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Research article

Microbial composition and bioremediation in frass fertilizers from insect-based agri-food waste valorization

María Gómez-Brandón [b,*,1], Dennis Beesigamukama [a,**,1], Maraike Probst [c,1], Thomas Klammsteiner [d,1], Jian-Qiang Su [e], Yong-Guan Zhu [e,f], Chrysantus Mbi Tanga [a,***,1]

[a] International Centre of Insect Physiology and Ecology, P. O. Box 30772-00100, Nairobi, Kenya
[b] Grupo de Ecología Animal (GEA), University of Vigo, Vigo, 36310, Galicia, Spain
[c] Universität Innsbruck, Department of Microbiology, University of Innsbruck, Technikerstrasse 25d, Innsbruck, A-6020, Austria
[d] Universität Innsbruck, Department of Ecology, Technikerstrasse 25, Innsbruck, A-6020, Austria
[e] Key Lab of Urban Environment and Health, Institute of Urban Environment, Chinese Academy of Sciences, Xiamen, 361021, China
[f] State Key Laboratory of Urban and Regional Ecology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing, 100085, China

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ABSTRACT

Insect frass fertilizer is emerging as a sustainable and novel input for improving soil health and crop production; however, research attention on its safety and microbial properties remains limited. Here, we evaluated the levels of heavy metals, pathogens, diversity, abundance, composition and functional roles of bacteria and fungi in frass fertilizer produced by eight edible insect species. Our results revealed the absence of *Salmonella* spp. In the frass fertilizers produced by all insect species, while the levels of other pathogens and heavy metals were within permissible limits for organic fertilizers. We found that 79–86 % of the variations in bacterial and fungal communities in the frass fertilizers were influenced by the species of insects used in waste recycling. The highest richness of bacteria and fungi was recorded in the frass fertilizers generated from *Oryctes rhinoceros* and *Pachnoda sinuata*. Taxonomic classification revealed 36 bacteria phyla across the frass fertilizers, with most belonging to Firmicutes (43 %), Proteobacteria (23 %), and Actinobacteriota (18 %), whereas the main fungal phyla were Ascomycota (80 %) and Basidiomycota (10 %). Functional profiling revealed that most fungi were sapotroph-symbiotrophs, pathogenic saprotrophs, pathotrophs, and symbiotrophic saprotrophs, which are key in organic matter decomposition, nutrient recycling and pathogen suppression. In contrast, the bacteria were mostly associated with antibiotic and phytohormone production, biosynthesis of plant growth regulators, nitrogen metabolism, nitrification, nitrogen fixation, especially in frass fertilizers derived from *P. sinuata*, *Schistocerca gregaria, and Hermetia illucens*. Our findings demonstrate the potential of insects to recycle low-value organic wastes into hygienic organic fertilizer and highlight the role of beneficial microbes, which could be harnessed for bioremediation, sustainable soil health management, improved crop productivity and food security.

1. Introduction

Insects offer a promising solution within the bioeconomy for resource utilization and waste minimization (Liu et al., 2022; Beesigamukama et al., 2023). They possess complex digestive systems that confer them the ability to process a broad variety of waste streams (Holtof et al., 2019), including low-grade feedstock like municipal organic waste or livestock manure. The highly flexible adaptations of insects to the consumed diets have opened new avenues to exploit their potential for bioremediation applications (Lalander et al., 2016; Tepper et al., 2024); as well as, for biomanufacturing purposes as a rapid means of converting large quantities of waste into high-value products such as industrial biomolecules (i.e., enzymes, lipids and proteins; Van Huis and Gasco, 2023; Tepper et al., 2024), and improved fertilizer (Beesigamukama et al., 2023). Based on this, insect larval-based bioconversion known as entomocomposting has seen a significant

* Corresponding author.
** Corresponding author.
*** Corresponding author.
E-mail addresses: mariagomez@uvigo.es (M. Gómez-Brandón), dbeesigamukama@icipe.org (D. Beesigamukama), ctanga@icipe.org (C.M. Tanga).
[1] Contributed equally to this paper.

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surge in recent years as a bio-based and low-impact alternative to circular management to convert organic waste into nutrient-rich insect biomass for food, feed, fertilizer and other products (Hénault-Ethier et al., 2024; Siddiqui et al., 2024).

Insect frass, one of the main outputs of the bionconversion process, is being promoted as a high-quality and affordable organic fertilizer or soil amendment (Poveda et al., 2019; Beesigamukama et al., 2020a, 2021, 2022a, 2022b), with potential to enhance soil health and crop productivity (Klammsteiner et al., 2020a; Amorin et al., 2024; Nurfikari et al., 2024). Besides plant-accessible nutrients, growth hormones, and plant growth-promoting microbes (Beesigamukama et al., 2022a; Lopes et al., 2022), the presence of chitin-rich exuviae fragments in the frass offers solutions against biotic and abiotic stresses and stimulates beneficial soil microbes with biocontrol activity (Barragán-Fonseca et al., 2022). Thus, effective and sustainable utilization of the insect frass can support the transition of entomocomposting toward a net zero-waste process (Beesigamukama et al., 2023).

Nevertheless, in some countries, increasing concerns regarding biosafety, as expressed by regulatory authorities, have led to more stringent requirements for processing frass intended for use as soil amendment and organic fertilizer. Within the European Union, frass is categorized as processed animal manure, which requires heat treatment (70 °C for a minimum of 60 min) before it can be made commercially available (European Commission, 2018). This procedure, while challenging the preservation of beneficial microbes, effectively reduces the risk of pathogen propagation. Heat treatment does not adversely affect the frass plant nutrient content; however, there is a reduction in microbial activity, albeit with promising prospects for recovery following application to soil (Praeg and Klammsteiner, 2024).

Putting the accent on the (micro)biological aspects of insect frass is, therefore, crucial to unravel how its posterior addition into soil may enhance nutritional efficiency and/or crop quality traits, and to provide a more holistic view of its stability and maturity status prior to field application. Filling this knowledge gap is critical for envisaging and promoting the diversification of this residual stream as an alternative to conventional fertilizers to restore soil health and improve crop productivity. It is likely that insect frass acts not only as a source of soil nutrients but also as a bioinoculum providing a community of microorganisms that contribute to the functioning of the soil system and the maintenance of crop productivity (Insam et al., 2023). Organic amendments are known to contain an endogenous active microbiome that may exert long-term effects on the productivity and sustainability of agro-ecosystems (Mas-Carrió et al., 2018). Nonetheless, to date, there is still a paucity of information on the (micro)biological properties of frass fertilizers from different mass-reared insects, except for those from BSF (*Hermetia illucens* L.) and mealworm (*Tenebrio molitor* L.), which have received more attention as alternative fertilizers for soil amendment and crop production (Beesigamukama et al., 2023; Salomon et al., 2025). For instance, soil amendment with black soldier fly (BSF) frass and exuviae has been found to boost plant health by suppressing soil-borne pathogens, such as *Fusarium* spp., *Ralstonia solanacearum, Rhizoctonia solani*, among others in cowpeas (Quilliam et al., 2020); beans (Choi and Hassanzadeh, 2019); and tomatoes (Kemboi et al., 2022). Beesigamukama et al. (2022a) also revealed higher concentrations of nitrogen (N) and potassium (K) in BSF frass fertilizer, while frass from other edible insects such as *Gryllus bimaculatus* had superior phosphorus (P) content. This further indicates that the nutritional value of frass is largely influenced by the insect species used in bioconversion, along with the bioconversion time and composition of the rearing substrate (Praeg and Klammsteiner, 2024).

