Applied Soil Ecology 188 (2023) 104854

Contents lists available at ScienceDirect

Applied Soil Ecology

journal homepage: www.elsevier.com/locate/apsoil

Soil amendment with insect exuviae causes species-specific changes in the rhizosphere bacterial community of cabbage plants

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ARTICLE INFO

Keywords:
Insect production
Organic substrates
Plant growth
Agricultural soil
Brassica oleracea
Acheta domesticus

ABSTRACT

Insect exuviae are a chitin-rich by-product of insect farming that is considered to have great potential for contributing to sustainable agriculture. When used as soil amendment, insect exuviae have been suggested to promote plant growth and health by stimulating naturally occurring beneficial microbes. In a greenhouse experiment, the exuviae of black soldier fly larvae (*Hermetia illucens* L.), house crickets (*Acheta domesticus* L.), and yellow mealworms (*Tenebrio molitor* L.) were added to soil from an organically managed field. Brussels sprouts, *Brassica oleracea* L., plants were grown in amended soil to assess effects on plant growth and investigate bacterial abundance, diversity, and community composition in the rhizosphere. All soil amendments increased plant shoot biomass and stimulated bacterial growth. At the same time, bacterial diversity was diminished and the different amendments resulted in distinct bacterial communities. Most notably, soil amendment with house cricket exuviae increased the relative abundances of the genera *Lysinibacillus* and *Paenibacillus*, whereas the other amendments did not. The exuviae of black soldier fly larvae, however, stimulated the genus *Pseudomonas* and different genera belonging to the *Burkholderiaceae* for a longer period of time than the exuviae of either other insect species. In view of the differential enrichment of potentially plant growth-promoting or plant-protective bacteria, soil amendments with the exuviae of different insect species might have specific uses in agriculture. The present study provides a basis for investigating the combined application of insect exuviae with beneficial bacteria that are commonly used in crop production.

1. Introduction

Farming insects for food and feed is a fast-developing industry that can produce high-quality animal protein using organic residual streams as input. Some of the most important species currently reared are the black soldier fly, *Hermetia illucens* L., the house cricket, *Acheta domesticus* L. and the yellow mealworm *Tenebrio molitor* L., among others (Van Huis, 2021). As commercial insect production is increasing worldwide, more of the by-products it generates are becoming available for potential applications in agriculture. These insect-derived products include exuviae (molted exoskeletons) and frass, a mixture of insect feces, exuviae and unconsumed feed. The use of insect residual streams for soil amendment has been suggested to stimulate beneficial microorganisms that can promote plant growth, induce plant resistance or have biocontrol activity against plant pathogens and pests (Barragán-Fonseca et al., 2022). In this respect, insect exuviae are particularly interesting as they contain a relatively large proportion of chitin, which is one of the most abundant biopolymers in nature and represents an important substrate for soil microbes.

Soil amendment with chitin has been widely researched for its influence on soil microbial communities and its suppressive effects on plant pathogens (Cretoiu et al., 2013; Ootsuka et al., 2021; Randall et al., 2020). Indeed, the addition of chitin to soil has often been found to increase the relative abundances of various bacterial taxa that are commonly associated with plant protection or plant growth promotion (Andreo-Jimenez et al., 2021; Debode et al., 2016). Although the effects of insect exuviae on soil microbial communities have been only scarcely investigated, one study found that mealworm exuviae, unlike other chitin resources, resulted in a strong increase in the abundance of Bacilli (Bai, 2015). This class of bacteria includes several beneficial species from genera such as *Bacillus*, *Lysinibacillus* or *Paenibacillus*, many of which are used for crop protection or plant growth promotion (Ahsan

Abbreviations: ASV, amplicon sequence variant; LM, linear model; EMM, estimated marginal means; PCoA, principal coordinate analysis; PERMANOVA, permutational multivariate analysis of variance; BSF, black soldier fly; HC, house cricket; MW, mealworm.

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https://doi.org/10.1016/j.apsoil.2023.104854
Received 30 October 2022; Received in revised form 19 December 2022; Accepted 21 February 2023
Available online 10 March 2023

and Shimizu, 2021; Borriss, 2015; Grady et al., 2016). Interestingly, soil amendment with the exuviae of black soldier fly larvae has been found to reduce the biomass of cabbage maggots, whereas mealworm exuviae did not negatively affect the performance of this insect pest (Wantulla et al., 2022). Such differences are likely related to the composition of the exuviae, as proportions of chitin, different proteins and other components vary between insect species. While mealworm exuviae have a chitin content of 7.9–8.6 % and are rich in lipids, exuviae of black soldier fly larvae contain 10.9–11.1 % chitin and are thought to have a relatively low lipid content based on their lower hydrophobicity (Nurfikari and de Boer, 2021). Thus, though the exuviae of different species are expected to influence soil microbiomes in a similar way, they may also be colonized and degraded by specific microbes that can affect other soil organisms differentially.

