

Microbial community dynamics during decomposition of insect exuviae and frass in soil

Azkiya Nurfikari^{a,b}, Márcio Fernandes Alves Leite^{a,c}, Eiko Eurya Kuramae^{a,c}, Wietse de Boer^{a,b,*}

^a Department of Microbial Ecology, Netherlands Institute of Ecology (NIOO-KNAW), 6708, PB, Wageningen, the Netherlands

^b Soil Biology Group, Wageningen University & Research, PO Box 47, 6700, AA, Wageningen, the Netherlands

^c Department of Biology, Ecology and Biodiversity, Institute of Environmental Biology, Utrecht University, 3584, CH, Utrecht, the Netherlands

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ABSTRACT

With the expected growth of insect mass rearing industry, residual streams are set to become increasingly available. However, before these residues can be used as organic amendments, more knowledge of the decomposition dynamics and the associated microbial communities is needed. This study aimed to investigate the decomposition, N-mineralization, and fungal/bacterial community composition over a 16-week incubation period of exuviae and frass from BSF, mealworm, and house cricket in pots containing arable soil. The decomposition of insect residues in litterbags was rapid, with over 50% weight loss observed within two weeks. The release of mineral nitrogen from dispersed insect materials was highest during the initial two weeks, particularly evident in soils treated with exuviae compared to their frass counterparts. Moreover, soil amendment with insect residues enriched chitinolytic soil microbial inhabitants belonging to Gammaproteobacteria, Bacilli, Actinobacteria, and Mortierellomycetes. A comparison of soil amendments with sterilized and non-sterilized mealworm exuviae indicated minimal influence of microbial propagules on the composition of bacterial decomposers, though a notable impact on the fungal community was observed. These findings suggest that amendments with insect residues show promise in enhancing natural biocontrol by stimulating microbial antagonists of plant-pathogenic fungi, thereby presenting a potential tool for integration into soil-borne disease management strategies.

1. Introduction

With the growing world population and increasing public awareness of the environmentally-harmful side effects of agricultural intensification, there is a need to produce proteins in a sustainable manner. In this respect, commercial production of insects has recently received considerable attention. Insects have the ability to convert organic by-products into high-value commodities such as proteins and biofuels (van Huis, 2021). Several major advantages of insect-based bioconversion over conventional livestock systems have been well-documented, including higher feed conversion efficiency, reduced greenhouse gas emissions, and lower water and land use (Tabassum et al., 2016; van Huis and Oonincx, 2017). As detritivores and herbivores, insects thrive on various agro-industrial by-products as food sources, making their mass rearing a potential solution for organic residue management (Fowles and Nansen, 2020; Ojha et al., 2020).

Mass rearing of insects not only yields insect biomass but also generates residual streams, mainly consisting of excrements (frass) and, to a lesser extent, molting skins (exuviae). These residual streams are considered promising organic materials for agricultural purposes (e.g., as biofertilizers, soil improvers, and organic amendments) (IPIFF, 2019; Poveda, 2021; Barragán-Fonseca et al., 2022). An essential component of insect exuviae is chitin, a biopolymer also found in crustacean exoskeletons and the cell walls of fungi (Gooday, 1990). Chitin-containing soil amendments have been shown to enrich chitinolytic bacteria from several taxa (e.g., Actinobacteria, Bacilli, Bacteroidetes, and Gammaproteobacteria) and fungi (mainly Mortierellomycetes) (Cretoiu et al., 2013; Jacquioud et al., 2013; Debode et al., 2016; Wieczorek et al., 2019). In several greenhouse and field studies, the stimulation of these microbes coincided with the decreased occurrence of diseases caused by root-infecting fungal pathogens (Cretoiu et al., 2013; Randall et al., 2020; Andreo-Jimenez et al., 2021). Microbial chitinases can

* Corresponding author. Department of Microbial Ecology, Netherlands Institute of Ecology (NIOO-KNAW), 6708, PB, Wageningen, the Netherlands.

E-mail addresses: A.Nurfi@nioo.knaw.nl (A. Nurfikari), M.Leite@nioo.knaw.nl (M.F.A. Leite), E.Kuramae@nioo.knaw.nl (E.E. Kuramae), W.deBoer@nioo.knaw.nl (W. de Boer).

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disintegrate the chitin-containing cell wall of fungi (Beyer and Diekmann, 1985). However, other lytic enzymes and antibiotics also appear to weaken the vital structures of fungal pathogens (de Boer et al., 1998). In addition to anti-fungal effects, chitin degradation in the soil may release short-chain chitin oligomers, which can promote plant growth (Winkler et al., 2017; de Tender et al., 2019). Most studies exploring the possibility of chitin amendments have used waste from shellfish processing industries (Sharp, 2013). To date, there is limited information on soil amendment with insect exuviae for this purpose.

In nature, chitin rarely occurs in a pure form (Tsurkan et al., 2021). Instead, it is often intricately bound with other compounds, the composition of which varies depending on its role in terrestrial or aquatic environments (Tamone and Harrison, 2015). For instance, in insect exuviae, chitin forms a part of a complex with proteins and melanin, shielded by cuticular lipids to prevent desiccation (Merzen-dorfer, 2011). On the other hand, crustacean shells integrate chitin with proteins and a substantial amount of calcium carbonate to strengthen mechanical strength (Tamone and Harrison, 2015; Bentov et al., 2016). In fungal cell walls, the major source of chitin in the terrestrial ecosystem, chitin tightly associates with glucan, providing protection against adverse environmental factors (Nwe et al., 2010). Given these structural differences among chitin-containing materials, we hypothesize that applying chitinous amendments from different origins may incite different responses from resident chitinolytic soil microorganisms. Such variations can potentially influence the efficacy of these amendments in suppressing pathogens. Therefore, understanding the shifts in soil microbial communities following the application of novel organic soil amendments is essential before their implementation in agricultural settings. Next to the knowledge of the stimulation of beneficial microbes, it is also imperative to mitigate the potential risk of pathogen proliferation (Bonanomi et al., 2018).

In insect farming, frass refers to a mixture of excrements, shed exoskeletons, and undigested feed (Fowles and Nansen, 2020). Frass contains plant-available nutrients, mainly N, P and K (Lovett and Ruesink, 1995; Beesigamukama et al., 2021; Watson et al., 2021b). In nature, frass plays a vital role in nutrient cycling. Its decomposition results in a short-term pulse of plant-accessible nutrients, accelerating the decomposition of recalcitrant organic matter (Lovett and Ruesink, 1995; Zimmer and Topp, 2002; Frost and Hunter, 2008). The fertilizer potential of frass derived from insect mass rearing has recently been assessed for black soldier fly (Beesigamukama et al., 2020; Gärtling et al., 2020; Tan et al., 2021; Gebremikael et al., 2022) and mealworm (Poveda et al., 2019; Houben et al., 2020; Watson et al., 2021a). While plant-accessible nutrients primarily drive the positive effects of frass, frass-associated microorganisms are also likely to play a role (Poveda et al., 2019). Members of Gammaproteobacteria, Bacilli, and Ascomycota are the most frequent microorganisms found in frass (Boiocchi et al., 2017; Poveda et al., 2019). Despite previous studies on the fertilizer properties and microbial composition of frass, little attention has been paid to the compositional dynamics of frass and exuviae degraders in soil.

The aim of the present study is threefold: (1) to investigate the decomposition of various insect residues and the associated nitrogen dynamics in arable soil, (2) to examine the composition and dynamics of bacterial and fungal decomposer communities involved in this process, and (3) to assess the impact of microbes present in insect residues on the assembly of microbial decomposers. The materials studied were exuviae and frass generated during the mass rearing of three insects: black soldier fly (*Hermetia illucens*), mealworm (*Tenebrio molitor*), and house cricket (*Acheta domesticus*). Our previous study showed that the exuviae of these insects contained approximately 8–11% chitin (Nurfikari and de Boer, 2021). Therefore, milled mushrooms (*Agaricus bisporus*) were included as an additional treatment for comparison with insect residue treatments, owing to their similar chitin content (6–9%) (Hassainia et al., 2018).

Table 1
Detailed information about soil used in the experiment.

Soil parameters	Unit	Results
Total nitrogen (N)	kg/ha	4760
C/N ratio		12
Available nitrogen	kg/ha	80
Total sulfur (S)	kg/ha	680
C/S ratio		86
Available sulfur	kg/ha	8
Total phosphorus (P)	kg/ha	740
Total potassium (K)	kg/ha	335
Total calcium (Ca)	kg/ha	1705
Total magnesium (Mg)	kg/ha	300
Total sodium (Na)	kg/ha	30
pH		6
Water holding capacity	%	29
Organic carbon (SOC)	%	1.7
Organic matter (SOM)	%	3.2
Carbonated lime	%	<0.2
Clay	%	2
Silt	%	14
Sand	%	81

Table 2
Materials used for soil amendment and litterbag experiments and their carbon and nitrogen contents.