In the present study, we aimed to provide an in-depth characterization of the composition and functionality of bacterial and fungal communities as well as the pathogenic content of frass fertilizers generated from eight insect species. Growing evidence has shown that the gut of insects is a major shaper of microbial communities (Klammsteiner et al., 2020b) and acts as a selective filter for microorganisms in rearing substrates (Kariuki et al., 2023). In addition, potentially human pathogenic bacteria may be acquired by the insects during feeding, and they may transit through the larval gut into the frass (Tanga et al., 2021). We hypothesized that different insects and organic wastes will produce frass fertilizers with varying biological properties in terms of microbial community composition and functionality as well as pathogenic load. Ultimately, these insect-specific effects on microbial communities might have important implications for impoverished soils because varying the insect species and rearing conditions could result in specific frass fertilizers that could be employed for distinct practical applications. This information and detected differences are a prerequisite to define the potential usefulness of each type of frass as a soil conditioner and/or plant growth promoter. Moreover, it will be essential to pave the way towards the establishment of guidelines for the consideration of insect frass in existing agro-input markets and farming practices.

2. Material and methods

2.1. Experimental set up and sample collection

The eight insect species were mass-produced at the International Centre of Insect Physiology and Ecology (*icipe*) including BSF (*H. illucens* L.), two-spotted crickets (*G. bimaculatus* De Geer and *Scapsipedus* icipe Hugel and Tanga), silk moth (*Bombyx mori* L.), mealworm (*T. molitor* L.), desert locust (*Schistocerca gregaria Forsskal*), African fruit beetle (*Pachnoda sinuata* L.), and rhinoceros beetle (*Oryctes rhinoceros* L.).

H. illucens frass fertilizer was obtained by feeding the larvae on a mixture of Irish potato waste and brewery spent grain for two weeks following the procedure described by Beesigamukama et al. (2021). *S. gregaria* frass fertilizer was obtained from a colony of locusts fed on diet consisting of wheat and barley seedlings and wheat bran, while the cricket frass fertilizer samples were produced by feeding neonates on a diet consisting of wheat bran, soybean, sweet potato vines and weeds as reported by Magara et al. (2018). Frass fertilizer samples of *P. sinuata* and *O. rhinoceros* were obtained by feeding the larvae on fresh cattle dung for four weeks. *T. molitor* frass fertilizer was generated by feeding mealworms on wheat bran and chayote (*Sechium edule*) for five weeks (Thévenot et al., 2018). *B. mori* frass fertilizer was obtained by rearing silkworms on mulberry tree leaves (*Morus* spp.) for six weeks (Nguku et al., 2007; Hailu, 2016). Further details of frass fertilizer production from various edible insect colonies are presented in our sister paper (Beesigamukama et al., 2022a). Frass fertilizer samples collected from the various insect species were stored at −20 °C prior to laboratory analyses.

2.2. Physicochemical parameters

Physicochemical characterization of the frass samples was carried out using standard laboratory methods, as described by Beesigamukama et al. (2022a). The moisture content was assessed gravimetrically after drying the samples for 24 h at 105 °C. Measurements of pH and electrical conductivity (EC) were performed on aqueous extracts of 1:10 (weight/volume, w/v) sample to distilled water by using a pH (AD1000, Adwa, Romania) and EC meter (AVI, Labtech, India), respectively.

2.3. Heavy metal concentration

The total concentration of heavy metals was determined after microwave-assisted acid digestion of 0.2 g of ground frass sample with 4 mL HNO_3 and 2 mL H_2O_2 in Teflon-PFA microwave digestion vessels. The vessels were tightly sealed and placed in a scientific microwave oven (Anton Paar GmbH Multiwave-3000) operating at a maximum output of 800 W. After cooling for approximately 30 min, the supernatants from each sample were transferred to 100-mL volumetric flasks in which MilliQ water was added up to 50 mL. The extracts were analyzed for arsenic (As), cadmium (Cd), cobalt (Co), chromium (Cr), copper

(Cu), molybdenum (Mo), nickel (Ni), and lead (Pb) using inductively coupled plasma optical emission spectrometry (Thermo Scientific™ iCAP PRO XP Duo ICP-OES). Mercury (Hg) was quantified using cold vapor-phase atomic absorption spectrometry.

2.4. Pathogen cultivation

Twenty-five grams of each frass sample (fresh weight, fw) was placed in a sterile stomacher bag and mixed with 200 mL buffered peptone water (BPW). Subsequently, the homogenized samples were subjected to ten-fold serial dilutions in BPW for pathogen cultivation and identification according to the ISO standard methods. *Escherichia coli* was detected and quantified by incubating the samples on Trypton Bile X-lucuronide and 5-bromo-4-chloro-3-indolyl beta-D-glucuronide at 37 °C for 3 h and then at 44 °C for 18–24 h (ISO 16649-2, 2001). Total and faecal coliforms were determined after incubation at 37 °C and 44 °C, respectively, for 24 h on violet red bile agar (ISO 4832, 2006). Total enterococci were enumerated after incubation on Slanetz Bartley medium at 37 °C for 48 h (ISO 7899-2, 2000). To detect *Bacillus cereus*, the samples were inoculated on mannitol yolk polymyxin B (MYP) agar and incubated at 30 °C for 18–24 h (ISO 7932, 2005). Various presumptive colonies were picked from the MYP agar plates for further confirmation by monitoring complete haemolysis on blood agar plates. Total *Clostridium perfringens* counts (vegetative cells and spores) were determined on tryptose sulphite cycloserine agar under anaerobic conditions for 24 h at 44 °C (ISO 7937, 2004). Presumptive identification was based on colony morphology, with black colonies with a white halo considered to be positive. The detection of *Salmonella* spp. was assessed by pre-enrichment of the samples in BPW for 16–20 h at 37 °C based on the horizontal method described in ISO 6579 (2002), followed by selective enrichment in selenite-cysteine broth over a period of 24 h at 37 °C. After the enrichment step, aliquots of the broth medium were streaked on xylose lysine deoxycholate (XLD) agar and suspected colonies (black color) were confirmed by the cross-streaking method using CHROMagar™ *Salmonella*.

