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Research Paper

Garden fruit chafer (*Pachnoda sinuata* L.) accelerates recycling and bioremediation of animal waste

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ABSTRACT

Bioconversion of livestock wastes using insect larvae represents an emerging and effective strategy for waste management. However, knowledge on the role of the garden fruit chafer (Pachnoda sinuata L.) in waste recycling and influence on the diversity of microbial community in frass fertilizer is limited. Here, we determined whether and to what extent the conversion of cattle dung into insect frass fertilizer by P. sinuata influences the frass' microbial community and its associated antibiotic resistance genes abundance. *Pachnoda sinuata* larvae were used to valorise cattle dung into frass fertilizer; samples were collected weekly to determine the composition of bacteria and fungi, and antibiotic resistant genes using molecular tools. Results revealed that bioconversion of cattle dung by P. sinuata larvae significantly increased the richness of beneficial bacteria in the frass fertilizer by 2.5-folds within 28 days, but fungal richness did not vary during the study. Treatment of cattle dung with P. sinuata larvae caused 2 – 3-folds decrease in the genes conferring resistance to commonly used antibiotics such as aminoglycoside, diaminopyrimidine, multidrug, sulfonamide and tetracycline within 14 days. Furthermore, the recycling cattle dung using considerably reduced the abundance of mobile genetic elements known to play critical roles in the horizontal transfer of antibiotic resistance genes between organisms. This study highlights the efficiency of saprohytic insects in recycling animal manure and suppressing manure-borne pathogens in the organic fertilizer products, opening new market opportunities for innovative and safe bio-based products and achieving efficient resource utilization in a circular and green economy.

1. Introduction

Globally, more than 2 billion tons of municipal solid waste are produced annually but only 16 % is recycled, while > 46 % is discarded (Kaza et al., 2018). This threatens environmental and human health (Nweke and Sanders, 2009; Kimani, 2007). The growing demand for animal products leads to increasing volumes of manure that must be treated, disposed of or recycled in a proper manner (Awasthi et al., 2022). If poorly managed, land-application of increasing volumes of manure may exert a pronounced deleterious effect on soil and environmental health. In most developing countries, animal manure is used as a source of nutrients to boost soil organic matter contents and crop yields (Gram et al., 2020; Rufino et al., 2007), saving farmers from the costly mineral fertilizer (WFP, 2022) and rejuvenating degraded soils (Stewart et al., 2020). Although developed countries have policies and legal framework on appropriate use of manures, manure pollution control and recycling, most treatment technologies cannot eliminate all the contaminants (IAEA, 2008; Brandjes et al., 1996).

The overuse of antibiotics in livestock husbandry for disease control and/or growth promotion is accompanied by the discharge of a considerable percentage (30–90 %) of antibiotics in the urine and feces. Consequently, livestock manure is considered a potential reservoir for antibiotic-resistance genes (ARGs) and antibiotic-resistant bacteria (ARB) (Zhu et al., 2019). Although barriers restricting the flow of both,

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microbes and genes, between environments exist (Larsson and Flach, 2022), manure application may result in ARGs to be horizontally transferred from manure-borne bacteria to the native soil microbiota through mobile genetic elements (MGEs). Soil itself also contains intrinsic ARBs that can be enriched in response to fertilizer (Keenum et al., 2021). The spread of ARGs may lead to their accumulation and prevalence in the environment with significant consequences for both human and animal health through uptake by plants or release into water bodies via runoff, representing a threat under the One Health framework (White and Hughes, 2019).

Despite possible economic trade-offs for farmers, effective strategies must be applied to suppress pathogens, MGEs and ARGs prior to utilization of manure for soil fertility management and crop production. Up to now, many studies have focused on ordinary composting as a means of minimising the spread of ARGs and ARBs in agricultural soils upon application of manure (Keenum et al., 2021) but with limited success (Brinton et al., 2009). Recently, the use of insect larvae represents an efficient and low-cost technology for suppressing manure-borne pathogens (Klammsteiner et al., 2020a; Lalander et al., 2013, 2015) through several mechanisms including ingestion as well as lysis through the insect gut (Tanga et al., 2021a). Saprophytic insects such as BSF can also secrete new antimicrobial peptides, which are responsible for elimination of multidrug-resistant pathogens contained in organic wastes (Mudalungu et al., 2021). Besides, insect larvae have the capability to recycle organic waste into nutrient-rich organic fertilizer with potential positive effects on soil health and crop productivity (Beesigamukama et al., 2021a, 2021b, 2022; Liu et al., 2022; Insam et al., 2023). Previous findings have shown that the black soldier fly (*Hermetia illucens* L.) (BSF) and yellow mealworms (*Tenebrio molitor* L.) require only five weeks to bioconvert organic waste into mature and stable insect frass organic fertilizers, while conventional composting methods require longer time of between 8 and 24 weeks (Heussler et al., 2022; Beesigamukama et al., 2021a,b). In parts of Africa, the prices of synthetic fertilizer have increased by 64 – 113 % due to Ukraine-Russia war that has disrupted global supply chains (WFP, 2022). Therefore, recycling organic waste into insect frass fertilizer could provide a local, sustainable and regenerative strategy for transforming food systems through circular economy.

Insect frass fertilizer is one of the value-added products derived from insect farming, and has potential to serve as an alternative to conventional and commercial fertilizers (European Commission, 2021; Liu et al., 2022; Beesigamukama et al., 2022) due to multiple benefits on soil fertility and plant health (Klammsteiner et al., 2020a; Barragán-Fonseca et al., 2022; Beesigamukama et al., 2022). The beneficial effects of frass fertilizer have primarily been ascribed to plant-accessible nutrients and moderated contents of chitin (Barragán-Fonseca et al., 2022), but frass-associated microbes are also likely to play a role. Bearing this in mind, it is crucial to shed light onto the compositional changes and functional capabilities of the microbial communities over the course of the entomocomposting process to broaden the understanding about the potential usefulness of insect frass as biofertilizer. Although there exists previous knowledge on bacterial communities during bioconconversion of different waste streams using saprophytic insects (Cai et al., 2018; Zhang et al., 2021), fungal communities have been underexplored in this context (Ziganshina et al., 2018). Furthermore, there is still sparse information on how the changes in the microbiome might be related to antibiotic resistome suppression during entomocomposting. Previous studies found an increase in the prevalence of genes resistant to tetracycline and erythromycin in insect biomass and associated frass products (Milanović et al., 2016; Osimani et al., 2017). However, the efficiency of insects to attenuate ARGs might vary with insect species and type of organic waste. As such, the question of whether treating organic waste with insect larvae may reduce the abundance of ARGs and how this is linked to compositional community changes remains unresolved (Rawat et al., 2023). The BSF larvae can suppress pathogens in organic wastes, but its potential is limited by low breakdown efficiency of lignocellulosic waste such as cattle dung, one of the most available organic wastes with challenges of microbial contamination (Klammsteiner et al., 2020b).