Material	C%	N%	C:N	Description
Mealworm exuviae	46.0 ± 0.3	9.7 ± 0.2	4.8	Exuviae of <i>Tenebrio molitor</i> larvae (Nijenkamp Voedseldieren, Hellendoorn, NL)
Mealworm frass	39.3 ± 0.3	3.1 ± 0.1	13.1	Frass of <i>Tenebrio molitor</i> larvae (Nijenkamp Voedseldieren, Hellendoorn, NL)
BSF larval exuviae	41.1 ± 0.1	4.3 ± 0.1	9.4	Exuviae of <i>Hermetia illucens</i> larvae (Bestico, Berkel en Rodenrijs, NL)
BSF frass	38.0 ± 0.4	2.9 ± 0.1	13.3	Frass of <i>Hermetia illucens</i> larvae (Bestico, Berkel en Rodenrijs, NL)
House cricket exuviae	44.4 ± 0.1	8.0 ± 0.2	5.6	Exuviae of <i>Acheta domesticus</i> at various instar stages (Fair Insects, Dongen, NL)
House cricket frass	38.0 ± 0.1	3.2 ± 0.1	12.0	Frass of <i>Acheta domesticus</i> at various instar stages (Fair Insects, Dongen, NL)
Mushroom	38.7 ± 0.2	4.7 ± 0.1	8.3	Fruiting bodies of <i>Agaricus bisporus</i> (local supermarket)

2. Materials and methods

2.1. Soil collection and soil characteristics

Soil samples were collected in September 2018 at the Unifarm experimental field, Wageningen, The Netherlands (51.9865° N, 5.6634° E) from the 0–10 cm layer. The soil (disturbed Hortico Podzol) is sandy (texture composition: 81% sand, 14% silt, and 2% clay) with a moderate organic matter level (~4%) and a pH (H₂O) of 6.0. The sampling area had been planted with black mustard (*Brassica nigra*) the previous summer, and residual plant matter was still evident. After collection, the soil was sieved (4 mm) to remove coarse materials, homogenized and stored at 4 °C until further use. Soil characteristics were assessed by Eurofins Agro (Wageningen, The Netherlands), a soil and plant analysis laboratory, using their standard methodology (Table 1).

2.1.1. Raw materials and pre-processing

Exuviae and frass were obtained from the following insect species: yellow mealworm (*Tenebrio molitor*), black soldier fly (*Hermetia illucens*), and house cricket (*Acheta domesticus*) (Table 2). The samples were

kindly supplied by Nijenkamp Voedseldieren (Hellendoorn, NL), Bestico (Berkel en Rodenrijs, NL), and Fair Insects (Dongen, NL), where the insects were reared on a variety of agro-industrial waste materials. Mushroom (*Agaricus bisporus*) was obtained from a local supermarket. Before use, all raw materials were air-dried with forced convection at 60 °C for 24 h (Binder Model FED-260, Binder GmbH, Tuttlingen, Germany), milled (cutting mill SM 100, Retsch B.V., Haan, Germany), sieved (2-mm mesh size), and stored at room temperature. The total carbon and total nitrogen contents of each material were determined with Thermo Flash EA 1112 (Thermo Fisher Scientific, Waltham, Massachusetts, United States) (Table 2). Exuviae and frass of each insect species, as well as mushroom powder, were tested separately to examine their individual decomposition and associated microbial decomposers.

2.1.2. Experimental design

2.1.2.1. Soil amendment experiment. For each treatment, the amended soil was placed in twenty replicate plastic pots (10 cm × 10 cm × 8 cm). These twenty pots per treatment comprised five sets of four replicates, with each set being destructively sampled at distinct time points (1, 2, 4, 8, and 16 weeks) after the start of the experiment. Control treatments consisted of soil incubations without additions of organic amendment. Before being transferred into pots, each material was mixed with soil (320 g dry weight equivalent) at a rate of 1% (10 g kg⁻¹ dry weight soil). The amendment rate was selected based on the rates used in previous chitin amendment studies, such as 1% (crustacean shell chitin, w/w) (Hallmann et al., 1999), 1.8% (shrimp waste chitin, w/w) (Cretoiu et al., 2013; Kielak et al., 2013), and 2% (crab shell chitin, w/w) (Debode et al., 2016), ensuring comparability with prior research. Soil moisture was adjusted to 60% water holding capacity by adding sterile water. In total, 160 pots were prepared for this experiment ((6 types of insect materials (mealworm exuviae, mealworm frass, BSF exuviae, BSF frass, cricket exuviae, cricket frass), mushroom powder, and unamended control) × 5 incubation times × 4 replicates). The pots were randomly distributed and incubated in a dark climate chamber at 20 °C and relative air humidity of 85% (Micro Clima-Series, Snijders Labs, Tilburg, NL). They were sprayed with water three times a week to maintain initial moisture levels based on measurements of pot weight. Pot trays were randomly repositioned twice weekly to minimize the potential impact of uneven air circulation inside the climate chamber.

At each sampling time, soil subsamples from each treatment were destructively collected. This was done by removing the top 3 cm layer of soil, which experienced the largest moisture fluctuation during incubation, and mixing the rest of the pot contents before soil subsamples were collected. Part of the collected soil samples was stored at -20 °C for further DNA extraction. To measure ergosterol (a fungal biomass indicator), sampled soil was stored in 10% KOH in methanol at -20 °C for two months until extraction (de Ridder-Duine et al., 2006). For the measurement of pH and mineral nitrogen (ammonium-N and nitrate-N), part of the sampled soil was transferred into glass jars, air-dried at 40 °C for five days, and stored at room temperature for up to four months until extraction.

2.1.2.2. Litterbag decomposition experiment. In addition to the soil amendment experiment described above, we conducted a litterbag experiment to assess decomposition based on weight loss. This experiment mirrored the setup of the soil amendment experiment and used the same insect and mushroom materials. Litterbags (9 × 9 cm) were crafted from nylon filters with a 30-μm mesh size (Sefar, Switzerland). This mesh size was selected to prevent the loss of milled materials into the soil. Each material was precisely weighed (3.0 g) and enclosed in sterile nylon litterbags. Soil moisture was adjusted to 60% water holding capacity by adding sterile water. Each litterbag was horizontally laid in individual pots (10 cm × 10 cm × 8 cm) containing 200 g of moist soil (at a depth of 4 cm) and buried by adding 150 g of moist soil on top. In total,

140 pots were prepared (7 types of material × 5 incubation times × 4 replications). The incubation conditions were identical to those used in the soil amendment experiment. The litterbags were carefully retrieved and weighed during each sampling time (1, 2, 4, 8, and 16 weeks). The remaining mass was freeze-dried for 48 h (Labconco Freezone 12, Kansas City, United States), and the weight was recorded for a second time to determine the water content. Mass loss for each time point was expressed as a percentage of the initial mass.

2.1.2.3. Gamma-sterilization experiment. A follow-up soil amendment experiment was conducted with mealworm exuviae to examine the impact of existing microbes in insect residues on the assembly of microbial decomposers. The experiment followed a randomized design with three incubation periods (2, 4, and 8 weeks) and four replicates for each combination of amendment type and incubation period. Soil samples for this experiment were collected in March 2020 from the same site described in the “2.1 Soil collection and soil characteristics” section. The materials included in this additional experiment were: (1) mealworm exuviae (as described in Table 2) and (2) mealworm exuviae which had been sterilized by gamma irradiation gamma-radiation (at a minimum of 25 kGy, Syngenta BV, Ede, the Netherlands). In this experiment, our focus was solely on mealworm exuviae to investigate the effect of sterilizing insect residues on the development of microbial decomposers, rather than comparing different insect materials. Soils without amendments were prepared as a control. The treatments were prepared and incubated following the same procedures as described in the soil amendment experiment. Each material was mixed with soil (320 g dry weight equivalent) at a rate of 1% (10 g kg⁻¹ dry weight soil) and transferred into pots. After 2, 4, and 8 weeks of incubation, soil subsamples were collected destructively for bacterial and fungal community analysis. Fresh soil samples were stored at -20 °C for further DNA extraction.

2.1.3. Fungal biomass

Fungal biomass was assessed by measuring the ergosterol concentration in samples obtained from the soil amendment experiment, following the method described by de Ridder-Duine et al. (2006). Soils amended with mushroom powder were omitted from this analysis because the method could not differentiate ergosterol derived from soil fungi from that originating from the mushroom powder added as an amendment. In brief, fresh soil samples (1 g) stored in 4 ml 10% KOH in methanol were subjected to vortexing (10 s) and sonication (47 kHz, 15 min) to release ergosterol from fungal membranes. Alkaline hydrolysis was conducted by adding 1 ml of sterile distilled water and 2 ml of *n*-hexane. The resulting solution was vortexed (30 s) and then centrifuged (10 min at 11,000 rev·min⁻¹), with the top phase containing ergosterol collected. The remaining solution underwent another extraction with *n*-hexane, and the combined hexane fractions were evaporated overnight in a water bath at 50 °C. After evaporation of the *n*-hexane, the precipitated ergosterol was dissolved in methanol, filtered through a 0.2 μm PTFE syringe filter, and quantified using LC-MS/MS (UHPLC 1290 Infinity II, Agilent Technologies and 6460 Triple Quad LC-MS, Santa Clara, California, United States).

2.1.4. Mineral-N and pH

The concentration of soil nitrate-N and ammonium-N, which serves as a proxy for N mineralization, was measured by performing KCl extraction of soil obtained from the amendment experiment. Initially, air-dried soil (10 g) was suspended in 25 ml of demi water and placed in a shaker (250 rev·min⁻¹) with a linear movement for 2 h. Following pH measurement, 25 ml of 2M KCl was added, and the suspensions were placed in a shaker (250 rev·min⁻¹) for another 2 h. After allowing the suspensions to settle for 15 min, the aqueous phase was transferred into Eppendorf tubes and centrifuged at 10,000×g for 10 min. The clear supernatant was collected for the measurement of nitrate-N and

ammonium-N using an AutoAnalyzer (QuAatro SFA system, Beun-de Ronde B.V., Abcoude, the Netherlands).