To understand the effects of insect exuviae on soil organisms or plants and specify their potential uses, it is essential to compare how the exuviae of different species affect soil microbial communities. Differential enrichment or depletion of specific microbes by the exuviae of one species can indicate a particular suitability of those exuviae for certain agricultural applications or for a combination with commonly used microbial agents. In this regard, effects on communities in the plant rhizosphere are particularly relevant, as it represents the environment in which interactions with root pathogens and pests but also with most beneficial microbes occur. Positive effects of soil amendment with chitin on plant growth and health have been related to the increased abundance of beneficial microbes in the rhizosphere (Debode et al., 2016). To our knowledge, no studies have yet examined the effects of insect exuviae on rhizosphere microbial communities. While the addition of exuviae to soil may stimulate both bacteria and fungi, bacteria in particular are expected to be important colonizers of these materials. Bacterial communities are known to respond more quickly and strongly to chitin inputs and are generally regarded as key drivers of chitin degradation in soil (Cretoiu et al., 2013; Kielak et al., 2013). The aim of the present study was thus to investigate whether soil amendment with the exuviae of different insect species stimulates bacterial growth and to determine how it influences the diversity and composition of the bacterial community in the rhizosphere of cabbage plants. As exuviae from different species differentially affect the performance of root maggots on cabbage plants, they were expected to induce distinct changes in the rhizosphere bacterial community of this crop (Wantulla et al., 2022).

2. Materials and methods

2.1. Insect exuviae and soil

The exuviae of three different insect species were used in this study: Exuviae of black soldier fly larvae, *Hermetia illucens* (Bestico, Berkel en Rodenrijs, the Netherlands), exuviae of house crickets, *Acheta domesticus* (Protix, Bergen op Zoom, the Netherlands) and exuviae of yellow mealworms, *Tenebrio molitor* (Nijenkamp Voederdieren, Hellendoorn, the Netherlands). All materials were inspected for the presence of insects or insect fragments other than exuviae, which were removed. Although current EU regulations for insect production residues require heating at 70 °C for 1 h (European Union, 2021), such guidelines did not exist when the experiment reported here was conducted. For the present study, the exuviae were oven-dried at 60 °C for 24 h to allow for soil amendment on a dry matter basis. The dried exuviae were subsequently ground to a powder with an SM 100 cutting mill (Retsch, Haan, Germany).

Agricultural soil was collected from the upper 10–20 cm mineral layer of an organically managed field in Wageningen, the Netherlands in October 2018. The field had been used to grow various brassicaceous plants since 2011 and black mustard (*Brassica nigra* L.) had recently been grown at the location from which the soil was collected. Soil composition as assessed for the same field by Eurofins Agro (Wageningen, the Netherlands) in 2018 was 81 % sand, 14 % silt and 2 % clay, while the soil organic matter content was 3.2 %. The soil was homogenized by sieving (particle size < 4 mm) and stored at 4 °C for 1 month before being mixed with black soldier fly, house cricket or mealworm exuviae at a ratio of 1 or 10 g/kg of dry soil.

2.2. Plants and growth conditions

Brassica oleracea L. var. *gemmifera* cv. Cyrus (Brussels sprouts) plants were kept in a greenhouse compartment at 20 ± 3 °C, 50–70 % relative humidity and 16 h light/8 h dark photoperiod. In a completely randomized design, 15 plants per treatment were grown on a single greenhouse bench. Plants were grown in 1 L plastic pots, which were individually placed in saucers. Two seeds were sown per pot and gently pressed down. If both seeds germinated, one seedling was randomly removed from each pot after one week. Excess seedlings were transplanted to pots of the same treatment in which no seeds had germinated or were discarded together with ungerminated seeds if not needed. All plants were watered three times per week. Rhizosphere samples were collected from five different plants of each treatment at three different time points as described below. At the end of the experiment, shoots of the eight-week-old plants were harvested separately during rhizosphere sampling and weighed.

2.3. Rhizosphere sampling and DNA isolation

Five plants per treatment were uprooted after two, four and eight weeks of growth to collect rhizosphere samples according to Lundberg et al. (2012). After eight weeks, three or four, instead of five, plants were sampled for some treatments because in total three plants had died towards the end of the experiment. These three plants had died for unknown reasons. Before the collection of each sample, gloves were cleaned with 70 % ethanol. Roots were manually separated from loose soil by kneading and shaking. Entire root systems with a soil layer of ca. 1 mm thickness attached to their surface were then collected in 50 mL tubes containing 25 mL of sterile phosphate buffer (6.33 g $NaH_2PO_4 \cdot H_2O$, 10.96 g $Na_2HPO_4 \cdot 2H_2O$ and 200 μL Silwet L-77 per L). Tubes were vortexed at maximum speed for 15 s, roots were removed and the resulting suspensions were centrifuged for 15 min at 1800*g*. Supernatants were discarded and pellets were stored at −25 °C until further processing for DNA isolation. DNA was extracted from 50 mg of each rhizosphere sample using the DNeasy PowerSoil Pro Kit (QIAGEN, Venlo, the Netherlands). DNA quantity and quality were checked using the DeNovix DS-11 Fluorometer and dsDNA Broad Range assay (DeNovix, Wilmington, Delaware, USA). Rhizosphere samples that were too small for the extraction of sufficient DNA were not processed further and excluded from analyses. At each time point, DNA was extracted from 3 to 5 samples per treatment and all replicates were used for subsequent 16S rRNA gene quantification and sequencing.

