *vanA* and *vanB* genes among vancomycin-resistant enterococci in ICU-hospitalized patients in Ahvaz in southwest of Iran. Infection and Drug Resistance 11: 2269-2275.

Naser El Deen, S., van Rozen, K., Elissen, H., van Wikselaar, P., Fodor, I., van der Weide, R., Hoek-van den Hil, E.F., Rezaei Far, A. and Veldkamp, T., 2023. Bioconversion of different waste streams of animal and vegetal origin and manure by black soldier fly larvae *Hermetia illucens* L. (Diptera: Stratiomyidae). Insects 14: 204.

Niu, S.-H., Liu, S., Deng, W.-K., Wu, R.-T., Cai, Y.-F., Liao, X.-D. and Xing, S.-C., 2022. A sustainable and economic strategy to reduce risk antibiotic resistance genes during poultry manure bioconversion by black soldier fly *Hermetia illucens* larvae: larval density adjustment. Ecotoxicology and Environmental Safety 232: 113294.

Osimani, A., Ferrocino, I., Corvaglia, M.R., Roncolini, A., Milanović, V., Garofalo, C., Aquilanti, L., Riolo, P., Ruschioni, S., Jamshidi, E., Isidoro, N., Zarantoniello, M., Cocolin, L., Olivotto, I. and Clementi, F., 2021. Microbial dynamics in rearing trials of *Hermetia illucens* larvae fed coffee silverskin and microalgae. Food Research International 140: 110028.

Pei, Y., Sun, M., Zhang, J., Lei, A., Chen, H., Kang, X., Ni, H. and Yang, S., 2023. Comparative metagenomic and metatranscriptomic analyses reveal the response of black soldier fly (*Hermetia illucens*) larvae intestinal microbes and reduction mechanisms to high concentrations of tetracycline. Toxics 11: 611.

Price, M.N., Dehal, P.S. and Arkin, A.P., 2010. FastTree 2 – approximately maximum-likelihood trees for large alignments. PLOS ONE 5: e9490.

Querejeta, M., Hervé, V., Perdereau, E., Marchal, L., Herniou, E.A., Boyer, S. and Giron, D., 2023. Changes in bacterial community structure across the different life stages of

black soldier fly (*Hermetia illucens*). Microbial Ecology 86: 1254-1267.

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

Rawat, N., Shreyata, A., Sabu, B., Jamwal, R., Devi, P.P., Yadav, K., Raina, H.S. and Rajagopal, R., 2023. Understanding the role of insects in the acquisition and transmission of antibiotic resistance. The Science of the Total Environment 858: 159805.

Rehman, K.U., Hollah, C., Wiesotzki, K., Rehman, R.U., Rehman, A.U., Zhang, J., Zheng, L., Nienaber, T., Heinz, V. and Aganovic, K., 2023. Black soldier fly, *Hermetia illucens* as a potential innovative and environmentally friendly tool for organic waste management: a mini-review. ISWA 41: 81-97.

Roberts, M.C. and Schwarz, S., 2016. Tetracycline and phenicol resistance genes and mechanisms: importance for agriculture, the environment, and humans. Journal of Environmental Quality 45: 576-592.

Ruan, L., Ye, K., Wang, Z., Xiong, A., Qiao, R., Zhang, J., Huang, Z., Cai, M. and Yu, C., 2024. Characteristics of gut bacterial microbiota of black soldier fly (Diptera: Stratiomyidae) larvae effected by typical antibiotics. Ecotoxicology and Environmental Safety 270: 115861.

Salahuddin, M., Abdel-Wareth, A.A.A., Hiramatsu, K., Tomberlin, J.K., Luza, D. and Lohakare, J., 2024. Flight toward sustainability in poultry nutrition with black soldier fly larvae. Animals 14: 510.

Salas, B., Conway, H.E., Schuenzel, E.L., Hopperstad, K., Vitek, C. and Vacek, D.C., 2017. *Morganella morganii* (Enterobacteriales: Enterobacteriaceae) is a lethal pathogen of Mexican fruit fly (Diptera: Tephritidae) larvae. Florida Entomologist 100: 743-751.

Schreven, S.J.J., De Vries, H., Hermes, G.D.A., Zeni, G., Smidt, H., Dicke, M. and van Loon, J.J.A., 2022. Black soldier fly larvae influence internal and substrate bacterial community composition depending on substrate type and larval density. Applied and Environmental Microbiology 88: e00084-22.