2.1.5. DNA extraction and qPCR analysis

Changes in bacterial and fungal community structure associated with insect residual stream degradation were analyzed by combining quantitative PCR and Illumina MiSeq sequencing approaches. Total genomic DNA was extracted from 0.25 g of soil samples collected in the soil amendment experiment using the PowerSoil Pro DNA Isolation kit (MoBio Laboratories, Carlsbad, California, USA) according to the manufacturer's protocol. Total DNA concentration and quality were determined using NanoDrop® spectrophotometer and Qubit® fluorometer.

The total DNAs were diluted 100-fold and used to quantify bacteria using the Bio-Rad qPCR system (Bio-Rad Laboratories, Hercules, California, USA). Primer sets used were Eub338 (5' ACTCCTACGGGAGG-CAGCAG 3') and Eub518 (5'ATTACCGGGCTGCTGG 3') for the bacterial 16S rRNA gene (Fierer et al., 2005). Standard curves were generated using 10-fold dilution series of a plasmid containing a full-length copy of the 16S rRNA gene of *Collimonas fungivorans* using QIAgility™ robot instrument (Qiagen, Hilden, Germany). Each 15 µl-reaction mixture contained 7.5 µl of iTaq Universal SYBR Green Supermix (2x) (Bio-Rad Laboratories, Hercules, California, USA), 0.5 µl of forward primer, 0.5 µl of reverse primer, 2.5 µl of PCR-grade water, and 4 µl of DNA template. Negative controls were included with each reaction using PCR-grade water. PCRs were run with the following cycling program: 3 min at 95 °C; 40 cycles of 95 °C for 30 s, 53 °C for 30 s, and 72 °C for 45 s; and 72 °C for 5 min. Bacterial gene copy numbers were quantified using a regression equation based on the cycle threshold (*C_t*) value to the known number of copies in the standards.

In light of the importance of the chitinase-possessing group during the decomposition of insect residue, qPCR amplifications targeting the group A bacterial chitinase *chiA* gene were performed. qPCR runs were performed using the f primer set GA1F (5' CGTCGACATCGACTGGGARTDBCC' 3) and GA1R (5'ACGCCGGTCCAGCCNCKNCCRTA 3') (Williamson et al., 2000). Standard curves were generated using a 10-fold dilution series of a plasmid containing a full-length copy of family 18 *chiA* gene of *Streptomyces coelicolor*. PCRs cycling program consisted of 5 min at 95 °C; 40 cycles of 95 °C for 30 s, 63 °C for 30 s, and 72 °C for 60 s; and 72 °C for 5 min. The reaction efficiencies ranged from 80% to 101%, with *R*² values between 0.90 and 0.99.

2.1.6. 16S and ITS rRNA gene amplicon sequencing

Temporal shifts in bacterial and fungal communities during the decomposition of insect residue amendments were examined using the same genomic DNA extracts used for the qPCR analyses. DNA extracts were subjected to 16S rRNA gene and ITS region amplicon sequencing using the Illumina MiSeq platform (Illumina MiSeq PE250) (Genome Quebec Innovation Centre, Montreal, Quebec, Canada). We used the primer set 515F/806R targeting the V4 hypervariable region of the bacterial 16S rRNA gene (Caporaso et al., 2012) and the primer set fITS9/ITS4R flanking the ITS2 region (Ihrmark et al., 2012) for fungi. The 16S rRNA and ITS amplicon sequences were processed using DADA2 pipeline (v1.21) (Callahan et al., 2016) in R environment (Team, 2021). Raw sequencing reads were quality-filtered. Forward and reverse PCR primers were removed using the *truncLen* function and the *cutadapt* plugin (v3.5) (Martin, 2011) for bacterial and fungal sequences, respectively. Paired-end reads were merged, and chimeric sequences were discarded. Taxonomy was assigned using SILVA version 138.1 (McLaren, 2020) and UNITE (v2019) for bacterial and fungal data, respectively. An amplicon sequence variants (ASV) table was constructed with taxonomic information for each ASV. Singletons, doubletons and reads belonging to taxa outside bacterial or fungal origin (e.g., chloroplasts, mitochondria, and protists) were discarded. The raw sequences were deposited at European Nucleotide Archive under the accession number PRJEB54065.

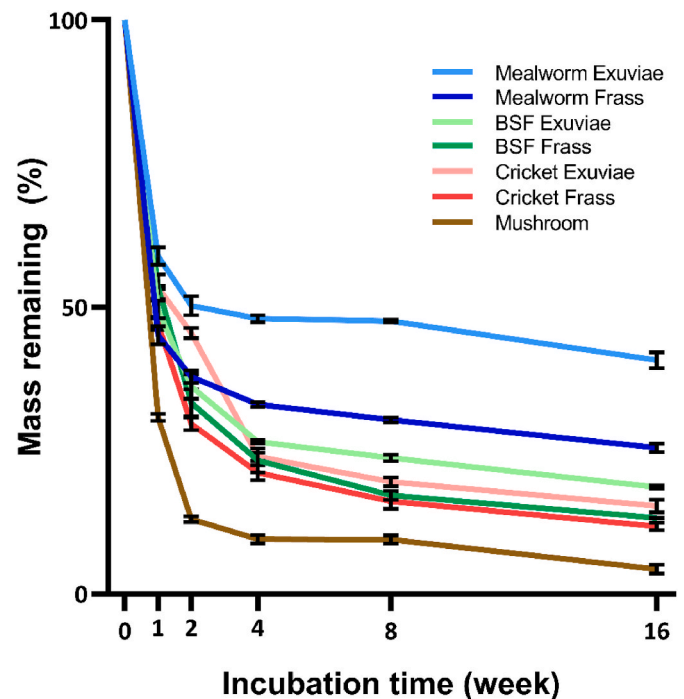


Fig. 1. Mass loss of insect and mushroom materials enclosed in litterbags during a 16-week incubation period in sandy arable soil (mean \pm SE, *n* = 4).

2.1.7. Data analysis

Data processing and statistical analysis were conducted using SPSS version 29 (SPSS Inc., Chicago, IL) and R version 4.1.2 (Team, 2021) in RStudio (2023.12.1.402). Repeated measures ANOVA (RM_ANOVA) were conducted to ascertain whether the addition of organic amendments had a significant effect on the measurement of mass loss, soil mineral N, and fungal biomass over time. For mass loss and mineral N, variables were log-transformed to achieve a normal distribution of residuals. If a significant interaction between treatment and time was identified by RM_ANOVA, follow-up analysis was conducted with individual ANOVAs for each time interval, following the method outlined by Park et al. (2009). For the analysis of fungal biomass data, the Greenhouse-Geisser correction (Park et al., 2009) was applied, as Mauchly's test indicated a violation of the assumption of sphericity (Nagarsenker and Pillai, 1973). Comparisons of repeated measures data were conducted using Tukey's HSD test, except in cases where the sphericity assumption was not met, in which instances Bonferroni or Tukey's WSD approach was employed (Park et al., 2009). Additionally, the linear relationship between soil pH and mineral nitrogen concentration was assessed using Pearson's correlation, based on 168 independent pairs of observations. Data for mineral nitrogen were log-transformed to meet the normality assumptions for Pearson's correlation.

Sequencing data obtained from DADA2 pipeline were analyzed using the following R packages: *phyloseq* (McMurdie and Holmes, 2013), *ade4* (Dray and Dufour, 2007) and *metamicrobiomeR* (Ho et al., 2019). ASVs were transformed by centered log-ratio (CLR) due to the compositional nature of amplicon sequencing data (Gloor et al., 2017) and analyzed at both genus and class level. Principal Component Analysis (PCA) with duality diagram functions was used to perform Between-Class Analysis (BCA) to explore the dissimilarity between treatments during the different decomposition stages (Escoufier, 1987; Kenkel, 2006; Leite et al., 2021). BCA enables the measurement of the amount of variability the treatment can explain. Monte Carlo tests with 999 permutations were performed to verify the significance of the groups. P-values were adjusted using FDR, as implemented in the *metamicrobiomeR* package. Generalized additive model for location, scale, and shape (GAMLSS)

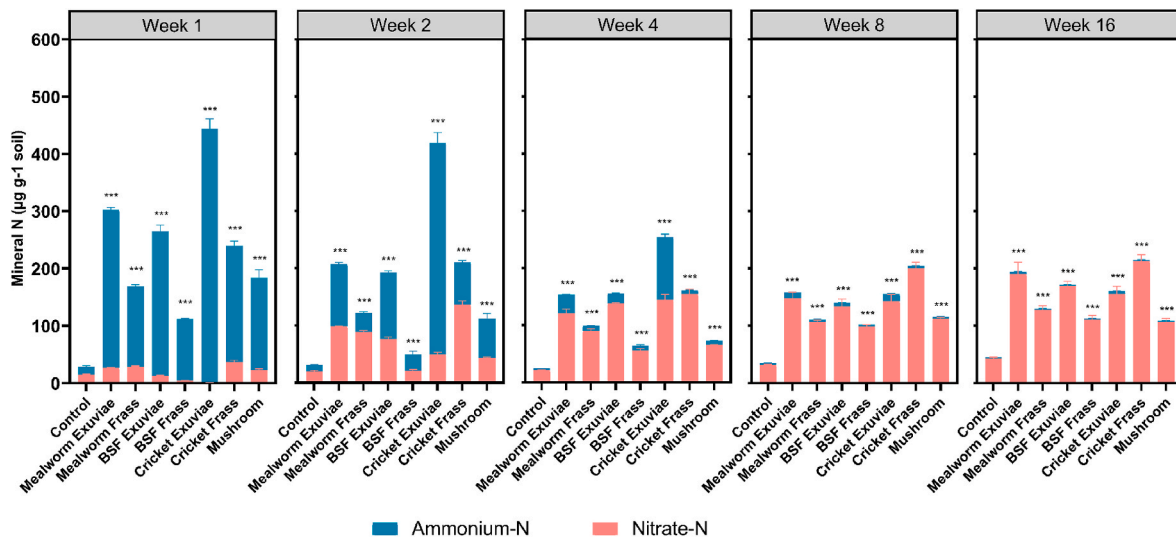


Fig. 2. Concentration of mineral N (ammonium-N and nitrate-N) in unamended soil and soil amended with insect and mushroom materials over a 16-week incubation period (mean \pm SE, $n = 4$). Significant difference between treatments and the respective controls is indicated with asterisks (Tukey's HSD; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, ns: not significant).

combined with a zero-inflated beta (BEZI) family was used to obtain regression coefficients and identify the taxonomic group responsible for the shifts in the soil bacterial and fungal community while taking into account the random effects of different sampling times (Leite et al., 2021). The difference in bacterial and fungal community structure between soils amended with sterile and non-sterile mealworm exuviae was examined by permutational multivariate analysis of variance (PERMANOVA) using Bray-Curtis as a distance matrix. For all statistical analyses, statistical significance was accepted when $P < 0.05$.