Beetles provide multiple ecosystem services, including bioconversion of waste especially cattle dung, seed dispersal, nutrients recycling, incorporation of soil organic matter, and soil erosion control through increased soil porosity, movement of nutrients along the soil profile, and reduction in parasitic nematodes (Nervo et al., 2017). Despite their economic importance, most studies have largely focused on the dung beetle, thereby leaving a knowledge gap on the role of other species including the garden fruit chafer (*Pachnoda sinuata* L.) in the bioconversion of organic waste.

Most research efforts on *P. sinuata* have focused on its control as a pest, particularly on horticultural crops (Auerswald and Gäde, 2000). Exploration of the potential of domesticated *P. sinuata* to convert lignocellulose-rich, fibrous agricultural byproducts such as cattle dung into organic fertilizer has received little attention to date. Although previous knowledge on the gut microbiome of beetles exists (Thiyonila et al., 2018), the impact of *P. sinuata* composting on the abundance of microbial community diversity and ARGs of the frass fertilizer generated from cattle dung is unknown. Such information is necessary for developing recommendations on the application of *P. sinuata*-based technologies for frass fertilizer production, environmental management, and bioremediation to suppress pathogens and reduce transfer of ARGs.

Therefore, this study aims to evaluate the compositional changes of bacterial and fungal communities during the bioconversion of cattle dung into frass fertilizer by *P. sinuata* larvae, and explore the resistome of the frass fertilizer as well as the relationships between the microbiome and the antibiotic resistome. We tested whether the bioconversion of cattle dung into insect frass fertilizer using *P. sinuata* would cause a reduction in the abundance of ARGs and MGEs, to generate recommendations for industrial use of saprophytic insects in bioremediation, fertilizer production, and sustainable animal manure management.

2. Material and methods

2.1. Colony of garden fruit chafer (Pachnoda sinuata L.)

The mother colony of *P. sinuata* was set up in the Duduville campus of the International Centre of Insect Physiology and Ecology (*icipe*) (in the Animal Rearing and Quarantine Unit (ARQU) at Kasarani area, Nairobi County, Kenya. Wild specimens of *P. sinuata* were captured on mango flowers employing a sweep net and transported to the ARQU through well aerated Perspex cages that contained mango peels, tree branches and fresh cattle dung. The experimental cages were revised twice a week to monitor egg deposition in the feedstock. Afterwards, the eggs were moved to plastic trays in which newly hatched neonates were reared with fresh cattle dung. The larvae developed into pupae were placed into new Perspex cages for adult beetles to emerge. The moisture and temperature conditions in the rearing rooms were monitored with portable digital thermo-hygrometers. A temperature of 28 ± 2 ℃, moisture levels of 60 ± 10 % and a photoperiod exposure of L12:D12 were maintained throughout the experimental period.

2.2. Experimental set-up and sample collection

The fresh cattle dung was sourced from a dairy farm near *icipe* (Table 1). The five-day old *P. sinuata* larvae were obtained from a colony described above. The insects were reared in four-liter rectangular plastic containers (22.0 × 15.6 × 16.0 cm) (Rectangular Food Mate No. 3, Kenpoly Manufacturers Ltd., Nairobi, Kenya) in the larvarium at the ARQU of *icipe*. To allow for gas exchange, each experimental container had an opening (18 × 12.5 cm) on the lid that was further covered with netting organza material. One hundred 10-day old beetle larvae were kept in plastic containers and fed with 200 g of dry cattle dung. The experiment was performed in four replicates under the same

Table 1
Physico-chemical properties of the cattle dung used in the experimental set-up. Values are means ± standard error (n = 4).

Parameter	Value
pH	8.3 ± 0.12
Electrical conductivity (mS cm^{-1})	3.0 ± 0.51
Moisture (%)	80.7 ± 0.53
Carbon (%)	40.6 ± 0.56
Nitrogen (%)	1.9 ± 0.04
Phosphorus (%)	0.7 ± 0.08
Potassium (%)	1.2 ± 0.19
Calcium (%)	1.9 ± 0.02
Magnesium (%)	0.5 ± 0.02
Sulphur (%)	0.3 ± 0.02
Manganese (mg kg^{-1})	1303.3 ± 54.9
Iron (mg kg^{-1})	5670.0 ± 245.8
Zinc (mg kg^{-1})	128.7 ± 4.7
Copper (mg kg^{-1})	25.8 ± 0.38
Boron (mg kg^{-1})	24.0 ± 0.84
Sodium (mg kg^{-1})	850.0 ± 95.2
C to N ratio	21.4 ± 0.27

temperature and moisture conditions as described in section 2.1. Feeding was done *ad libitum* until pupal stage when the larvae formed cocoons and stopped feeding. Exhaustion of diet was indicated by the formation of pellets, and the quantity of cattle dung provided per feeding was recorded and used to calculate the total amount consumed giving a waste reduction index of 1.46. Samples of cattle dung and/or frass were collected weekly from each experimental container for a time period of 28 days, placed in ziplock bags before storing at −20 ℃ for further analysis.

2.3. Physico-chemical properties

The physico-chemical characterization of the samples was carried out following the procedures as reported in Beesigamukama et al. (2022). Moisture levels were assessed by weight loss when the samples were dried at 105 °C for a period of 24 h. For the electrical conductivity (EC) and pH values, measurements were conducted in aqueous extracts at a ratio of 1:10 (weight by volume) by utilizing a pH device (AD1000, Adwa, Szeged, Hungary) and EC meter (AVI Scientific, Labtech, Mumbai, India), respectively. Wet oxidation method was performed to determine total organic carbon; while the total nitrogen content was quantified based on the Kjedahl distillation and titration procedure. Other macronutrients (K and P), secondary (Ca, Mg and S) and micronutrients (Mn, Fe, Zn, Cu, B, Na) were extracted by acid digestion and measured by optical emission spectrometry with inductively coupled plasma.