Shelomi, M., 2020. Nutrient composition of black soldier fly (*Hermetia illucens*). In: Mariod, A.A. (ed.) African edible insects as alternative source of food, oil, protein and bioactive components. Springer International Publishing, Cham, Switzerland, pp. 195-212.

Shi, Z., Zhang, J., Jiang, Y., Wen, Y., Gao, Z., Deng, W., Yin, Y. and Zhu, F., 2022. Two low-toxic *Klebsiella pneumoniae* strains from gut of black soldier fly *Hermetia illucens* are multi-resistance to sulfonamides and cadmium. Environmental Pollution 312: 120062.

Soomro, A.A., Cai, M., Laghari, Z.A., Zheng, L., Ur Rehman, K., Xiao, X., Hu, S., Yu, Z. and Zhang, J., 2021. Impact of heat treatment on microbiota of black soldier fly larvae reared on soybean curd residues. Journal of Insects as Food and Feed 7: 329-343. https://doi.org/10.3920/JIFF2020.0108

Tang, D., Chen, M., Huang, X., Zhang, G., Zeng, L., Zhang, G., Wu, S. and Wang, Y., 2023. SRplot: a free online platform for data visualization and graphing. PLOS ONE 18: e0294236.

Tegtmeier, D., Hurka, S., Mihajlovic, S., Bodenschatz, M., Schlimbach, S. and 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: 1642.

Tewari, A. and Abdullah, S., 2015. *Bacillus cereus* food poisoning: international and Indian perspective. Journal of Food Science and Technology 52: 2500-2511.

Van Huis, A., 2020. Insects as food and feed, a new emerging agricultural sector: a review. Journal of Insects as Food and Feed 6: 27-44. https://doi.org/10.3920/JIFF2019.0017

Van Looveren, N., Ijdema, F., van der Heijden, N., Van Der Borght, M. and Vandeweyer, D., 2024. Microbial dynamics and vertical transmission of *Escherichia coli* across consecutive life stages of the black soldier fly (*Hermetia illucens*). Animal Microbiome 6: 29.

Vandeweyer, D., De Smet, J., Van Looveren, N. and Van Campenhout, L., 2021. Biological contaminants in insects as food and feed. Journal of Insects as Food and Feed 7: 807-822. https://doi.org/10.3920/JIFF2020.0060

Varotto Boccazzi, I., Ottoboni, M., Martin, E., Comandatore, F., Vallone, L., Spranghers, T., Eeckhout, M., Mereghetti, V., Pinotti, L. and Epis, S., 2017. A survey of the mycobiota associated with larvae of the black soldier fly (*Hermetia illucens*) reared for feed production. PLOS ONE 12: e0182533.

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. and 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: 913-930.

Yang, F. and Tomberlin, J.K., 2020. Comparing selected life-history traits of black soldier fly (Diptera: Stratiomyidae) larvae produced in industrial and bench-top-sized containers. Journal of Insect Science 20: 25.

Yu, Y., Zhang, J., Zhu, F., Fan, M., Zheng, J., Cai, M., Zheng, L., Huang, F., Yu, Z. and Zhang, J., 2022. Enhanced protein degradation by black soldier fly larvae (*Hermetia illucens* L.) and its gut microbes. Frontiers in Microbiology 13: 1095025.

Zhao, Z., Yang, C., Gao, B., Wu, Y., Ao, Y., Ma, S., Jiménez, N., Zheng, L., Huang, F., Tomberlin, J.K., Ren, Z., Yu, Z., Yu, C., Zhang, J. and Cai, M., 2023a. Insights into the reduction of antibiotic-resistant bacteria and mobile antibiotic resistance genes by black soldier fly larvae in chicken manure. Ecotoxicology and Environmental Safety 266: 115551.

Zhao, Z., Yu, C., Yang, C., Gao, B., Jiménez, N., Wang, C., Li, F., Ao, Y., Zheng, L., Huang, F., Tomberlin, J.K., Ren, Z., Yu, Z., Zhang, J. and Cai, M., 2023b. Mitigation of antibiotic resistome in swine manure by black soldier fly larval conversion combined with composting. The Science of the Total Environment 879: 163065.

Zheng, L., Crippen, T.L., Singh, B., Tarone, A.M., Dowd, S., Yu, Z., Wood, T.K. and 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: 647-658.