2.5. DNA extraction and microbiome analysis

DNA was extracted from 0.25 g of each frass sample using the DNeasy PowerSoil Kit (Qiagen) following the manufacturer's instructions. The purity and quality of the extracted DNA were assessed using a BioTek Take3TM Multi-Volume Plate (Sinergy™ Multi-Mode Microplate Reader, Bio-Tek Instruments, Inc.). The 16S V4 gene region and internal transcribed spacer 2 (ITS2) region were amplified using the respective primer sets 515F/806R (Caporaso et al., 2011) and ITS3/ITS4 (White et al., 1990) for characterization of bacterial and fungal microbial communities. Equimolar amounts of purified and barcoded PCR products were sequenced on an Illumina MiSeq instrument using a 2 × 300 bp paired-end approach (Microsynth AG, Switzerland). At Microsynth, the sequences were trimmed (primers and adapters were removed) and demultiplexed.

The DADA2 pipeline (version 1.8, Callahan et al., 2016) was used to infer amplicon sequence variants (ASVs) of each frass sample from the trimmed, demultiplexed fastq files, following the standard protocol provided by the developers on GitHub (version 1.16). Briefly, forward/reverse read pairs were filtered, no ambiguous bases were allowed, and each read was required to have fewer than two expected errors, based on their quality scores. ASVs were independently inferred from the forward and reverse sequences of each sample using run-specific error rates. Read pairs were then merged, followed by chimera removal. For bacteria, reads longer than 299 bp or shorter than 294 bp were discarded, and taxonomic assignment of bacterial ASVs was performed against the SILVA reference database v.138.1, using the *assignTaxonomy* function in DADA2, which implements the RDP naive Bayesian classifier (Quast et al., 2013). The length of the fungal merged sequences did not exceed 521 bp, indicating that for all fungal sequences there was sufficient overlap of forward and reverse reads to be considered for further analysis. For fungi, length filtering was not applied. Taxonomy assignment of the fungal ASVs was based on UNITE reference database (version May 10, 2021). For both bacterial and fungal datasets, a frequency ASV table was generated, yielding the relative read abundance of ASVs in each sample. A total of 2693 and 679 ASVs were assigned to bacteria and fungi, respectively, across all frass samples (Tables S1 and S2). Sequencing depth did not differ among the sample groups with regard to bacterial communities ($F_{4,15} = 0.696$; $p = 0.675$, mean ± standard deviation = 40,000 ± 18,000 reads). For fungi (grand mean ± standard deviation = 30,000 ± 14,000 reads), lower values were recorded in the frass samples obtained from *H. illucens* (10,000 ± 7400 reads) compared to those from *S. icipe* (50,000 ± 17,000 reads) ($F_{4,15} = 3.13$; $p = 0.0278$).

The functional composition of the metagenomes was predicted using the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States software package (PICRUSt2) according to the developers' instructions available on Github (Douglas et al., 2020). FUNGuild was used to taxonomically parse fungal ASVs by ecological guild, as instructed by the developers' protocol available on Github (Nguyen et al., 2016).

2.6. Statistics

Principal component analysis of the heavy metal concentrations of frass was performed using the *princomp* function and visualized with the *fviz_pca_biplot* function of the factoextra R package (Kassambara and Mundt, 2020). Microbial community analysis was performed using the R package vegan (2.6–2; Oksanen et al., 2019). Richness and α-diversity were estimated as the number of observed ASVs and the Shannon index, respectively. Taxonomic β-diversity at the ASV level was estimated by Non-Metric Multidimensional Scaling (NMDS) on Bray-Curtis distances to visualize the compositional differences in bacterial and fungal communities among the frass samples obtained from the eight edible insects. Permutational analysis of variance was performed using the *adonis2* function to test for differences in microbial community composition among the insect frass fertilizers. All statistical analyses were performed using R 4.2.1 (R Core Team, 2022). The significance among pairwise sample groups were determined using ANOVA and post-hoc Tukey HSD test. Colors were chosen from the RColorBrewer package (Neuwirth, 2014).

3. Results

3.1. Pathogen levels in frass fertilizers

The number of pathogenic bacteria, as determined by cultivation methods on selective media, varied greatly among the frass samples generated by the eight insect species (Fig. 1). From the palette of investigated pathogens, *Salmonella* spp. was the only species not detected in any of the samples. However, the plates obtained from *T. molitor*, *S. gregaria*, and *O. rhinoceros* contained significant numbers of CFUs from five of the seven screened pathogens, whereas *B. cereus* was the pathogen present in all frass samples except from *B. mori*. The latter only showed significant growth of enterococci but no other potential pathogens (Fig. 1).

3.2. Heavy metal concentrations in frass fertilizers

Principal component analysis of the frass samples revealed distinct patterns of heavy metal concentrations (Fig. 2). Notably, the first two principal components collectively accounted for 97.4 % of the total variance in the data. Particularly distinctive was the frass from *O. rhinoceros*, which exhibited the highest concentrations across nearly all measured heavy metals (As, Cd, Co, Cr, Ni, and Pb), marking it as a notable outlier. In addition, the ordination analysis identified two

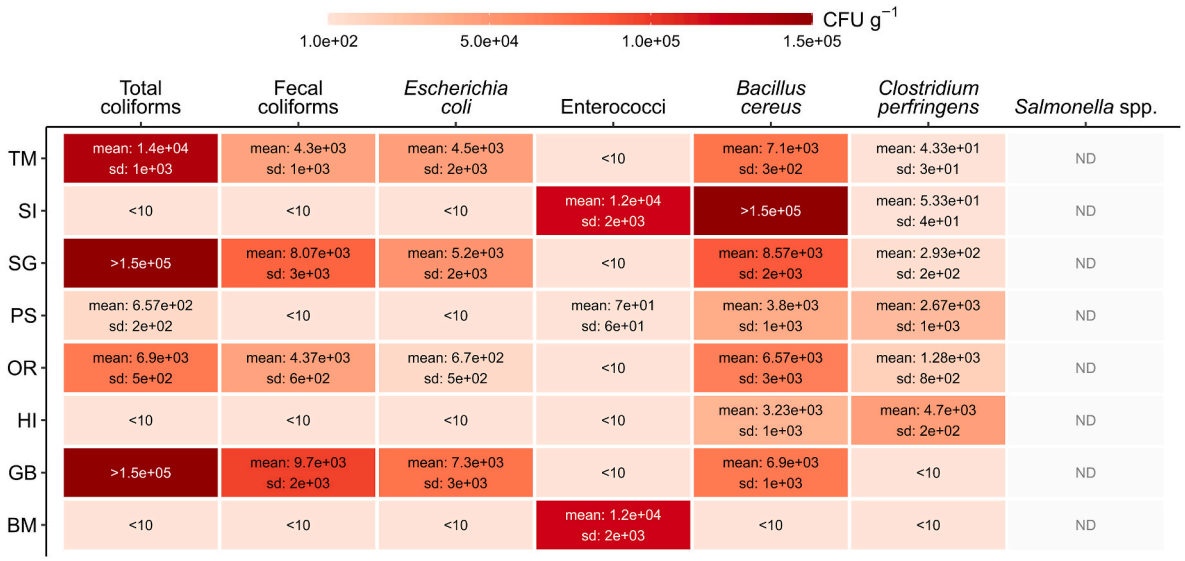

Fig. 1. Counts of colony forming units (CFU) of selected pathogens per gram of frass obtained from the eight edible insect species. *Salmonella* was determined as presence/absence in 25 g of frass sample. sd = standard deviation, nd = not detected, BM: *Bombyx mori*; GB: *Gryllus bimaculatus*; HI: *Hermetia illucens*; OR: *Oryctes rhinoceros*; PS: *Pachnoda sinuata*; SG: *Schistocerca gregaria*; SI: *Scapsipedus icipe*; TM: *Tenebrio molitor*.