2.4. DNA extraction, amplification and sequencing

The DNA extraction process was performed on cattle dung and frass (0.25 g, fw) with DNeasy PowerSoil Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. DNA purity and quality was evaluated by using the BioTek's Take3TM Multi-Volume Plate (Bio-Tek Instruments, Inc.). The 16S rRNA V4 and the internal transcribed spacer 2 (ITS2) gene regions were targeted via PCR with the primer pairs 515F/806R (Caporaso et al., 2011) and ITS3/ITS4 (White et al., 1990), respectively. Prior to sequencing, the purified PCR products were pooled in equimolar concentrations. An Illumina MiSeq instrument (San Diego, CA, USA) was employed by applying 2 × 300 bp paired-end run (Microsynth AG, Balgach, Switzerland). Microsynth provided trimmed (primers and adapters removed) and demultiplexed data. The data from the sequences were kept in a repository in SRA under project number PRJNA868198.

Dada2 pipeline (version 1.18, Callahan et al., 2016) was used to obtain a table of amplicon sequence variants (ASV) from the trimmed, demultiplexed fastq files following Github developers' protocol (version 1.16; https://benjjneb.github.io/dada2/tutorial.html). Firstly, sequences with ambiguous base pairs and those sequences exceeding an expected error rate of > 2 (maxEE) were excluded from the dataset. Prediction of error models was done considering randomly selected samples, and the error profiles were taken into account to filter both forward and reverse reads prior to merging them. Longer (>299 bp) and shorter (<294 bp) bacterial reads were excluded. After chimera removal, filtered sequences were then aligned to SILVA reference database v.138.1 based on the *assignTaxonomy* function depicted in dada2 (Quast et al., 2013). Taxonomically, fungal amplicon sequence variants were assigned by employing UNITE reference database (version 10.05.2021). For the bacterial dataset, amplicon sequence variants not annotated to a bacterial phylum were excluded from the final dataset. A total of 2,489 and 656 amplicon sequence variants were assigned to bacteria and fungi across all samples, respectively. For bacteria, the sequencing depth did not differ among sample groups ($F_{4,15} = 0.88$; $p = 0.499$). In the case of fungal communities, a higher sequencing depth was recorded on day 7 in comparison with the 14- and 28-day sample groups ($F_{4,15} = 4.225$; $p = 0.0173$).

2.5. High-throughput quantitative polymerase chain reaction (HT-qPCR)

High-throughput qPCR was performed with the WaferGen SmartChip Real-time PCR system (WaferGen Inc., Fremont, CA, USA) that allowed for the detection of 308 ARGs, 8 taxonomic genes, 10 heavy metal resistance genes, 36 MGEs marker genes, 10 transposase genes, 3 integrase genes, 8 insertional sequences, and the 16S rRNA gene (see Table S1 for further details). The HT-qPCR amplifications were replicated thrice (triplicate), taking into consideration the non-template control, in a 100-nL reaction that contained LightCycler 480 SYBR Green I Master, bovine serum albumin, primers, PCR grade sterile water and the sample DNA template, previously diluted to equimolar concentration with sterile water (Zhu et al., 2020). The cycling conditions were as those depicted in Zhu et al. (2020). The melting curves and data treatment were generated using the WaferGen software and SmartChip qPCR Software (2.7.0.1 version), respectively. We ensured that wells that had multiple melting peaks and low amplification efficiencies (<1.8) were excluded. Threshold cycle (Ct) of 31 was set as the limit of detection of amplification and absolute copy value of each ARG was normalized to the absolute 16S rRNA gene copy number (Zhu et al., 2020). For each biological sample, the mean gene copy number of the three technical replicates was considered for further data analysis. An ARG was considered positive when all 3 technical replicates were found to be amplified. If an ARG was recorded in anyone out of the 3 technical replicates, the ARG was recorded absent. In the case of an ARG being found in two out of three technical replicates, we considered it as positive if the ARG was detected in ten out of the twelve biological samples across all time points, which equals to three out of four biological replicates.

2.6. Statistical analysis

Data on microbial communities were analyzed using the R package vegan (2.6–2; Oksanen et al., 2019). The estimation of richness and α-diversity was assessed as the number of observed amplicon sequence variants and Shannon index, respectively. Non-metric multidimensional scaling (NMDS) on Aitchison distances of clr-transformed count data was used to estimate taxonomic β-diversity at the amplicon sequence variant level in order to visualize the compositional differences in bacterial and fungal communities between the cattle dung and the frass samples during the bioconversion process. Genus-level heatmaps based on relative abundance data for bacteria and fungi were generated using the ampvis2 R package (v.2.7.4) (Andersen et al., 2018). The influence of time and environmental variables on the microbial community composition was analysed with Adonis2 (Permutational Multivariate Analysis of Variance Using Distance Matrices). The number of amplicon

sequence variants unique to a sample group or those shared among sample groups were visualized by Venn diagrams (Hulsen et al., 2008). The effect of time on individual variables was analysed using ANOVA and posthoc Tukey HSD test. The heatmap of the ARGs was created using heatmap2 function of the R package gplots (Warnes et al., 2020).

To link the ARG and MGE copy numbers to the bacterial microbiome, a network was calculated using SpiecEasi (Kurtz et al., 2021). Meinshausen-buhlmann's neighborhood selection was used as method, "stars" used as selection criterion. The resulting network was transformed into an edgelist using the package igraph (Csardi and Nepusz, 2006). Bacterial-bacterial and ARG-ARG associations were removed from the list. The frequency of associations among bacterial phyla and resistance genes classes was visualized. To test if amplicon sequence variants with a higher abundance tended to associate with different antibiotic classes when compared to amplicon sequence variants with lower abundances, we extracted the edgelist of all amplicon sequence variants-antibiotic resistant gene associations from the network. We calculated the relative abundances of all amplicon sequence variants across the entire dataset and categorized them into quartiles. The frequency distribution of amplicon sequence variant quartiles and antibiotic classes of the ASV-associated ARGs was tested using chi-square test. This statistical test was also used to determine if the number of associations with regards to the different antibiotic classes was dependent on the bacterial phylum. The abovementioned statistical tests were carried out in R 4.2.1 (R Core Team, 2022); and RColorBrewer (Neuwirth, 2014) was used for the color selection in the graphs.