3. Results

3.1. Litterbag incubation experiment

The mass loss of litterbag contents was significantly affected by the type of organic material ($F_{6,105} = 266.9$, $p < 0.001$), time ($F_{4,105} = 324.2$, $p < 0.001$) and their interaction ($F_{24,84} = 13.5$, $p < 0.001$) (Fig. 1, Table S1). More than 50% of the initial amount of all insect residues, except mealworm exuviae, had decomposed within the first two weeks. Subsequently, a gradual but consistent reduction in mass was observed over the ensuing weeks. At the end of the incubation period, the remaining mass was constantly lower for frass compared to exuviae (Table S2). Moreover, mushroom powder exhibited a greater mass loss relative to exuviae and frass, with minimal biomass remaining by the end of the incubation period.

3.2. Soil amendment experiment

3.2.1. Mineral N and pH

The soil amendment with insect residues and mushroom powders at a rate of 1% (10 g kg⁻¹ dry weight soil) increased soil mineral N concentration depending on the type of organic material ($F_{6,84} = 393$, $p < 0.001$), time ($F_{4,84} = 299$, $p < 0.001$) and their interaction ($F_{24,84} = 13.5$, $p < 0.001$) (Fig. 2, Table S3). Ammonium-N concentration surged immediately after the first week of incubation across all treatments. Amendments with exuviae from the three different insects led to a higher release of ammonium-N compared to their frass counterpart. The most substantial increase of mineral N was observed in soils treated with house cricket exuviae (443 $\mu\text{g N g}^{-1}$ soil at week 1). Subsequently, ammonium-N concentrations began to decline. Conversely, nitrate-N levels exhibited a contrasting pattern, gradually increasing over time and maintaining elevated concentrations until the end of the

experiment. The highest nitrate-N accumulation was observed in soil treated with cricket frass (212 $\mu\text{g N g}^{-1}$ soil), while the lowest was observed in soils amended with mushroom (106 $\mu\text{g N g}^{-1}$ soil). In all amended soils, total mineral N decreased from week 1 until week 4 (Table S4). By the end of the incubation period, the available mineral N was solely present as nitrate.

While the pH in the unamended control remained relatively stable (5.8–6.0) throughout the incubation period, amended soils experienced a notable increase in pH by 0.5–2 units after one week of incubation (Fig. S1). Among all treatments, soils treated with cricket exuviae showed the most significant pH increase. After the initial first week, pH levels in all treatments declined rapidly and stabilized within the range of 4.5–6.5. A positive linear correlation was observed between soil pH and ammonium-N levels ($r(166) = 0.85$, $p < 0.001$, Fig. S2A). The converse was observed for nitrate-N concentration ($r(166) = -0.7$, $p < 0.001$, Fig. S2B).

3.2.2. Fungal biomass, total bacteria, and chitinase-possessing bacteria

Soil amendment with insect exuviae and frass significantly increased soil fungal biomass, depending on the type of added materials ($F_{6,42} = 102$, $p < 0.001$), incubation time ($F_{2,42} = 102.8$, $p < 0.001$) and their interaction ($F_{12,42} = 11.5$, $p < 0.001$) (Fig. S3; Table S5). Ergosterol levels rose in all amended soils until week 8, with the highest stimulation observed in soil treated with BSF frass and exuviae (1.9 $\mu\text{g g}^{-1}$ dry soil). By week 16, ergosterol concentration had declined in all amended soils, reaching levels not significantly different from the unamended control for BSF frass, cricket exuviae and cricket frass (Table S6).

Bacterial abundances in amended soils, assessed for three incubation times (week 2, 4 and 16), were increased relative to the unamended control (Fig. 3A, Table S7). The highest numbers of bacteria were found at week 4, with values ranging from 2.4 to 3.8 $\times 10^{10}$ (gene copies g⁻¹ dry soil). The initial bacteria-stimulating effect (2 weeks) was more prominent in soils supplied with exuviae than with frass. After 16 weeks of incubation, bacterial numbers had decreased in all amended soils, but the values were still significantly different from the control (TukeyHSD, $p < 0.001$). In the unamended control, the quantities of soil bacteria carrying *chiA* genes were constantly within the range of 3–3.8 $\times 10^8$ (gene copies g⁻¹ dry soil). Significant enrichment of chitinolytic bacteria was seen in all amended soils during the entire time points, which peaked at week 4 (Fig. 3B–Table S8). The highest increase of chitinolytic bacteria was seen in soils supplied with BSF exuviae (4.5 $\times 10^9$ (gene copies g⁻¹ dry soil)). The ratio of *chiA* gene possessing bacteria versus

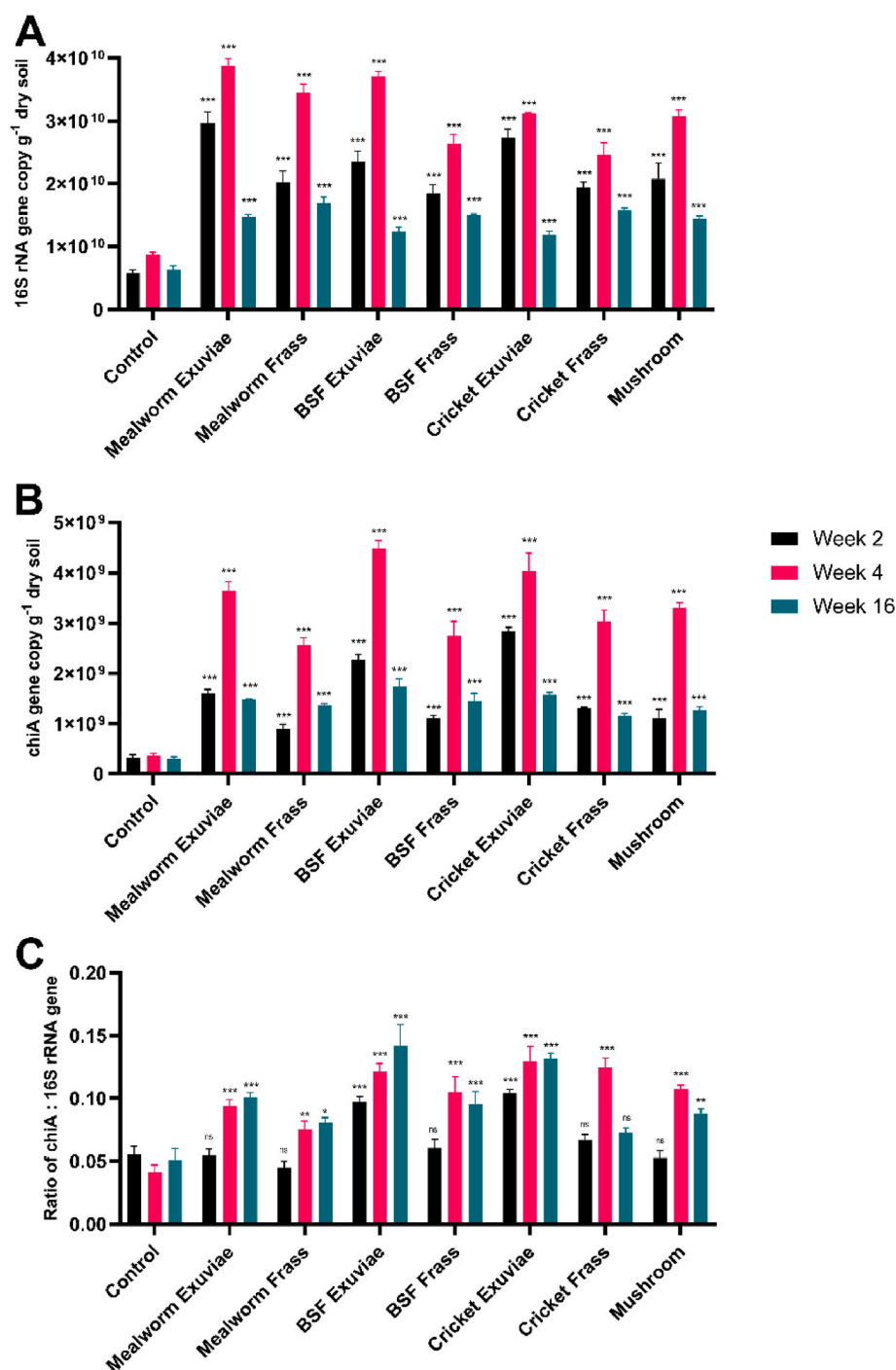


Fig. 3. Total bacteria (16S rRNA gene copies) (A), bacteria possessing *chiA* genes (B), and *chiA* : 16S rRNA gene copy ratio (C) in soils amended with insect residues and mushroom powder and sampled after 2, 4, and 16 weeks (mean \pm SE, $n = 4$). Significant differences between treatments and the respective controls are indicated with asterisks (Tukey's HSD; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, ns: not significant).

total bacteria was significantly increased compared to the control for all insect residue amendments after 4 and 16 weeks of incubation, but not after 2 weeks (Fig. 3C). The growth dynamics of total and chitinolytic bacteria seen for insect residue amendments were also evident for the amendment with mushroom powder.