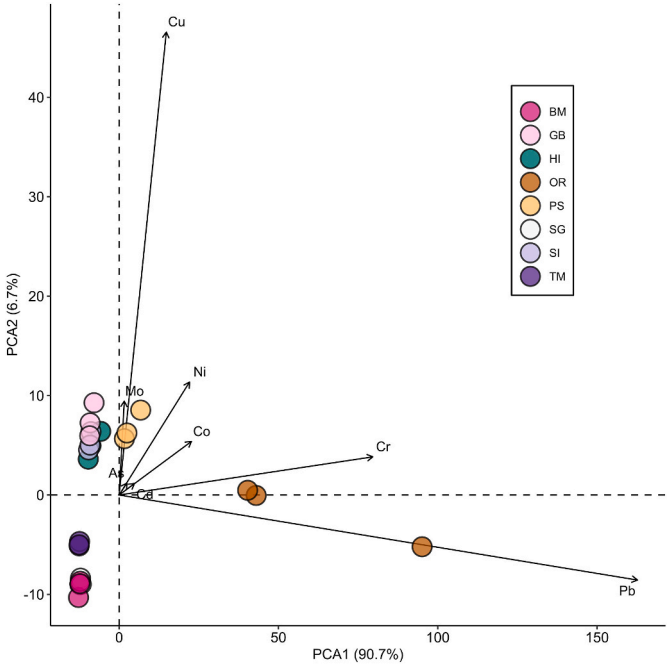

Fig. 2. Principal component analysis (PCA) of heavy metal concentrations measured in the frass of eight insect species. BM: *Bombyx mori*; GB: *Gryllus bimaculatus*; HI: *Hermetia illucens*; OR: *Oryctes rhinoceros*; PS: *Pachnoda sinuata*; SG: *Schistocerca gregaria*; SI: *Scapsipedus icipe*; TM: *Tenebrio molitor*.

discernible clusters among the frass samples. Cluster one, comprising *T. molitor*, *B. mori*, and *S. gregaria* frass, demonstrated either negligible or isolated low concentrations of heavy metals. In contrast, cluster two, comprising frass from *G. bimaculatus*, *H. illucens*, *P. sinuata*, and *S. icipe*, exhibited slightly increased heavy metal concentrations. The frass within cluster two was particularly characterized by elevated levels of Cu, Mo, and Ni. The key drivers influencing the spread of the data were Pb, Cu, and Cr (Fig. 2).

3.3. (Dis)similarity of bacterial and fungal communities in frass fertilizers

Differences among the eight insect frass fertilizers were evident in terms of both bacterial and fungal beta diversity, as shown by NMDS ordination based on Bray-Curtis dissimilarity (Fig. 3A–D). Analysis of the bacterial and fungal communities revealed that 86 % and 79 % of the explained variance was attributed to the type of insect frass, respectively (Adonis2, bacteria: $F_{7,23} = 13.52$, $p = 0.001$; fungi: $F_{7,23} = 8.627$, $p = 0.001$). For bacteria, the frass fertilizers produced by *P. sinuata* and O. *rhinoceros* were comparable (Fig. 3A), yet distinct from those generated by *H. illucens*. The other types of frass obtained from *G. bimaculatus*, *T. molitor*, *S. gregaria*, *S. icipe,* and *B. mori* were more comparable to each other, but still grouped distinctly from each other (Fig. 3A). In terms of fungal community compositional differences, there was a clear discrimination between the *P. sinuata* frass fertilizer and all other sample groups (Fig. 3D). Moreover, the frass samples generated by *O. rhinoceros* clustered separately from those produced by other insect species, which appeared to group closely together along the negative side of the first axis (Fig. 3D). Among the environmental variables, pH had the largest effect on both the bacterial and fungal ordinations, considering the Bray-Curtis dissimilarity (bacteria: $R^2 = 0.192$, fungi: $R^2 = 0.166$; $p = 0.001$).

3.4. Richness and diversity of microbial communities in frass fertilizers

The bacterial alpha diversity (Fig. 3B), assessed as ASV richness, varied among the frass fertilizers produced by the different insect species ($F_{7,16} = 34.48$, $p = 1.72e\text{-}08$). The highest bacterial richness was reported for *O. rhinoceros* and *P. sinuata* samples, with mean values of 650 ± 45 and 540 ± 27, respectively (Fig. 3B), followed by those from *H. illucens* and *G. bimaculatus* whose richness was reduced between two and three times compared to that from *O. rhinoceros* (Fig. 3B). In the case of the frass fertilizers obtained from *S. icipe*, *S. gregaria,* and *T. molitor,* the bacterial richness ranged from 120 ± 14 to 80 ± 2, and the lowest value was recorded for *B. mori* samples (20 ± 4; Fig. 3B). The differences among frass fertilizers in terms of bacterial diversity (estimated using the Shannon index) were comparable to those reported for ASV richness ($F_{7,16} = 128.8$, $p = 7.28e\text{-}13$; Fig. 3C).

As for bacterial communities, the fungal alpha diversity differed significantly among the frass fertilizers ($F_{7,16} = 8.296$; $p = 0.000247$; Fig. 3E). *O. rhinoceros* and *P. sinuata* frass samples also had the highest fungal richness, with average values of 120 ± 50 and 80 ± 5 (Fig. 3E).