3. Results

3.1. Microbial richness, diversity and community composition during the bioconversion of cattle dung using Pachnoda sinuata larvae

After quality filtering, we obtained 50 k ± 18 k and 20 k ± 11 k reads per sample with respect to fungal and bacterial communities. Bacterial α-diversity assessed as amplicon sequence variant richness was 299 ± 31 in the fresh cattle dung (Fig. 1A). The bioconversion of cattle dung into insect frass by *P. sinuata* larvae led to a 2.5-fold increase in bacterial richness until day 7 ($F_{4,15}$ = 24.18, p = 2.16 e-06). Higher levels of bacterial diversity assessed by Shannon index were also recorded at this early time point ($F_{4,15}$ = 24.18, p = 2.16 e-06; Fig. 1B). After the first 7 days, the community composition in the immature frass likely reflected the status of bacteria that might have recently processed and passed through the gut of the beetle larvae and then expelled by *P. sinuata* larvae without being eliminated in the digestion process.

No further noticeable changes in bacterial richness and diversity were found on days 14 and 21 in the presence of *P. sinuata* larvae, followed by a 1.5-fold decrease on day 28 (Fig. 1A). In contrast to bacteria, neither fungal richness nor diversity significantly varied over time (richness: $F_{4,15}$ = 1.845, p = 0.173; diversity: $F_{4,15}$ = 2.937, p = 0.0561; Fig. 1D, E).

3.2. Effect of Pachnoda sinuata larvae on bacterial and fungal community composition of cattle dung and frass fertilizer

In total, 35 bacterial phyla (Table S2) were detected, with most of the annotated reads in the fresh cattle dung belonging to Firmicutes (78 %) and to a lesser extent to Bacteroidota (7 %), Proteobacteria (6 %) and Actinobacteriota (5 %) (Fig. 1C). Once processed by *P. sinuata* larvae,

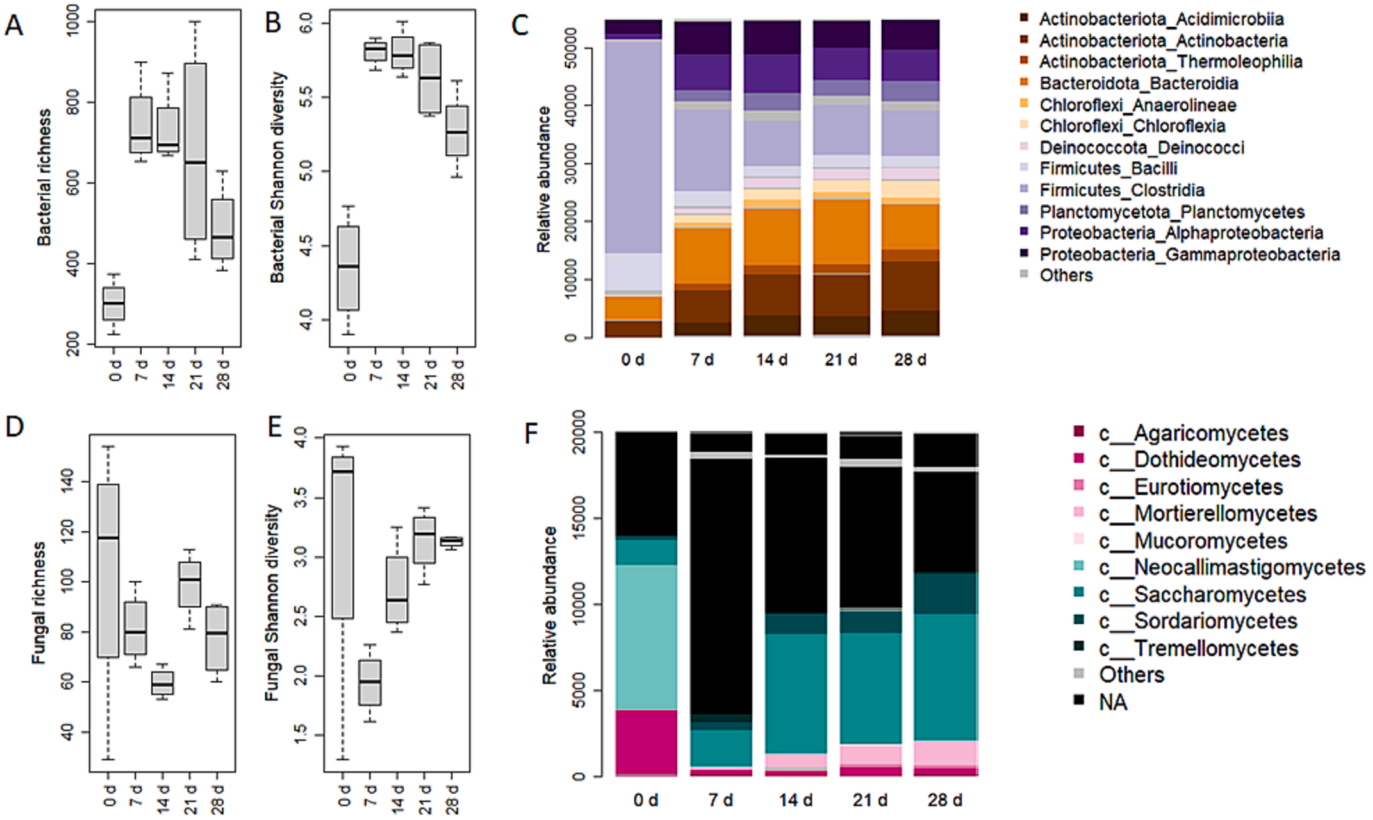

Fig. 1. Bacterial and not fungal richness and diversity rapidly increased during bioconversion of cattle dung into insect frass fertilizer using *Pachnoda sinuata* larvae. (A, D) Bacterial and fungal richness estimated as the number of observed amplicon sequence variants; (B, E) Bacterial and fungal diversity assessed as the Shannon index; (C, F) Taxonomic composition of fungal and bacterial communities based on relative read abundances.

Actinobacteriota (22 %), Firmicutes (21 %), Proteobacteria (20 %) and Bacteroidota (17 %) were detected in comparable relative abundances in the frass samples (days 7 to 28; Table S2). Over time, we observed that other bacterial phyla exceeded the initially abundant Firmicutes in relative abundance and that this compositional change further came with an increase in alpha-diversity (Fig. 1A-C). This study showed that the relative abundance of Firmicutes was reduced by approximately four-fold compared to the fresh cattle dung. On the level of class (Fig. 1C), Clostridia represented the most substantial proportion of the bacterial reads (66 %) in the fresh cattle dung, with a lower representation of the classes Bacilli (12 %), Bacteroidia (7 %), Actinobacteria (5 %), Gamma-(5 %) and Alphaproteobacteria (2 %). After treatment with *P. sinuata*, Clostridia were pronouncedly reduced in relative abundance, reaching average values of 26 % on day 7 and 15 % on days 14 to 28 (Fig. 1C; Table S2). The other abovementioned bacterial classes were comparable in terms of abundance (10–20 %) across time points within the 28-day timeframe (Fig. 1C). Other classes such as Acidimicrobiia, Anaerolineae, Bacilli, Chloroflexia, Deinococci, Planctomycetes and Thermoleophilia were also present in the *P. sinuata* frass samples on a lower percentage (≤5%; Fig. 1C, Table S2).