3.2.3. Bacterial community dynamics

Between-class analysis was performed to identify the proportion of variability that can be attributed to the applied amendment treatment (Table S9). Changes in soil bacterial community structure were significantly explained by the amendment type (27%; $p < 0.001$; Fig. S4A;

Table S9) and incubation time (14%; $p < 0.001$; Fig. S4B; Table S9). Together, these factors accounted for 57% of the total variability in the soil bacterial composition dynamics ($p < 0.001$).

The bacterial community profiles of soils amended with exuviae and frass from the same insect species clustered together, except for mealworm-amended soil (Fig. 6A). Specifically, the bacterial community profile in soil amended with mealworm frass showed a closer relationship to that of BSF exuviae and frass, whereas soil treated with mealworm exuviae formed a distinct cluster with that of mushroom powder. We investigated which microbes most strongly contributed to the difference between amended and unamended soils. The native soil

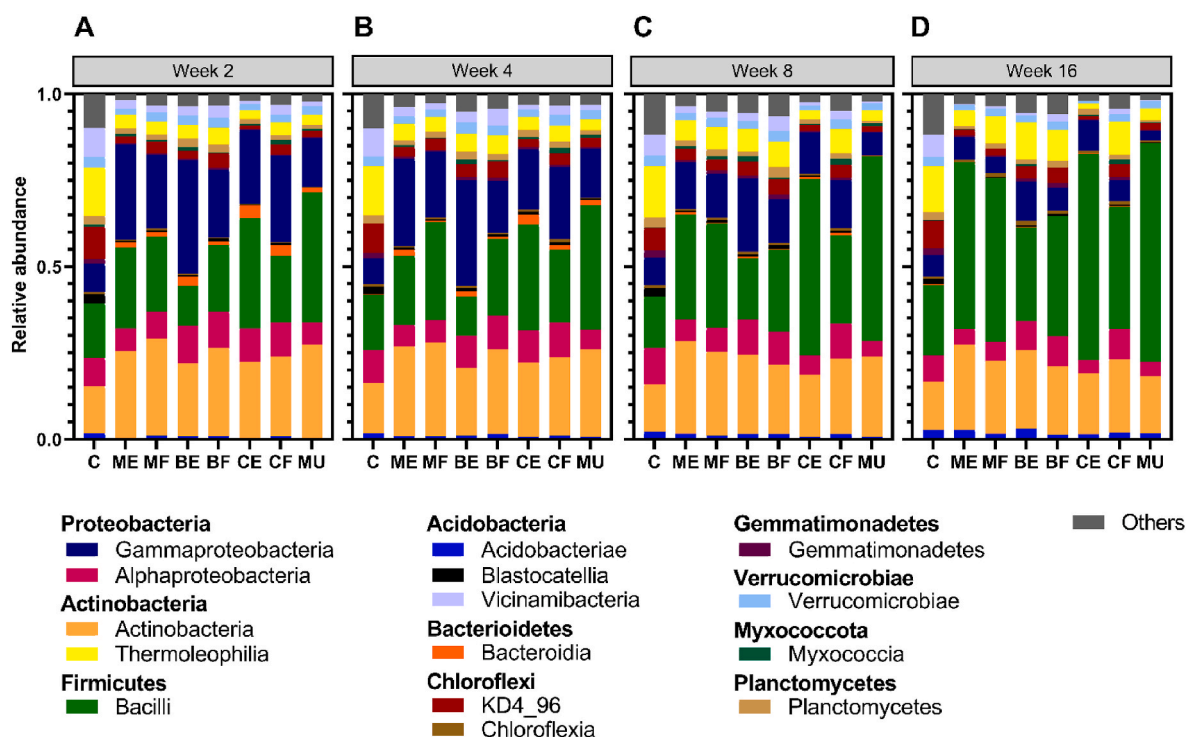


Fig. 4. Bacterial community composition in unamended soil (C), soil amended with mealworm exuviae (ME), mealworm frass (MF), BSF exuviae (BE), BSF frass (BF), cricket exuviae (CE), cricket frass (CF), or mushroom (MU) at a rate of 10 g kg^{-1} . Relative abundances of the different bacterial classes in the soil are presented for each treatment after (A) 2, (B) 4, (C) 8 and (D) 16 weeks of incubation. Phyla representing less than 1% of the total community are bundled in the group “Others”.

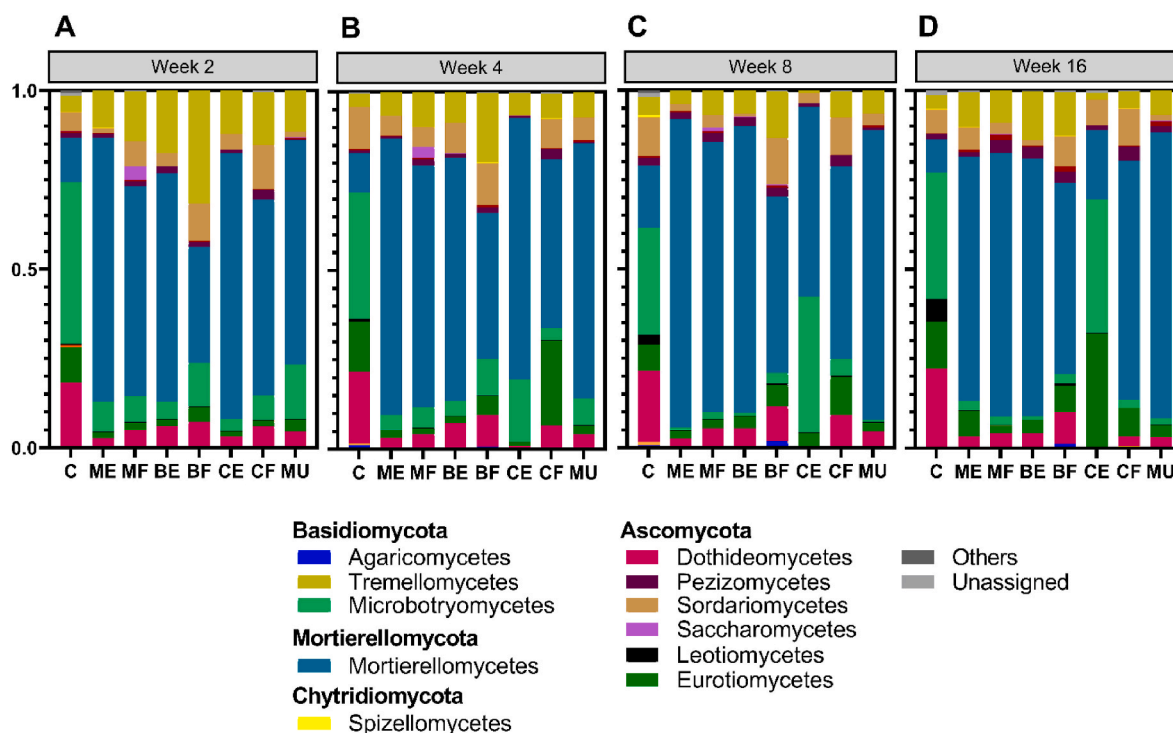


Fig. 5. Fungal community composition in unamended soil (C), soil amended with mealworm exuviae (ME), mealworm frass (MF), BSF exuviae (BE), BSF frass (BF), cricket exuviae (CE), cricket frass (CF), or mushroom (MU) at a rate of 10 g kg^{-1} . Relative abundances of the different fungal classes in the soil are represented for each treatment after (A) 2, (B) 4, (C) 8 and (D) 16 weeks of incubation. Phyla representing less than 1% of the total community are bundled in the group “Others”.

bacterial community was predominated by Gamma- and Alphaproteobacteria, Actinobacteria, Acidobacteria, and Bacilli (Fig. 4A; Table S10). A total of 37 bacterial classes significantly responded to the different

amendments. For all insect residue and mushroom amendments, a significant positive response was seen for Gammaproteobacteria, Bacilli, Actinobacteria, Bacteroidia, Myxococcia, Bdellovibrionia, and