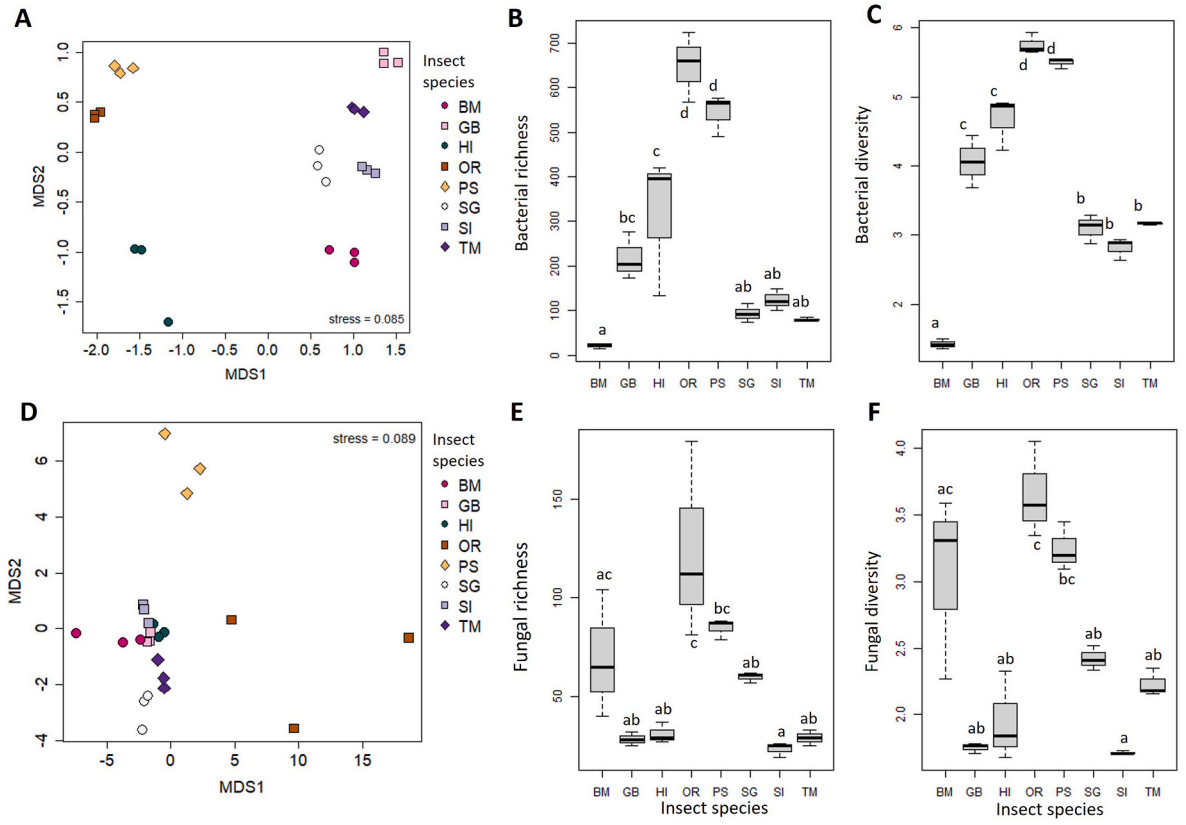

Fig. 3. Changes in beta diversity composition, richness and diversity of microbial communities in the frass samples generated by the eight insect species. (A, D) Distances of bacterial and fungal community compositions among the frass fertilizers illustrated by non-metric multidimensional scaling. (B, E) Bacterial and fungal richness estimated as the number of observed ASVs; (C, F) Bacterial and fungal diversity assessed as the Shannon index. BM: *Bombyx mori*; GB: *Gryllus bimaculatus*; HI: *Hermetia illucens*; OR: *Oryctes rhinoceros*; PS: *Pachnoda sinuata*; SG: *Schistocerca gregaria*; SI: *Scapsipedus icipe*; TM: *Tenebrio molitor*.

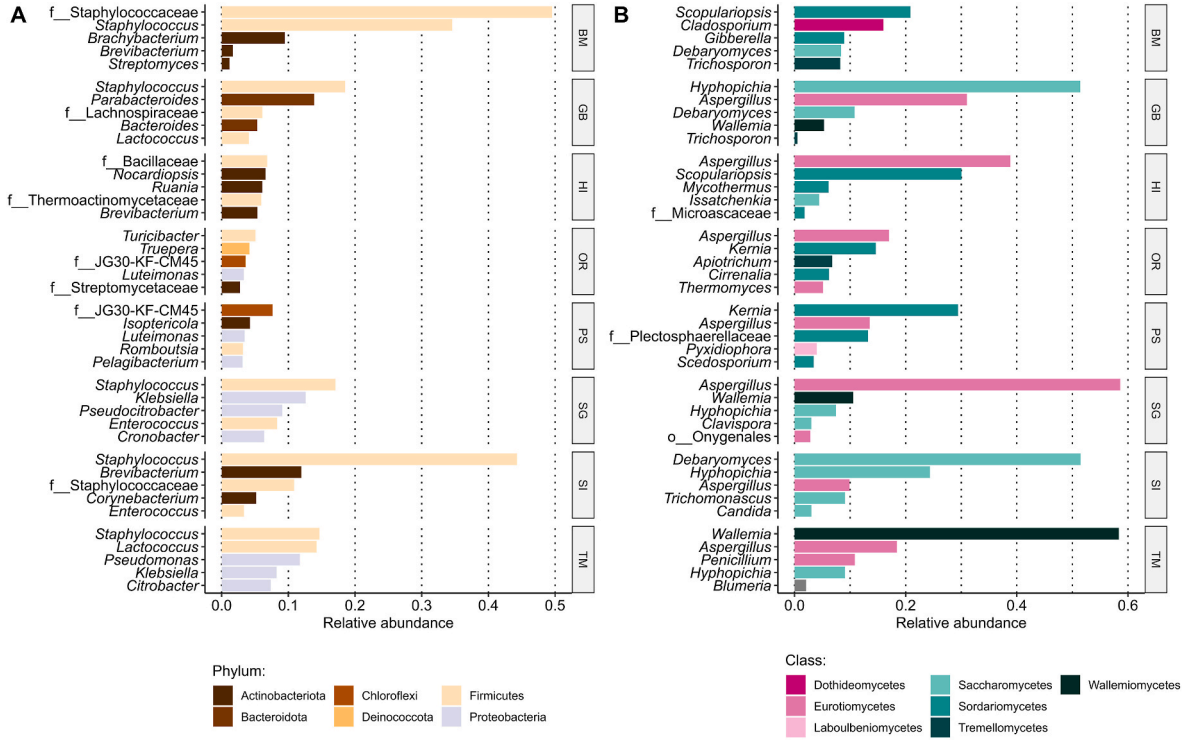

Fig. 4. Top 5 genera of A) bacteria and B) fungi identified in the frass samples generated by the eight insect species. BM: *Bombyx mori*; GB: *Gryllus bimaculatus*; HI: *Hermetia illucens*; OR: *Oryctes rhinoceros*; PS: *Pachnoda sinuata*; SG: *Schistocerca gregaria*; SI: *Scapsipedus icipe*; TM: *Tenebrio molitor*. Amplicon sequencing variants that were not taxonomically identified at order level were filtered from the data.

However, in contrast to bacteria, *B. mori* and *S. gregaria* frass showed greater values of fungal richness (70 ± 32 and 60 ± 3, respectively), doubling those obtained from the remaining insect species i.e., *G. bimaculatus*, *H. illucens*, *S. icipe,* and *T. molitor* (Fig. 3E). Fungal diversity followed a similar trend to that reported for ASV richness with regard to the different frass fertilizers ($F_{7,16}$ = 16.53; p = 3.17 e−06; Fig. 3F).

3.5. Composition of bacterial and fungal community profiles in frass fertilizers

As for beta and alpha diversity measurements (Sections 3.3 and 3.4), there were clear differences in the relative abundances of the frass fertilizers at the genus level (Fig. 4). Among the highest abundant bacterial genera, *Staphylococcus* sp. and unclassified Staphylococcaceae predominated most frass samples (*B. mori*, *G. bimaculatus*, *S. gregaria*, *S. icipe*, and *T. molitor*). With regards to fungi, *Aspergillus* sp. was highly abundant across all frass types. In the frass of *H. illucens*, *O. rhinoceros*, *S. gregaria,* it outweighed all other genera; in the frass of *G. bimaculatus*, *P. sinuata*, and *T. molitor,* it was the second most abundant genus (Fig. 4).