On a genus level (Fig. 2A; Table S2), initially dominant anaerobic Firmicutes such as *Romboutsia* (18 %), *Clostridium* sensu stricto 1 (7 %), *Paeniclostridium* (9 %) and *Clostridioides* (6 %) were rapidly reduced to low relative abundances being below or close to 5 % in the first week of dung colonization by *P. sinuata,* however, they were not completely removed until the end of the treatment. Solely the genus *Turicibacter*, which comprises obligate anaerobic, Gram-positive bacteria belonging to the phylum Firmicutes kept stable relative abundances of 2–5 % throughout the experiment both in fresh and treated dung. With progressing dung processing by *P. sinuata* larvae, aerobic genera primarily including *Knoellia*, *Truepera*, *Luteimonas*, *Iamia* and *Ornithinicoccus* appeared after the first week and gradually increased in numbers (Fig. 2A).

Evaluation of the fungal composition revealed that the dominant phyla in the fresh cattle dung were Neocallimastigomycota and Ascomycota, which represented about 41 % and 27 % of the sequences in this sample group (Fig. 1F; Table S3).

After treatment with *P. sinuata* larvae, Ascomycota became the most abundant phylum across all time points (Table S3). On the class level, Neocallimastigomycetes accounted for 60 % of the reads in the untreated dung (Fig. 1F), followed by the classes Dothideomycetes and Saccharomycetes (27 % and 10 % respectively; Fig. 1F). The most representative orders within these fungal classes were Neocallimastigales, Pleosporales and Saccharomycetales (Table S3). During bioconversion of the cattle dung by *P. sinuata*, both Neocallimastigomycetes and Dothideomycetes were drastically reduced in terms of abundance, accounting for only 0.8 % and 4 % of the reads across time points (days 7 to 28; Fig. 1F); whilst an increase was recorded for the classes Saccharomycetes (65 %), Sordariomycetes and Mortierellomycetes (Fig. 1F; Table S3).

On a genus level (Fig. 2B; Table S3), four genera identified as *Caecomyces* (30 %), *Pyromices* (27 %), *Paraconiothyrium* (10 %) and *Orpinomyces* (8 %) accounted for 75 % of the reads in the fresh cattle dung. Except for *Paraconiothyrium*, which was detected in low abundances of 2–3 % even after two weeks, the relative abundance of these genera dropped to values below 1 %. On the other hand, after the first week of dung treatment by *P. sinuata*, two clusters of genera with stable, but distinct relative abundances were detected. With proportions over 10 % each, *Candida*, *Barnettozyma*, *Kernia*, *Saccharomycopsis* and *Mortierella* made up for a first cluster that in sum accounted for at least 50 % of the reads in each sample processed by *P. sinuata*. The second cluster consisted of *Pichia*, *Debaryomyces* and *Chordomyces*, which appeared in lower abundances of 2–6 % each, but remained stable throughout the treatment.

3.3. Changes in beta-diversity composition and unique amplicon sequence variants during the bioconversion of cattle dung using *Pachnoda sinuata* larvae.

The analysis of bacterial and fungal communities revealed that 33 % and 34 % of the explained variance was attributed to the time factor (Adonis2, $p = 0.001$), clearly separating the fresh cattle dung (day 0) from the *P. sinuata* treated samples that clustered altogether on the positive side of the first axis (days 7 to 28; Fig. 3A, D). Among the environmental variables, pH had the largest effect on the bacterial and fungal ordinations considering Aitchison distances (bacteria: $R^2 = 0.140$, fungi: $R^2 = 0.147$; $p = 0.001$).

These time-dependent alterations in the microbial composition were not primarily induced by changes in the relative abundances of individual microbial units in the cattle dung, rather by qualitative changes in its microbial community composition (Fig. 3A). A higher number of both bacterial and fungal amplicon sequence variants were established over the time of *P. sinuata* larvae feeding on the cattle dung (Fig. 3B, E). Moreover, the number of amplicon sequence variants detected exclusively in the insect frass compared to those unique in the cattle dung was higher for bacteria (1450/307 amplicon sequence variants) than for fungi (397/164 amplicon sequence variants) (Fig. 3B, E).

The taxonomic composition of bacterial and fungal unique amplicon sequence variants throughout the 28-d entomocomposting trial included members of all major phyla (Fig. 3C, F). Therefore, it was similar to that shown for the overall microbial community composition (Fig. 1C, F). For the bacterial communities, especially members of different families within Proteobacteria, Actinobacteria and Bacteriodota gained in relative abundances (Table S4). Furthermore, members of the family Peptostreptococcaceae (Firmicutes) were frequent (Table S4). For fungi, two unannotated amplicon sequence variants were particularly

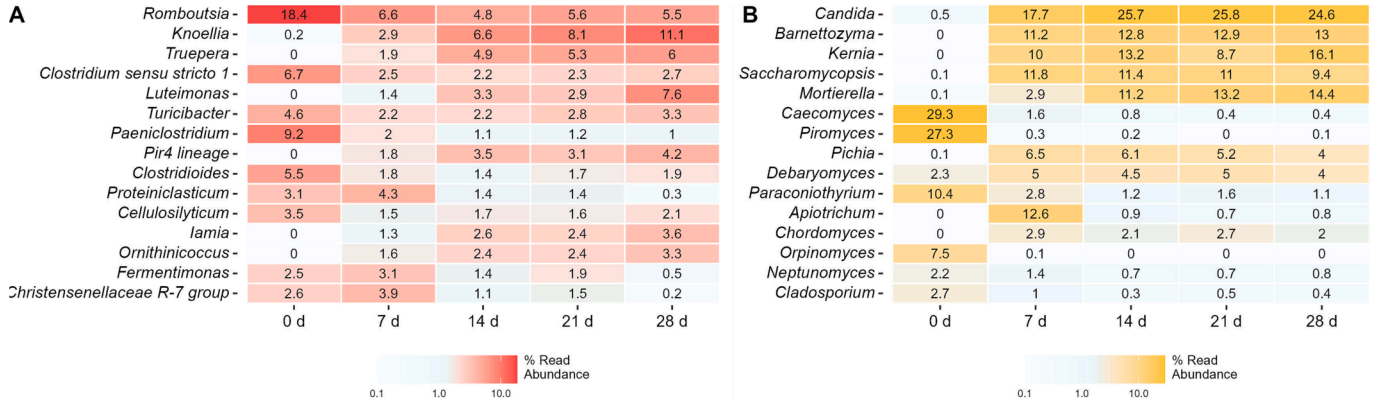

Fig. 2. Heatmap highlighting the genus-level differences in the relative abundances of bacteria (A) and fungi (B) between cattle dung and the resultant frass fertilizer after bioconversion using *Pachnoda sinuata* larvae.