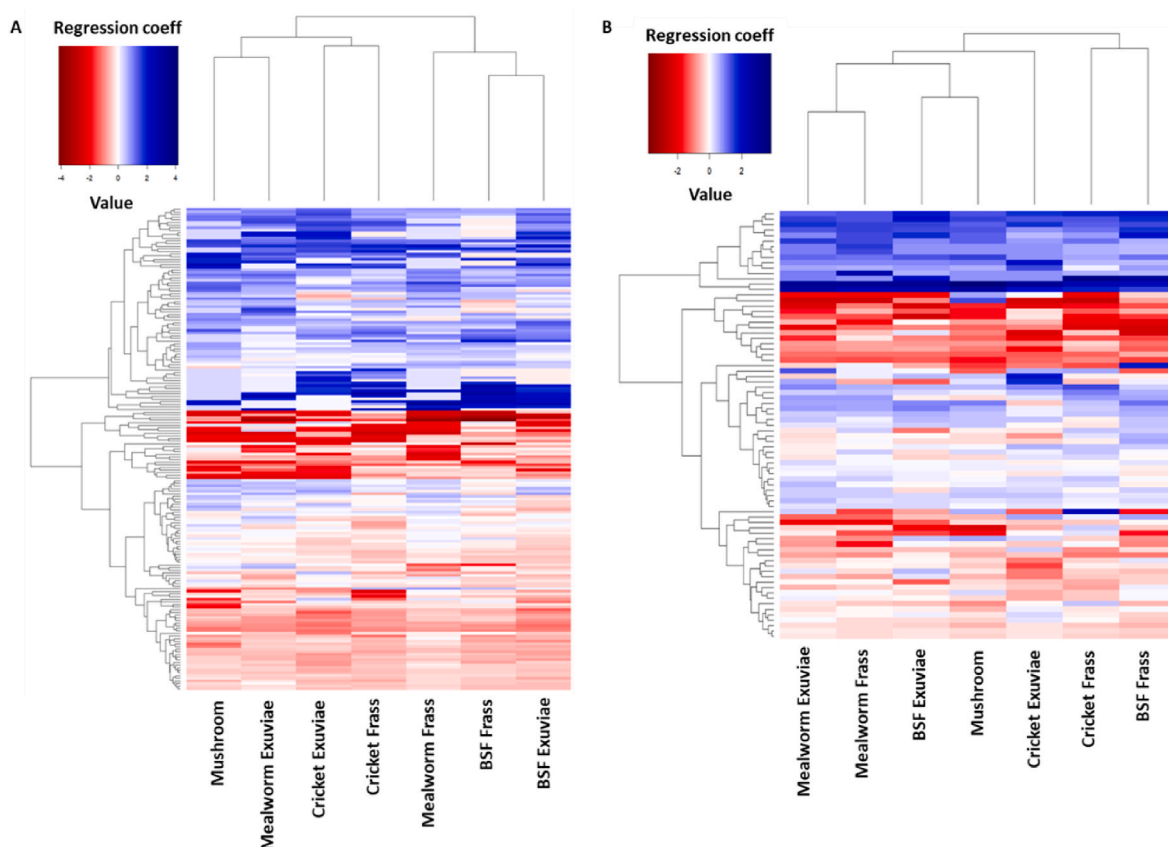


Fig. 6. Impact of insect residue and mushroom amendment on the bacterial (A) and fungal (B) community profile through a heatmap of regression coefficients. These coefficients indicate significant shifts in response to organic amendments, based on data collected after 2, 4, 8, and 16 weeks of incubation, with positive (blue) and negative (red) values corresponding to increased or decreased abundance of bacterial and fungal genera in amended soils compared to the unamended control. Further information regarding the genera that are increased or decreased per treatment is presented in Fig. S5(A–G) for bacteria and Fig. S7(A–G) for fungi.

Sumerlaeia (Figs. S5A–G). Bacterial classes not responding positively to the amendments included Acidimicrobiia, Acidobacteria, Verrucomicrobiae, Gemmatimonadetes, Vicinamibacteria, and Blastocatellia. On the genus level, changes in the relative abundance of 125 bacterial genera were observed (Fig. 6A). The enrichment of

Gammaproteobacteria in insect residue-amended soils was mainly attributed to *Pseudomonas*, *Massilia*, and *Lysobacter*, but these genera were less prevalent in mushroom powder-amended soils (Figs. S5A–G). All treatments showed strong stimulation of the genera *Bacillus* and *Pseudarthrobacter*. The enrichment of Bacteroidia in amended soils was

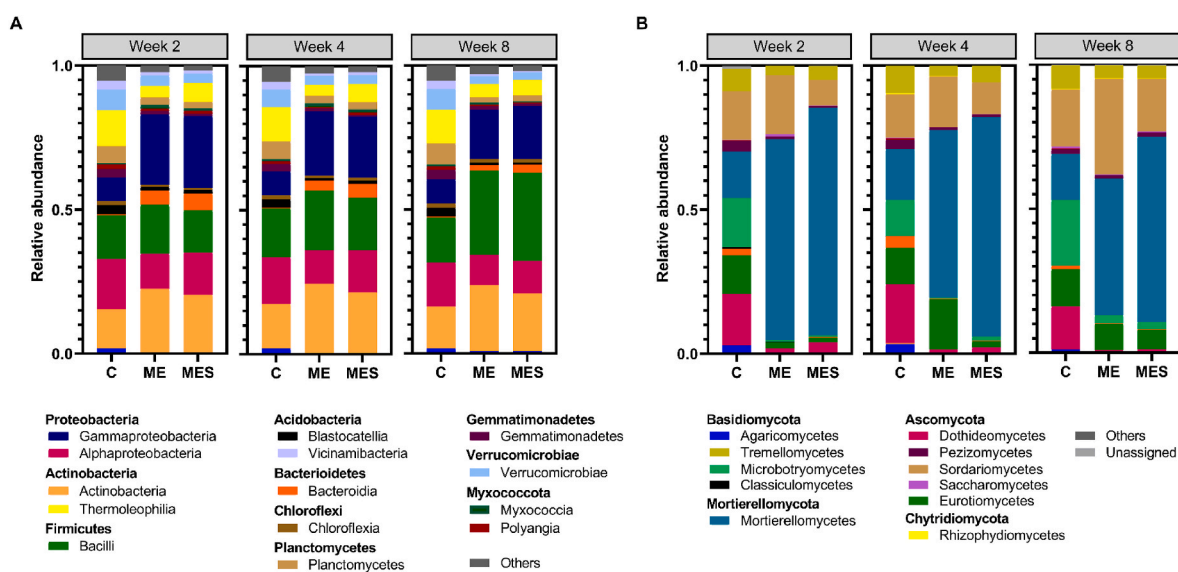


Fig. 7. (A) Bacterial and (B) fungal community composition in unamended soil (denoted as C) and soil amended with sterilized (MES) or non-sterilized (ME) mealworm exuviae. Relative abundances of the different bacterial and fungal classes in the soil are represented for each treatment after 2, 4, and 8 weeks of incubation. Phyla representing less than 1% of the total community are bundled in the group “Others”.

represented mainly by *Flavobacterium* and *Pedobacter*. As the decomposition progressed, the dominance of Gammaproteobacteria was gradually replaced by Bacilli (Fig. 4B–D, Table S10). By the end of the investigated period (week 16), Bacilli was the most prevalent bacterial class in amended soils (Fig. 4D).

3.2.4. Fungal community dynamics

Between-Class Analysis (Table S11) revealed a marked contribution of amendment type (23%; $p < 0.001$; Fig. S6A) as well as of the incubation time (7.8%; $p < 0.001$; Fig. S6B) in shaping the fungal community composition. The combination of both factors explained 49% of the total variability in the soil fungal community composition.

In the fungal community profile, soils amended with exuviae and frass from mealworms were observed to cluster together (Fig. 6B). Furthermore, soils treated with BSF exuviae, cricket exuviae, and mushrooms appeared closely related, whereas those treated with cricket frass and BSF frass formed a distinct cluster separate from other treatments. Changes in the relative abundance were observed for 79 fungal genera (Fig. 6B). Microbotryomycetes and Dothidiomycetes were the dominant resident fungal groups in the unamended soil (Fig. 5, Table S12). A strong enrichment by all amendments was seen for Mortierellomycetes (*Mortierella*). Relative abundances of Sordariomycetes (*Humicola*) and Tremellomycetes (*Saitozyma*) were also significantly increased (Fig. 5, Figs. S7A–G). Conversely, many other fungal genera belonging to Ascomycota and Basidiomycota did not positively respond to the added insect materials and mushroom powder. In soil treated with house cricket exuviae, the dominance of Mortierellales was gradually replaced by Eurotiomycetes and Microbotryomycetes. The genera *Trichoderma* and *Leucosporidium* were significantly enriched in soil treated with house cricket exuviae but not in other treatments (Fig. S7E).

3.2.5. Effect of gamma-sterilization on microbial community assembly

The possible involvement of microbes inhabiting insect residues in the assembly of residue-decomposing microbes in soil was investigated by comparing soils amended with gamma-sterilized and non-sterilized mealworm exuviae (Fig. 7A and B). To explore the dissimilarity and difference in community structure between the treatments, PERMANOVA was performed independently on the bacterial and fungal composition data for each time point (week 2, 4 and 8). The analysis showed that the bacterial community structures at the level of classes in non-sterile and sterile treatments were not significantly different ($Pseudo-F = 1.25$; $p = 0.096$ for week 2, $Pseudo-F = 1.1$; $p = 0.16$ for week 4, $Pseudo-F = 1.06$; $p = 0.32$ for week 8). Analysis of regression coefficients based on data obtained after 2, 4 and 8 weeks of incubation showed similar stimulation of 7 bacterial classes in soils amended with sterile and non-sterile mealworm exuviae (Bdellovibrionia, Gammaproteobacteria, Bacteroidia, Actinobacteria, Bacilli, Oligoflexia, and Myxococcia) (Fig. S8). Yet, a total of 11 and 8 bacterial genera were found to be differentially stimulated in soils amended with non-sterile and sterile mealworm exuviae, respectively (Fig. S10A).