On a broader taxonomic scale, 36 bacterial phyla were detected across the frass samples produced by the eight insect species (Table S1), with most of the annotated reads belonging to Firmicutes (43 %), Proteobacteria (23 %), and Actinobacteriota (18 %) (Fig. S1A). Evaluation of the fungal community composition revealed that the dominant phyla across the different insect frass fertilizers were Ascomycota and Basidiomycota, representing approximately 80 ± 14 % and 10 ± 18 %, respectively (Fig. S1B; Table S2).

3.6. Functional profiling of microbial communities in frass fertilizers

In addition to significant differences in bacterial community composition and diversity, metagenomic predictions using PICRUSt2 showed distinct microbiome functional profiles for the various frass fertilizers regarding the family genes involved in antibiotic and phytohormone production (Fig. 5B), and nitrogen metabolism pathways (Fig. 5D). More in detail, a first cluster comprised those samples obtained from *P. sinuata*, *S. gregaria* and *O. rhinoceros* which had higher estimated abundances of putative genes related to the biosynthesis of salicylic acid (except for *O. rhinoceros*) and certain antibiotics including tetracenomycin, bacilysin, cephamycin_C, pentalenolactone, pikromycin and tylosin compared to the mean across all insect species (Fig. 5B). Within this cluster, *O. rhinoceros* frass fertilizer was additionally characterized by an increase in the estimated abundance of erythromycin and clavulanic acid-related genes (Fig. 5B). The remaining frass samples grouped distinctly in a second cluster, in which putative genes associated with other antibiotics, such as sulfonamides, beta-lactamases, trimethoprim, fosfomycin, and spretomycin, generally gained in abundance along with phytohormones, such as zeatin-type cytokinin and ethylene (Fig. 5B). Analysis with PICRUSt2 also showed that the

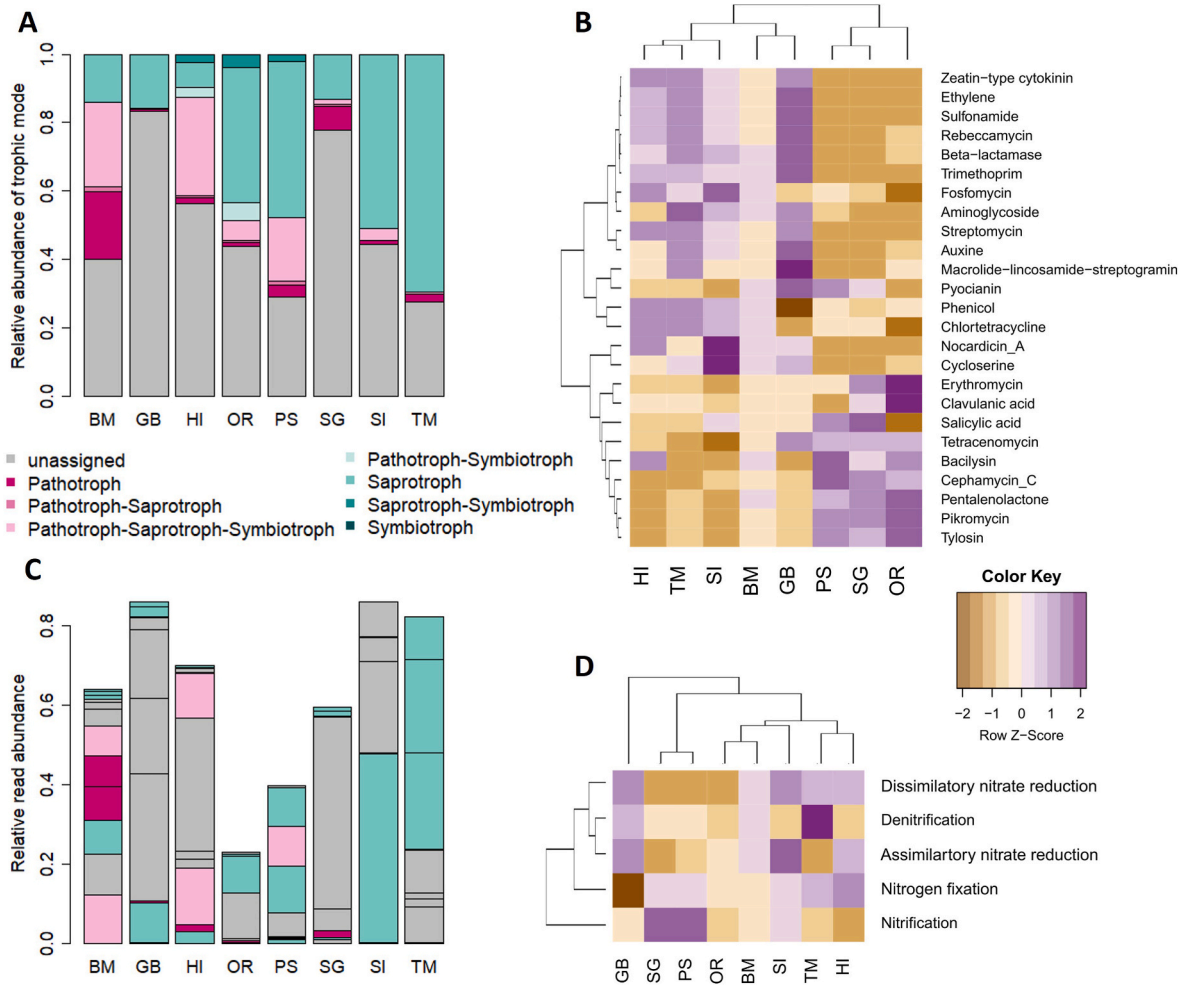

Fig. 5. Changes in the functional profiles of microbial communities in the frass samples generated by the eight insect species. (A, B) Guild annotation of all fungal ASVs and the top ones based on FUNGuild function prediction; (C, D) Heatmaps showing family genes involved in antibiotic and phytohormone production; and in nitrogen metabolism based on PICRUSt2 functional prediction. BM: *Bombyx mori;* GB: *Gryllus bimaculatus;* HI: *Hermetia illucens;* OR: *Oryctes rhinoceros;* PS: *Pachnoda sinuata;* SG: *Schistocerca gregaria;* SI: *Scapsipedus icipe;* TM: *Tenebrio molitor.*

metabolic pathways involved in nitrogen metabolism exhibited differences in terms of abundance across the frass fertilizers (Fig. 5D). For instance, the functional profiles related to nitrification were more prevalent in the samples obtained from *S. gregaria* and *P. sinuata*, whilst those involving nitrogen fixation appeared to be more predominant in the frass fertilizers generated by *H. illucens* in comparison to the mean across all insect species (Fig. 5D).