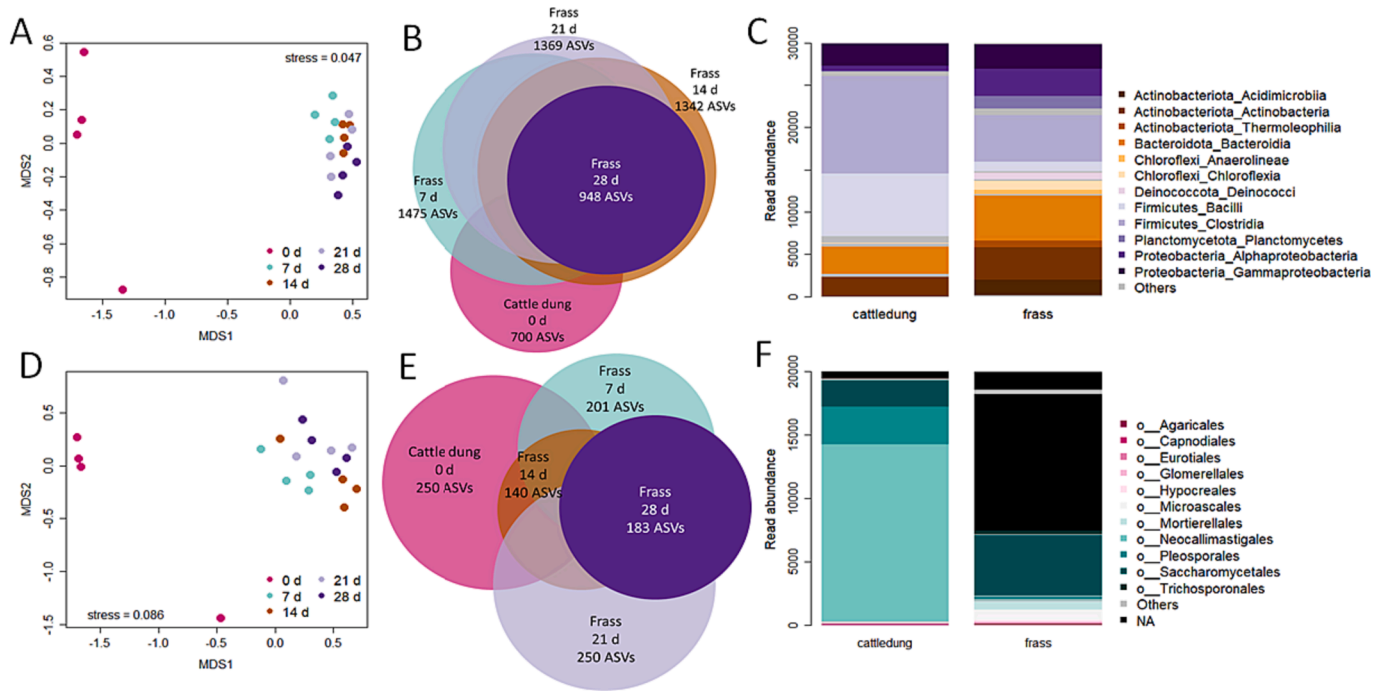

Fig. 3. Bioconversion of cattle dung into insect frass caused a qualitative shift in the microbiome, with a greater impact on bacteria than fungi. (A, C) Distances of bacterial and fungal community compositions between the cattle dung and the frass samples illustrated by non-metric multidimensional scaling on clr-transformed data; (B, E) Venn diagrams illustrating the shared and unique bacterial and fungal amplicon sequence variants across sample groups; (C, F) Taxonomic composition of amplicon sequence variants detected in only one sample group.

abundant (Table S5).

3.3. Changes in the resistance genes profiles during the bioconversion process of cattle dung by Pachnoda sinuata larvae

Here, we noticed that treating cattle dung with *P. sinuata* larvae resulted in a two- to three-fold decrease in the copy number of genes conferring resistance to aminoglycoside, diaminopyrimidine, multidrug, sulfonamide and tetracycline antibiotics within a 14 day-timeframe (Fig. 4A; Figure S1). The decrease in ARG copy numbers in the presence of *P. sinuata* larvae, however, did not hold for fluoroquinolone, glycopeptide, peptide, phenicol and heavy metal resistance genes (Fig. 4A); as higher abundances were measured on day 14 when compared to the cattle dung (Fig. 4A). No additional pronounced alterations were registered, except for heavy metal resistance genes whose abundances were much lower after 28 than 14 d (Fig. 4A).

To answer this, network analysis was carried out to establish the relationships of ARGs and MGEs with the bacterial community on a