Fungal community structure at the level of classes between sterile and non-sterile treatments was not significantly different at week 2 ($Pseudo-F = 4.32$; $p = 0.059$). However, at later time points (week 4 and 8), a significant difference was observed between the investigated fungal communities ($Pseudo-F = 12.23$; $p = 0.026$ for week 4, $Pseudo-F = 8.04$; $p = 0.035$ for week 8). Regression coefficients based on data obtained after 2, 4 and 8 weeks showed that the fungal class Mortierellomycetes responded positively to the amendment of both sterile and non-sterile mealworm exuviae (Fig. S9). When zooming in into the genus level, *Chrysosporium* was identified as the genus belonging to Eurotiomycetes enriched in non-sterile treatments (Fig. S10B). Four genera belonging to Sordariomycetes (*Acaulium*, *Phialemonium*, *Coniochaeta*, and *Fusarium*) were selectively stimulated in sterile mealworm exuviae amended soils (Fig. S10B).

4. Discussion

4.1. Decomposition of insect residual streams

Our litterbag study revealed a general trend in the decomposition of exuviae and frass: a rapid initial mass loss followed by an extended period of slower decay from the 2nd until the 16th week. This pattern is similar to previous findings on frass of gypsy moth and mealworm (Lovett and Ruesink, 1995; Houben et al., 2020) as well as remains of millipede and cricket (Seastedt and Tate, 1981; Fielding et al., 2013). The decomposition rate of non-processed organic inputs often correlates negatively with the C:N ratio (Enriquez et al., 1993). However, we found that the C:N ratio alone could not predict the observed changes in litterbag mass loss. Frass of the investigated insects exhibited a higher C:N ratio (12–13.3) compared to their exuviae counterparts (4.8–9.4). Yet, despite the higher C:N ration, the total mass loss for frass consistently exceeded that of exuviae. Similarly, Kagata and Ohgushi (2012) showed no clear relationship between the C:N ratio of cabbage armyworm frass samples and the decomposition rate. This underscores the limited predictive value of C:N for organic C cycling and N mineralization (Bonanomi et al., 2019). One major limitation is that C:N characterization alone does not account for the diverse chemistry of compounds, which can significantly vary even among materials with similar ratios. To address this limitation, conducting more detailed analyses, such as ^{13}C -NMR spectroscopy, on insect residual streams and mushrooms, would facilitate the identification of specific compounds responsible for the observed differences in decomposition.

4.2. Effect on soil nitrogen availability

The contribution to available nitrogen for plant uptake is a critical aspect to consider when using insect exuviae and frass as biofertilizers and soil improvers. In forest ecosystems, the accumulation of insect-derived inputs after intense herbivory infestation often coincides with increased soil mineral N availability and N content in plant tissues (Frost and Hunter, 2004; Kagata and Ohgushi, 2011). The impact of decomposing frass on soil nutrient dynamics varies with the quantity and quality of the inputs and the length of the decomposition period (Lovett et al., 2002; Kagata and Ohgushi, 2012). Furthermore, decomposition processes are influenced by soil physicochemical and biological properties (Gessner et al., 2010). The application of low-quality frass (C:N ratio >20) has also been reported to cause adverse effects due to increased competition between soil microbes and plants, resulting in immobilization and reduced N availability for plant uptake (Lovett and Ruesink, 1995).

Shortly after applying insect residues and mushrooms, changes in inorganic N concentration showed a similar pattern for all treatments: an increase in extractable ammonium levels followed by conversion into nitrate. This observation is similar to previous reports on insect-derived amendments (Rummel et al., 2021) and other organic amendments of animal origin (Tenuta and Lazarovits, 2004; Cayuela et al., 2009). However, the incomplete conversion of ammonium to nitrate suggests a partial loss of mineral nitrogen. An elevated pH level (>7.5), observed in soils amended with exuviae, shifts the ammonia-ammonium equilibrium from ammonium (NH_4^+) to ammonia (NH_3), potentially leading to ammonia volatilization and decreased ammonium levels in the soil (Beesigamukama et al., 2021).

In soils directly incorporated with insect residues, an increase in bacterial numbers and mineral N accumulation showed a different trend than expected based on the litterbag decomposition results. This discrepancy was most notable for mealworm exuviae, which decomposed slowly in litterbags but caused a strong initial response in bacterial increase and mineral N release upon incubation in soil. This suggests that the dispersed material in the soil follows a different decomposition pattern than materials compacted within litterbags. Substrate confinement in litterbags can alter microphysical conditions (e.g., moisture level

and temperature) and accessibility to soil decomposers, resulting in significantly different decomposition rates compared to non-compacted organic soil inputs (Kurz-Besson et al., 2005). To address this limitation, mixing the organic materials with inert non-degradable material (e.g., purified sand) may render the decomposition in litterbags more comparable to that in soil.

4.3. Effect on resident soil bacterial and fungal communities

Elevation of soil nutrient levels following the incorporation of N-rich organic materials has been shown to induce a shift in the prevailing microbial decomposers, favoring a more active copiotrophic microbial community, while oligotrophic taxa demonstrated the opposite pattern (Fierer et al., 2012; Leff et al., 2015; Liu et al., 2021). This trend was also observed shortly after incorporating exuviae, frass, and mushrooms into the soil. During the early decomposition period, members of Gammaproteobacteria (*Pseudomonas*, *Lysobacter*, and *Massilia*) immediately showed a marked growth response. These initial responders have been reported to possess copiotrophic traits, such as a high growth rate and the ability to utilize a broad range of substrates, allowing them to thrive under high-nutrient regimes (Fierer et al., 2012; Ho et al., 2017; Kurm et al., 2017). In contrast, resident soil bacteria belonging to Acidobacteria, Verrucomicrobia, and Chloroflexi did not show a positive growth response shortly after amendment; all these phyla are associated with oligotrophic life strategies (i.e., less responsive to abrupt resource availability) (Navarrete et al., 2015; Kielak et al., 2016; Ho et al., 2017). The shift in dominant life strategies of the responding soil microbes was most likely attributable to the presence of easily degradable compounds with high N content in insect residues, such as amino acids, protein and chitin, as copiotrophic taxa are frequently associated with lower biomass C:N ratios due to higher N demands than oligotrophic taxa (Fontaine and Barot, 2005; Fierer et al., 2007).

The *chiA* gene is described as the most commonly occurring bacterial gene involved in chitin degradation in soil (Williamson et al., 2000; Cretoiu et al., 2013). Following the addition of insect residues and mushrooms, we observed a positive temporal effect on the abundance of *chiA* gene, which was higher than the general stimulation of bacterial numbers (increased *chiA*:16S rRNA gene ratio). This indicates the selective enrichment of chitin-degrading bacteria. Based on the 16S rRNA gene amplicon sequencing data, Gammaproteobacteria, Bacilli, and Actinobacteria were likely responsible for the observed increase in *chiA* genes, consistent with the well-known occurrence of chitinolytic species within these bacterial classes (Cretoiu et al., 2012; Jacquiod et al., 2013; Bai et al., 2016; Debode et al., 2016; Wieczorek et al., 2019). The stimulation of chitin-degrading soil microbes can be attributed to the presence of chitin in the procuticle layer of exuviae and the cell walls of fungi (Gooday, 1990; Merzendorfer, 2011). The enrichment of *chiA* gene was also visible in soils treated with frass, likely due to the presence of fragments of chitin-containing exuviae. Degradation of the chitin component in exuviae and frass releases smaller chitin oligomers, which can serve as a nutrient source for soil microbes unable to degrade chitin themselves (Jacquiod et al., 2013; Wieczorek et al., 2019; Pontrelli et al., 2022). In the context of applying exuviae and frass as organic amendments, the release of short-chain chitin oligomers during degradation is important as these compounds are known to induce plant genes involved in growth and development (Winkler et al., 2017; de Tender et al., 2019).

As decomposition advanced, Bacilli gradually became the dominant class in amended soils, while Gammaproteobacteria began to decline. The later dominance of Bacilli may have resulted from their ability to use the remaining recalcitrant fraction of residues after the easily metabolizable compounds had been consumed. The enrichment of Bacilli, together with an increased abundance of Firmicutes-like *chiA* gene, has previously been observed in soils amended with mealworm exuviae (Bai, 2015). Interestingly, that study showed that the stimulation of Bacilli by mealworm exuviae was more pronounced than in soils

amended with purified shrimp chitin, suggesting that non-chitinous components of insect residues could also mediate the stimulation of Bacilli. Non-chitinous compounds commonly present in insect exuviae include proteins, lipids, and catechols (Kramer et al., 1995; Andersen, 2010).

The amendment with exuviae and frass resulted in a significant increase in ergosterol levels, which is consistent with a previous report on the soil incorporation of 2.5 % (w/w) BSF, buffalo worm, and mealworm frass (Watson et al., 2021b). However, the increase in fungal biomass is modest compared to soil amendments with cellulose-rich materials (Clocchiatti et al., 2020). The small response in fungal biomass based on ergosterol results can indicate that exuviae and frass decomposition are mainly bacterial-driven. Based on the ITS region amplicon sequencing results, the resident fungal community underwent less drastic temporal changes in community composition than bacteria. Irrespective of the added soil input, Mortierellomycota was consistently found to be the main responding fungal phylum. Fungi belonging to this phylum generally comprise fast-growing species and are known to respond strongly to the addition of chitin and other N-rich organic residues (Debode et al., 2016; Li et al., 2018; Clocchiatti et al., 2020; Ning et al., 2020).