From a functional perspective, 50 % of all ASVs (340 out of 679 ASVs) were assigned an ecological role using the FUNGuild predictive analysis. Saprotrophic fungi (151 in general, 17 sapotroph-symbiotrophs, 36 potentially pathogenic saprotrophs, and 81 potentially pathotropic, symbiotrophic saprotroph ASVs) accounted for 45 % of the reads and thus for the majority of functionally annotated ASVs across the frass samples (Fig. 5A–Table S3). However, clear differences in the functional annotation of the most abundant ASVs were observed between the sample groups (Fig. 5C–Table S3). The frass fertilizers obtained from *T. molitor* and *S. icipe* had a higher relative abundance of ASVs annotated as saprotrophs (59 % and 48 %, respectively), whereas much lower abundances were reported for the other insect species (*P. sinuata*: 22 %; *G. bimaculatus*: 14 %; *B. mori*: 11 %; *O. rhinoceros*: 10 %; *H. illucens*: 4 %; S. *gregaria*: 3 %; Fig. 5C–Table S3). According to FUNGuild functional prediction, *Debaromyces*, *Kernia*, *Wallemia,* and *Penicillium* were the most probable saprotrophic taxa across frass fertilizers (Table S3). A higher percentage of ASVs annotated as pathotroph-saprotroph-symbiotrophs was found in those generated by *B. mori* (20 %), *H. illucens* (26 %), and *P. sinuata* (10 %) than in the other frass samples (Fig. 5C–Table S3). Moreover, *B. mori* frass fertilizer was characterized by the greatest abundance of ASVs potentially classified as pathotrophs (16 %; Fig. 5C–Table S3), with *Giberella* and *Trichosporon* as the probable fungal taxa contributing to this trophic guild (Table S3).

4. Discussion

4.1. Safety levels of frass generated by different edible insect species

The insect-specific variations in pathogenic loads and heavy metal concentrations in frass fertilizers could be closely linked to the differences in waste degradation and bioremediation efficiencies of insect species substrate, the type rearing substrates, and rearing conditions (Beesigamukama et al., 2022a; Amorin et al., 2024). For example, frass from insects reared on animal manure tends to accumulate higher levels of certain heavy metals, pointing out the importance of substrate selection for safe fertilizer production (Köninger et al., 2021). This implies that while some insects possess mechanisms for immobilizing or excreting heavy metals, others may require targeted bioremediation strategies when processing contaminated substrates. In *H. illucens*, Pb is sequestered in metal-containing cells and transported to the exoskeleton, where it becomes immobilized until shedding, thus introducing Pb into the frass through shed larval skins (Diener et al., 2015). Wu et al. (2020) showed that *H. illucens* also exhibits high accumulation capacities for Cd, while efficiently excreting Cu, resulting in low bioaccumulation in larval biomass and consequently higher Cu concentrations in the resulting frass. On the other hand, *B. mori* frass which was derived from on mulberry leaves that had negligible heavy metal levels, and contained excessively low levels of heavy metals. Only copper was above the detection limit, and its concentration was substantially lower than in frass from the other insect species. A similar trend was recorded for the insect species *S. gregaria* and *T. molitor*, whose frass samples grouped closely together with those from *B. mori*.

Despite the aforementioned distinctions, the various insect frass samples collectively met the recommended standards and guidelines for quality compost fertilizers with regard to their heavy metal levels, according to the European Union (Brinton, 2000) and the Kenya Bureau of Standards guidelines for optimal commercial organic fertilizer (Kenya Bureau of Standards, 2017).

Previous studies have shown that BSF larvae can significantly suppress bacterial pathogens during bioconversion (Erickson et al., 2004; Lalander et al., 2013). In our study, only two of the seven screened potentially pathogenic bacteria were above the detection limits in the frass samples produced by *H. illucens,* demonstrating the ability of this insect species to suppress pathogens (Wu et al., 2024). During the bioconversion process, insect larvae, particularly BSFL, may exert several mechanisms involved in detoxification and host-pathogen resistance, including the excretion of proteins and peptides with antimicrobial activity in the gut (Van Moll et al., 2022), together with the secretion of an active digestive enzyme system (Jing et al., 2020). Recently, Tepper et al. (2024) proposed that engineering biological systems like BSF may provide opportunities for bioremediation of industrial pollutants. Additionally, saprophytic insects like the species *P. sinuata* have shown potential in suppressing antibiotic resistance genes in frass, further enhancing its safety and agronomic value (Gómez-Brandón et al., 2024).

Our results confirm earlier reports on the presence of spore-forming bacteria, such as *C. perfringens,* in certain frass types (Wynants et al., 2019; van Looveren et al., 2021). Most frass samples, except those from *B. mori*, contained detectable C. perfringens and *Bacillus cereus* colonies, with notably higher levels observed in *S. icipe*. The absence of *B. cereus* in *B. mori* frass suggests species-specific mechanisms that may target Gram-positive bacteria. The presence of spore-forming bacteria is very common in feedstocks of animal and vegetable origin (Osimani et al., 2018), highlighting the need for effective post-treatment to mitigate potential spoilage and health risks posed by toxin-forming species.

Insect-specific variability in the presence of certain pathogens, such as *E. coli* and fecal coliforms, was also observed among frass derived from the same feedstock, that is, between the cricket frass, as well as between *P. sinuata* and *O. rhinoceros* frass. While these two pathogenic bacteria were present in the frass produced by *G. bimaculatus* and *O. rhinoceros,* fecal coliforms and *E. coli* counts did not exceed the detection limit in the *S. icipe* and *P. sinuata* frass samples. Aligned with this, a recent study revealed that bioconversion of cattle dung by *P. sinuata* larvae significantly increased the richness of beneficial bacteria in the frass while reducing the abundance of antibiotic resistance genes after a four-week incubation period (Gómez-Brandón et al., 2024). Likewise, Zhao et al. (2022) reported a lower number of ARGs and MGEs in chicken manure-derived frass after conversion by *Protaetia brevitarsis* larvae. Additionally, these authors observed a reduction in the number and relative abundances of ARGs and MGEs following frass application into soil compared to the raw manure-amended treatments. *P. brevitarsis* larvae were also found to efficiently convert herbaceous and ligneous plant residues to plant growth-promoting frass with a high humic acid content (Li et al., 2019; Wang et al., 2022).

Considering these observations, and that regulations on pathogens in insect frass are still developing (OJEU, 2021), there is need to explore post-harvest treatment of frass to reduce the risk of spreading to the environment and food chains upon application of frass into the soil. Heat treatment (70 °C for 60 min) (Van Looveren et al., 2022; Praeg and Klammsteiner,2024) or thermophilic composting ($\geq$55 °C for 72 h) (Wichuk et al., 2011) are some of the effective strategies for eliminating potentially pathogenic microbes from insect frass. However, further research is needed to discern how this treatment affects the stability and maturity of frass for fertilizer use. Other post-treatment processes, such as composting or anaerobic digestion, must also be considered for improved biological stabilization of the frass, as previously recommended (Lopes et al., 2022, 2024).