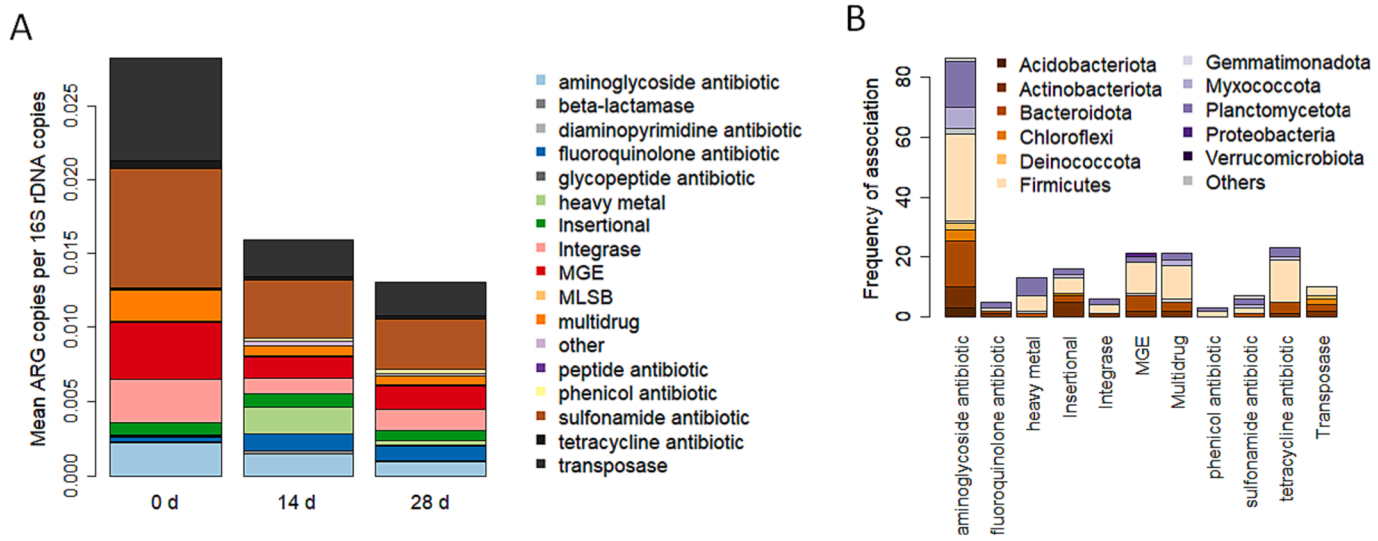

Fig. 4. *Pachnoda sinuata* reduced the abundances of antibiotic resistant genes and mobile genetic elements during bioconversion of cattle dung reducing the risk of horizontal transfer of antibiotic resistant genes between bacteria. (A) Abundance and composition of the detected antibiotic resistant genes and mobile genetic elements in the fresh cattle dung (day 0) and during the bioconversion process in the presence of *P. sinuata* larvae (14 and 28 days). MLSB: macrolide-lincosamide-streptogramin B; MGE: mobile genetic element. (B) Network analysis illustrating the frequency of association of antibiotic resistant genes and mobile genetic elements with the bacterial community on a phylum level.

phylum level (Fig. 4B). Firmicutes appeared to have the highest number of associations regardless of the antibiotic class (Fig. 4B), with frequency values of 29, 14 and 11 with regards to genes that confer resistance to aminoglycosides, tetracyclines and multidrug, respectively (Table S6). Amongst the bacterial phyla, Firmicutes was also found to have the greatest number of associations with the targeted MGE marker genes (Fig. 4B, Table S6). A frequency of association lower or equal than 5 was found between Firmicutes and other types of ARGs (i.e., sulfonamides, fluoroquinolones, phenicols; Fig. 4B, Table S6). On an amplicon sequence variant level and by categorizing them into quartiles (Table S7), we observed that those with higher relative abundances were not associated to different antibiotic classes when compared to amplicon sequence variants with lower abundances.

4. Discussion

4.1. Microbial richness, diversity and community composition during bioconversion of cattle dung by p. Sinuata larvae

The rapid and pronounced changes in the richness and diversity of bacterial communities observed during the study (Figs. 1 – 3) are in line with similar findings reported during the recycling of animal manure using other saprophytic insect species like BSF (Zhang et al., 2021), and the oriental edible beetle *Protaetia brevitarsis* (Du et al., 2022). The minimal changes in bacterial richness and diversity after the second and third week (Fig. 1) might be attributed to exhaustion of nutrients in the cattle dung. As reported for vermicomposting processes (Domínguez et al., 2019), the temporal shifts during entomocomposting are likely influenced by organic carbon levels in the initial feedstock.

The changes in bacterial community composition following larval treatment (Fig. 3) were consistent with previous studies that reported at least 65 % decrease in the percentage of Firmicutes after 12 days while using the BSF larvae to recycle animal manure (Cai et al., 2018). Similar trends have been reported while using other saprophytic insects such as *Musca domestica* (Wang et al., 2017a), *Oryctes nasicornis* (Ziganshina et al., 2018), *Dendroctonus armandi* (Wang et al., 2017b), *Copestylum* larvae (Martínez-Falcon et al., 2011), and red palm weevil (Montagna et al., 2015) to recycle different waste streams.

Here, *Luteimonas* was found to strongly increase in relative abundance during the conversion of cattle dung by *P. sinuata* (Fig. 3). *Luteimonas* spp. were found to process complex organic compounds, leading to an increased nitrogen or phosphorus bioavailability and the promotion of early plant development (Lee et al., 2022). This is an important aspect to bear in mind with regards to the potential use of the insect frass fertilizer for crop production and aligns with previous studies which have reported high levels of macronutrients, secondary nutrients and micronutrients in *P. sinuata* frass fertilizer (Beesigamukama et al., 2022).

The minimal changes in fungal richness and diversity (Fig. 1D – E) could be due to lower rates of turnover and nutrient use strategy associated with fungi (Domínguez et al., 2019). Our findings are in line with Ziganshina et al. (2018) who reported that Ascomycota to be the most prevalent phylum in the gut samples retrieved from larvae of different beetle species with distinct food preferences. Accordingly, Varotto-Bocazzi et al. (2017) found that *Saccharomyces*, *Candida*, *Cryptococcus* and *Pichia* are members in the intestine mycobiota of BSF larvae. Here we found several yeast genera, namely *Candida, Barnettozyma* and *Saccharomycopsis,* increasing in their relative abundance during the conversion of cattle dung (Fig. 3). Yeasts are capable of producing active antimicrobial compounds, which have active role in the biocontrol of plant pathogens. This is in line with previous studies which have reported the benefits of insect frass in suppressing plant pathogens (Lagat et al., 2021) and diseases (Kemboi et al., 2022Poveda et al., 2019).

As many amplicon sequence variants could not be taxonomically identified in the present study, it would be very interesting to conduct isolation experiments from these samples in order to shed light onto these taxa dominating the fungal frass communities. The continuous treatment of manure through larval digestion and fecal inoculation may help to rapidly establish and maintain a stable resident microbiota in the *P. sinuata* frass samples regardless of the time of processing. However, no conclusions can be made about the longevity of this community once larval activity has ceased and the frass is applied as soil amendment. Future studies will be necessary to investigate the impact of frass application on soil microbial survival, abundance and diversity.

4.2. Abundances of ARGs and MGEs in p. Sinuata frass fertilizer

The accumulation of antibiotic resistant genes in organic fertilizers is a major threat to food safety, and the health of humans and the environment. Mobile genetic elements have been reported to play a critical role in horizontal transfer of antibiotic resistant genes between microbes (Haudiquet et al., 2022). The reduced abundance of mobile genetic element marker genes achieved after 14 days (Fig. 4) provides critical evidence about the potential of insect larvae in alleviating the spread risk of antibiotic resistant genes during waste bioconversion into frass fertilizer. Our findings are congruent with earlier works showing an attenuation of manure-borne ARGs by insect larvae (Du et al., 2022). This is a further step towards the use of saprophytic insects in bioremediation.