Because the stimulation of fungal biomass is primarily ascribed to the phylum Mortierellomycota, the use of ergosterol measurement may have underestimated the actual level of fungal biomass. Desmosterol and cholesterol are the major sterols produced by Mortierellomycota (Weete and Gandhi, 1999; Poll et al., 2010). Thus, the enrichment of Mortierellomycota in exuviae- and frass-amended soils may not have been adequately reflected via ergosterol measurement.

4.4. Effect of microbes already present in the insect residue

Before application, the insect residues were oven-dried (60 °C for 24 h) to eliminate any remaining moisture. This step also facilitates the milling process to reduce particle size, which is important to ensure the even dispersion of materials in the soil. Thermal treatment also reduces the number of microbes present in insect residue, but spore-forming microbes can survive the mild conditions mentioned above. Heat treatment (70 °C for 1 h) is currently required to align with waste processing methods before insect residues can be used as organic amendments (IPIFF, 2019). However, this condition is not sufficient to eliminate spores. Therefore, disentangling the impact of microbes present in insect residue on the assembly of microbial decomposers of insect residues in the soil is necessary.

Analysis of the bacterial community composition in soils amended with sterilized and non-sterilized mealworm exuviae indicated that bacteria involved in the decomposition of these exuviae mainly originated from the soil. However, this was different for the fungal community. After 4 and 8 weeks of decomposition, the fungal community composition between sterile and non-sterile treatments showed a significant difference. Regression coefficient analysis revealed that soil amendment with non-sterile mealworm exuviae resulted in the stimulation of Sordariomycetes and Eurotiomycetes (*Chrysosporium*), suggesting that some fungal spores were carried over and subsequently proliferated in the soil. Fungal species belonging to the genus *Chrysosporium* are commonly found in insect guts and have often been shown to have entomopathogenic and antimicrobial activities (Mohanty and Prakash, 2009; Correa et al., 2019). Their presence in mealworm exuviae may have resulted from the continuous exposure of exuviae to frass during the rearing cycle. A study by Fuhrmann et al. (2022) showed that soil amendment with untreated BSF residues resulted in a strong difference in the bacterial and fungal community composition compared to sterilized residues. However, this effect was less pronounced in our study, probably due to the use of heat-treated materials (60 °C) instead of untreated materials.

4.5. Application perspective of insect residue for biological control and fertilization

This study indicates the potential of applying insect residue as organic amendments to steer toward a more protective soil microbiome. Bacterial and fungal taxa enriched following the amendment of exuviae and frass harbor many potential antagonists capable of suppressing soil-borne pathogens. Compared to the inoculation of biocontrol strains, the stimulation of naturally occurring antagonistic soil microbes for controlling soil-borne pathogens offers a clear advantage because the enriched microbes are well adapted to local soil conditions (Mazzola and Freilich, 2017). Bacilli are known for their antagonistic properties, including the production of a wide array of cell-wall-degrading enzymes (e.g., chitinases, β -glucanases, and proteases), antimicrobial peptides, and volatile organic compounds, contributing to the suppression of pathogenic fungi and oomycetes (Fira et al., 2018). Additionally, members of the bacilli class can secrete signaling molecules that act as elicitors to activate the host plant's induced systemic resistance (Kloepper et al., 2004). Actinobacteria are also known to antagonize pathogens via hyperparasitism and the production of anti-fungal peptides and lytic enzymes (Kinkel et al., 2012). Enrichment of *Pseudomonas*, *Pedobacter*, *Lysobacter*, and *Massilia* were observed in all amended soils, and these genera have often been associated with enhanced disease suppression against root-infecting fungal pathogens (de Boer et al., 2007; Postma et al., 2010; Gómez Expósito et al., 2015).

With respect to fungal enrichment, the stimulation of *Mortierella* has been shown to have positive effects on soil bioavailable P and phytohormone synthesis (Ning et al., 2020; Ozimek and Hanaka, 2021). *Mortierella* has also been reported to directly promote crop growth by altering root gene expression levels via interaction with indigenous rhizosphere bacteria (Li et al., 2020). In naturally suppressive soil, the hyper-dominance of *Mortierella* (37% of the total fungal sequences) was suggested as an indicator of suppressiveness against *Fusarium* wilt (Xiong et al., 2017).

Insect residue can also serve as an alternative to conventional fertilizers by providing nutrients (N, P, and K) and other compounds of interest for plant growth (Poveda, 2021). Because of the rapid mineralization and the presence of readily available nutrients, frass application has been shown to be more efficient than mineral NPK fertilizer in improving plant biomass and nutrient uptake by chard (Poveda et al., 2019), barley (Houben et al., 2020), and maize (Beesigamukama et al., 2020).

However, further studies are needed to carefully manage the outcomes of insect residue application, as undesirable effects of frass have also been reported. Similar to immature composts and organic inputs of animal origin, fresh insect-derived products may contain compounds detrimental to seed germination and plant growth (e.g., phenols and excess salts) (Song et al., 2021; Watson et al., 2021a; Lopes et al., 2022). To alleviate these potential negative effects, it is important to fine-tune the application strategies of insect residue, including the application rate, the time between application and sowing, and (partial) composting of the residues (Bonanomi et al., 2020; Song et al., 2021; Watson et al., 2021a).

Freshly-reared insects and their residual streams are densely populated by a diversity of microbes (Osimani et al., 2018; Garofalo et al., 2019). Thus, one of the challenges of applying animal-derived products as organic amendments is ensuring that the materials do not pose a risk of introducing human pathogens into the food chain and water sources (EU, 2011; Goss et al., 2013). Our findings showed that soil-borne pathogens commonly associated with insect-derived products, including *Salmonella*, *Enterococcus*, *Listeria*, *Campylobacter*, and *Escherichia*, were not detected in soils incorporated with exuviae and frass. Additional tests could be conducted to validate the microbial safety of insect residue, such as comparing the total viable microbial pathogen load before and after sanitizing treatment, as previously conducted by van Looveren et al. (2022). Concerning the stimulation of

Mortierellomycota in all amended soils, it is worth noting that pathogenic species are scarce within this taxon (Ozimek and Hanaka, 2021). However, in the case of Bacilli, this bacterial class comprises a diversity of species widely known as causative agents of human infections, such as *B. cereus*, *B. pumilus*, *B. subtilis*, *B. circulans*, *B. coagulans*, *B. brevis*, *B. macerans*, *B. sphaericus*, *B. thuringiensis* and *B. anthracis* (de Blackburn and McClure, 2009; Ehling-Schulz et al., 2019). In addition, several pathogenic and non-pathogenic Bacilli species have closely related phylogeny, and their distinguishing features are difficult to resolve using 16S rRNA gene sequences (Han et al., 2006). Therefore, before insect residue amendments can be used in practice, an in-depth investigation is warranted to identify which *Bacillus* species are enriched following insect residue application and to determine whether complete elimination of microbes (via gamma-irradiation or high-temperature treatment) is necessary. Further investigations are also necessary to identify soil functions related to soil nutrient cycling that are induced by the enriched soil microbiota.

5. Conclusions

Over a time frame of 16 weeks, the decomposition of insect residues showed a rapid and consistent stimulation of resident soil bacteria and fungi with potential disease-suppressive properties, namely Bacilli, Actinobacteria, Gammaproteobacteria, and Mortierellomycetes. The enrichment of taxa containing many chitinolytic antagonists of pathogens suggests the prospect of insect residues in managing soil-borne diseases and reducing reliance on synthetic pesticides. In line with the growing interest in using insect residues as bio-fertilizers, the decomposition of insect residues resulted in the release of plant-available N, more prominently so for exuviae than their frass counterparts. Our study also indicated that microbes present in insect residues did not significantly influence the composition of bacterial decomposers, but spores of insect-associated fungi could be carried over and subsequently proliferate in the soil. Although our study did not uncover the stimulation of pathogenic microbes by insect residues, this aspect warrants more detailed examination, especially concerning groups like Bacilli.

The current study uses bacterial and fungal phylogenetic markers to characterize the taxonomic profile of resident soil microbes, however, functional information on the involved soil degraders remains lacking. Additional studies are needed to unravel the potential soil and rhizosphere microbial functions induced by applying these novel soil inputs, particularly those related to nutrient cycle and plant protection. Further investigation is needed to address the issue of substrate confinement within litterbags to enhance the comparability of decomposition between litterbags and direct dispersal methods. Considering the lack of ergosterol in fungi belonging to Mortierellomycota, future research should explore alternative methods for measuring ergosterol to accurately estimate soil fungal biomass.

Studies on insect residues should be expanded before recommendations can be provided, as the current work was performed under controlled conditions and without the presence of plants. Further optimization is also needed to minimize the negative impacts associated with N-rich organic amendment, such as phytotoxicity. Collectively, our results suggest that insect exuviae and frass have a promising outlook as health-promoting soil amendments.

Ethics approval statement

This research did not contain any studies involving animal or human participants, nor did it take place in any private or protected areas.

CRediT authorship contribution statement

Azkie Nurfikari: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. **Márcio Fernandes Alves Leite:**

Conceptualization, Investigation, Methodology, Validation, Visualization. **Eiko Eurya Kuramae**: Investigation, Methodology, Supervision, Visualization, Writing – review & editing. **Wietse de Boer**: Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests. Azkia Nurfikari reports financial support was provided by Dutch Research Council.

Data availability

The data presented in this study are publicly available at the Dryad repository - DOI https://datadryad.org/stash/share/T6bAS_lge8RTMv1r5rkmHTGneI26k3kqYGTUbxNLoM – and at the European Nucleotide Archive – accession number PRJEB54065.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.soilbio.2024.109426>.

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