4.2. Diversity, composition, and functional roles of microbial communities in insect frass produced by different insect species

Exposure to different substrates with a high microbial diversity plays a critical role in shaping the insect larval gut and frass microbiota (Klammsteiner et al. (2020b). For example, the higher richness and diversity for both bacterial and fungal communities observed in

O. rhinoceros and *P. sinuata* frass could be attributed to the high microbial load in cattle dung used as substrate. On the other hand, *B. mori* frass showed comparable values of fungal diversity probably due to the high diversity of arbuscular mycorrhizal fungi associated with mulberry trees (Shi et al., 2016). Since part of the depicted frass microbiome composition is shaped by the microbial communities inherent in the rearing substrates, it is particularly challenging to draw a clear distinction between the portions of the microbiome introduced into the frass through the insect digestive process and those that are autochthonous to the substrate. The overlap between these microbial populations complicates our understanding of the origins and dynamics of the frass microbiome, making it difficult to determine the extent to which the substrate itself influences the observed microbial composition.

Additionally, our findings revealed compositional community differences across the studied frass types, with *H. illucens* frass samples grouping separately in the case of bacteria. Several authors have reported the existence of a predominant bacterial core community based on the screening of BSFL guts exposed to various organic waste (De Smet et al., 2018; Wynants et al., 2019; Klammsteiner et al., 2020b; Tanga et al., 2021). The mechanisms underlying these striking differences might be ascribed to the selective pressure of BSFL to establish a preferable gut microbiota when fed on different organic wastes (Gold et al., 2020; Chen et al., 2023), and/or the excretion of BSFL gut microbiota into frass (De Smet et al., 2018; Siddiqui et al., 2022). Nevertheless, whether the above-mentioned plausible mechanisms operate in a similar way for fungal communities and in other mass-reared insect species still needs further validation.

At a finer taxonomic level, we showed the presence of *Staphylococcus* sp. as the dominant bacterial genus across some of the tested frass types. Staphylococci have been found as symbionts in the guts of insects (Zhang et al., 2018); however, members of the *Staphylococcaceae* family, particularly those of the genus *Staphylococcus*, also encompass important human and animal pathogens, and some strains can act as reservoirs for antimicrobial resistance-encoding genes (Schoenfelder et al., 2017). In parallel, analysis of the mycobiome revealed the dominance of *Aspergillus* across the varying frass samples, as previously shown in *T. molitor* frass (Poveda et al., 2019). Aspergilli may comprise mycotoxin producers, such as *A. niger* and *A. flavus* (Pfliegler et al., 2020), as well as phosphorus solubilizers and biocontrol agents (Soliman et al., 2012) with the ability to produce siderophores (Patel et al., 2017). Taken together, further characterization of *Staphylococcaceae* and *Aspergillaceae* strains from a phenotypic and genotypic perspective remains a subject of interest to verify their pathogenic or commensal behaviour in the frass.

Furthermore, we cannot rule out that the observed differences in microbial composition could also be attributed to variations in the nutritional profiles and the chemical properties of the varying frass samples. This aligns with our sister papers (Beesigamukama et al., 2022a, 2023), which revealed significant differences in the concentrations of macronutrients, secondary nutrients, micronutrients, pH and C/N ratio across the tested frass types. These distinctions could have influenced the abundance and diversity of nitrifying bacteria, and key strains responsible for carbon transformation and mineralization of other nutrients. In fact, by using metagenome imputation, our findings revealed insect-specific variability on certain N-related routes, which are key for maintaining crop productivity. For instance, nitrification was strengthened in *P. sinuata* and *S. gregaria* frass, while nitrogen fixation was more prominent in BSF frass samples. Contrarily, other functions potentially involved in nitrogen loss (e.g., denitrification) were weakened in these frass types, as previously reported by Wu et al. (2024) during the bioconversion of pig and chicken manure by BSFL and the subsequent composting process. Nonetheless, it should be borne in mind that functional prediction tools such as PICRUSt2 or FUNGuild rely on databases and assumptions, which may not precisely depict the function of the frass's specific bacterio- and mycobiota, respectively. Integrating metagenomic and metatranscriptomic analyses in future studies could contribute to further verifying the actual microbial functions in the insect frass and revealing insect- and dietary-specific shifts at the functional gene level (Xu et al., 2022).

From an applied perspective, the high diversity of beneficial microbiota in insect frass reflects its potential to restore soil health by addressing loss in soil biodiversity (Montanarella et al., 2016), and boosting nutrient availability organic matter decomposition and immunity against biotic and abiotic stress (Poveda et al., 2019; Beesigamukama et al., 2020a; Anyega et al., 2021; Chepkorir et al., 2024). The beneficial microbes can also be harnessed to develop biostimulants, artificial plant growth regulators, and biofertilizers to enhance crop productivity and address specific soil-degradation challenges Furthermore, the combination of beneficial microbes and chitin contained in the frass is beneficial in suppressing crop pests and pathogens (Kemboi et al., 2022; Anedo et al., 2024; Kisaakye et al., 2024), leading to high crop yields (Beesigamukama et al., 2020b; Abiya et al., 2022). In general, the circular economy benefits of scaling insect farming innovations have revealed enormous benefits on sustainable waste management, fertilizers and animal feeds supply, food security, household income, poverty alleviation, and economic development worldwide (Abro et al., 2020, 2022; Verner et al., 2021; Siddiqui et al., 2024).

5. Conclusion

This study provides a comprehensive multispecies analysis of insect frass fertilizers, revealing insect-specific mechanisms in heavy metal excretion, pathogen suppression, and microbiome-driven nitrogen fixation, establishing a framework for optimizing entomocomposting in circular economies while addressing safety and functionality gaps in organic fertilizers. The levels of heavy metals and microbial pathogens in the frass fertilizers produced by various edible insects indicate their efficiency in bioremediation and in improving the maturity and stability of organic fertilizer products for field application, providing a sustainable alternative to conventional methods of compost production. Moreover, the high microbial diversity and numerous benefits of bacteria and fungi in various insect frass fertilizers could be harnessed to rejuvenate soil health, enhance plant growth, and protect against pests and diseases. Nonetheless, to explore the full potential of this insect residual stream, it will be necessary to: (i) develop quality standards for each frass fertilizer depending on the insect species, feedstock, and rearing conditions, among other factors; (ii) demonstrate the mid-to long-term effects of insect frass fertilizers on soil and plant health to discern how their addition might confer disease resistance in plants and heighten crop nutritional status; and (iii) develop a policy and legal framework to promote, scale up, and commercialize the use of insect frass fertilizers in farming practices.

CRediT authorship contribution statement

María Gómez-Brandón: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Methodology, Funding acquisition, Conceptualization. **Dennis Beesigamukama:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Methodology, Investigation, Funding acquisition, Conceptualization. **Maraike Probst:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Formal analysis, Data curation. **Thomas Klammsteiner:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Formal analysis, Data curation. **Jian-Qiang Su:** Writing – review & editing, Conceptualization. **Yong-Guan Zhu:** Writing – review & editing, Conceptualization. **Chrysantus Mbi Tanga:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jenvman.2025.125774.

Data availability

Data will be made available on request.

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