There are several mechanisms of resistome suppression associated with insect-based bioconversion of organic waste. A reduced relative abundance of certain bacterial taxa might be responsible for the decrease of antibiotic resistant genes and mobile genetic elements achieved during the study. For example, Firmicutes, which decreased in relative abundance over time in our study (Fig. 1C), was associated with multiple resistances, including aminoglycosides, tetracyclines, and especially multidrug resistance (Fig. 4). This is in line with previous studies that recognized Firmicutes as a common host harboring ARGs (Jiang et al., 2017). During the bioconversion process, manure-associated bacteria are exposed to the insect larval gut environment characterized by reduced oxygen levels, an active digestive enzyme system (Cai et al., 2018) and the presence of microbiota involved in detoxification and host-pathogen resistance (Jing et al., 2020). Additional mechanisms rely on the release of antimicrobial peptides or microbial antagonists, and digestive enzymes by the insect larvae (Mudalungu et al., 2021).

During waste conversion, high levels of heavy metals in the feedstock can apply a selective pressure on antibiotic resistant gene transmission via co-regulation, cross-resistance, and co-resistance processes (Zhou et al., 2022). It should be noted that only few studies have investigated the occurrence of antibiotic resistant genes and mobile genetic elements in such by-products (BSF: Cifuentes et al., 2020; Milanović et al., 2021; *T. molitor*: Osimani et al., 2018). Therefore, our knowledge on the content, spread and danger of antibiotic resistant genes and mobile genetic elements in frass is limited. While our study shows evidence for the reduction of potential pathogens and antibiotic resistant genes and mobile genetic elements carrying microbes, the spread and potential danger of remaining pathogens, antibiotic resistant genes and mobile genetic elements remains unanswered. This is a general lack of knowledge, which requires further attention with regards to frequency and mechanism. This is acknowledged and the EU has decided to increase safety measures. Now, the EU Regulation 142/2011 involves the use of heat treatment of insect frass at 70 °C for a minimum of 60 min to reduce the risk of ARG transmission for products entering the EU market.

4.3. Circular economy benefits of p. Sinuata frass fertilizer

The bioconversion time of 28 days achieved using *P. sinuata* has been previously reported, and highlights the critical role of insects in shortening period required to recycle organic wastes, compared to conventional composting which requires 3 – 6 months (Beesigamukama et al., 2022). The short composting time associated with insect bio-converters

implies less labor costs, reduced greenhouse gas emissions (Ermolaev et al., 2019) and lower global warming potential (Mertenat et al., 2019). However, future studies will be necessary to quantify the impacts of *P. sinuata* on greenhouse gas emissions during bioconversion of cattle dung.

Previous studies have demonstrated the benefits of insect frass fertilizer over compost (Anyega et al., 2021), biochar (Beesigamukama et al., 2020b) and synthetic fertilizers (Tanga et al., 2021b) in terms of higher nutrient levels and they supply both potential and ability to enhance soil fertility, crop yield and nutritional quality. The high nutrient levels in frass fertilizer and short bioconversion time could be largely attributed to effective microbiota, digestive enzymes, and high nutrient conservation associated with insects involved in entomocomposting (Beesigamukama et al., 2022). The increase in abundance and diversity of bacteria observed during the study provides evidence about the potential of entomocomposting in boosting the microbial regime of organic fertilizer, which is beneficial for improving biological soil fertility and rejuvenating degraded soils (Stewart et al., 2020). Due to higher nutrient concentrations of insect frass fertilizer, lower application rates are required to cause significant impacts in crop yield, saving farmers the burden of purchasing costly commercial fertilizers and increasing farm productivity (Beesigamukama et al., 2021c). Previous studies have shown that insect frass fertilizer has higher nitrogen fertilizer equivalence values and is cheaper compared to biochar, commercial organic and inorganic fertilizers (Beesigamukama et al., 2020a). Therefore, the availability of insect frass fertilizers could provide a local solution to the challenge of costly and imported fertilizers that has been exacerbated by the Russia-Ukraine war (WFP, 2022).

It is anticipated that the income and non-monetary benefits obtained from the recycling of cattle dung into high-value commercial products such as fertilizer will create an incentive towards the sustainable management of manure and other agro-industrial waste. Through circular economy, saprophytic insects are also excellent converters of low-value organic waste into high-quality insect-based animal feeds (Oonincx et al., 2015; Shumo et al., 2019), that have been found to improve the production of poultry (Sumbule et al., 2021), fish (St-Hilaire et al., 2007), and piggery (Chia et al., 2021). However, increasing the adoption and industrial application of saprophytic insects in organic waste management requires information on the profitability, environmental and socio-economic benefits of this technology in comparison with existing technologies. This strategy has been found effective in increasing the economic attractiveness of bio-based technologies such as biochar (Maroušek et al., 2023).

5. Conclusions

This study has demonstrated the high-potential of the garden flower chaffer (*P. sinuata*) to efficiently recycle animal waste into high-value frass (organic) fertilizer. Recycling recalcitrant organic waste through entomocomposting could be considered as a universal and economically profitable way of producing organic fertilizers, which are not only the source of soil nutrients, but also antagonistic against phytopathogens. The short bio-waste conversion time of 4 weeks achieved using *P. sinuata* larvae indicate that this technology is a crucial nature-based solution to overcome challenges of longer production time (12 – 24 weeks) associated with conventional composting methods. The ability of *P. sinuata* to reduce manure-borne antibiotic resistant genes and mobile genetic elements and increase beneficial bacteria in frass (organic) fertilizer confirms their potential to contribute to a circular and green economy and bioremediation of contaminated soils. The frass fertilizer obtained could play a critical role in rejuvenating degraded soils leading to innovative industries, opening new market opportunities for bio-based products and achieving efficient resource utilisation by the public–private sector partners through upscaling and commercialization of *P. sinuata* farming. However, potential social, economic and political barriers that might be encountered in the future must be overcome. Future research in terms of agronomic performance of the frass fertilizer generated by *P. sinuata* and its impact on greenhouse gas emissions, soil fertility and suppression of soil-borne pathogens would be necessary to propel this technology forward. It is clear that animal waste valorisation using *P. sinuata* would attract much attention from fundamental and applied fields, given its wide availability and versatility.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.wasman.2023.11.019.

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