2.4. Quantification of bacterial 16S rRNA gene numbers

Quantitative PCR of bacterial 16S rRNA genes was carried out in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, California, USA). Reaction mixes had a total volume of 20 μL and contained 10 μL SensiFAST SYBR No-ROX mix (Bioline Reagents, London, United Kingdom), 0.4 μL of each primer (25 μM; Eurofins Genomics, Ebersberg, Germany), 4.2 μL H_2O and 5 μL template DNA. Primers used were EUB338/EUB518 (Fierer et al., 2005). Reaction conditions were 3 min at 95 °C, followed by 40 cycles of 95 °C for 10 s, 59 °C for 10 s and 72 °C for 30 s. All DNA sample reactions were run in duplicate. A standard series of 10^3–10^8 *Bacillus circulans* 16S rRNA gene fragments was prepared for each plate by making triplicate 10-fold dilutions and target gene numbers were calculated for each sample using a standard curve.

2.5. Amplicon sequencing of the 16S rRNA gene V4 region

Rhizosphere samples from the high exuviae content treatments (10 g/kg) and control samples were submitted to the Centre d'expertise et de services Génome Québec (Montréal, Québec, Canada) for 16S rRNA gene amplicon sequencing on the Illumina MiSeq system. The primers 515F/806R (Caporaso et al., 2011) were used to target the V4 region of the gene. Generated FASTQ files were demultiplexed and non-biological nucleotides were removed by the sequencing provider. Primers used to amplify the V4 region were removed in R (Version 3.6.3; R Core Team, 2020) using the *filterAndTrim* function of the dada2 package and the sequencing data was further processed using the DADA2 pipeline following the standard operating procedure (Callahan et al., 2016). On average, 16,028 reads per sample remained after filtering, denoising, merging paired reads and the removal of chimeras. Taxonomy was assigned to amplicon sequence variants (ASVs) up to the genus level using the Silva reference database version 132 (Yilmaz et al., 2014).

2.6. Statistical analysis

All statistical analyses were carried out in R. The ASV table produced by the DADA2 pipeline was normalized by cumulative-sum scaling using the *cumNorm* function of the metagenomeSeq package (Paulson et al., 2013). Shannon indices were calculated as a measure of bacterial alpha diversity using the phyloseq package (McMurdie and Holmes, 2013) and were analyzed with linear models (LM) using the packages stats (R Core Team, 2020) and car (Fox and Weisberg, 2019). Pairwise comparisons were performed using estimated marginal means (EMM) with the package emmeans (Lenth, 2021). For beta diversity, Bray-Curtis dissimilarity matrices were used to perform principal coordinate analyses (PCoA) and permutational multivariate analyses of variance (PERMANOVA) using the packages phyloseq and vegan (Oksanen et al., 2020). Relative abundances of bacterial phyla, families and genera contributing >1 % to the total number of sequences as well as plant shoot biomass and bacterial 16S rRNA gene numbers were analyzed using LMs and pairwise comparisons of EMMs. LMs were validated by plotting residuals and homogeneity of variances and normality were confirmed with Levene's test and the Shapiro-Wilk test using the packages car and stats, respectively. Log transformations were applied to meet model assumptions where necessary and nonparametric tests were performed using the package dunn.test (Dinno, 2017) if the assumptions of homogeneity or normality were violated. Relative abundances of families and genera that were significantly different between the control and at least one of the soil amendments were used to order samples within heatmaps using PCoA ordination on Bray-Curtis dissimilarities. Heatmaps were created with the phyloseq implementation of the NeatMap approach (Rajaram and Oono, 2010).

3. Results

3.1. Plant growth

Soil amendment with insect exuviae significantly affected shoot fresh biomass of *B. oleracea* plants after eight weeks of growth (LM: $F = 312.6$, $df = 6$, $P < 0.001$). When soil was amended with exuviae at a ratio of 1 g/kg, shoot biomass was significantly increased by house cricket (EMM: $P < 0.001$; Fig. 1) and mealworm exuviae (EMM: $P = 0.008$; Fig. 1) compared to the control. Amendment with house cricket exuviae resulted in significantly higher shoot biomass than amendment with black soldier fly (EMM: $P < 0.001$; Fig. 1) or mealworm exuviae (EMM: $P = 0.028$; Fig. 1). When soil was amended with 10 g/kg, exuviae of all three insect species significantly increased shoot biomass compared to the control (EMM, $P < 0.001$; Fig. 1). Amendment with house cricket exuviae resulted in significantly higher shoot biomass than amendment with exuviae of either of the other two insect species (EMM: $P < 0.001$; Fig. 1). In all cases, shoot biomass of plants grown in soil amended with

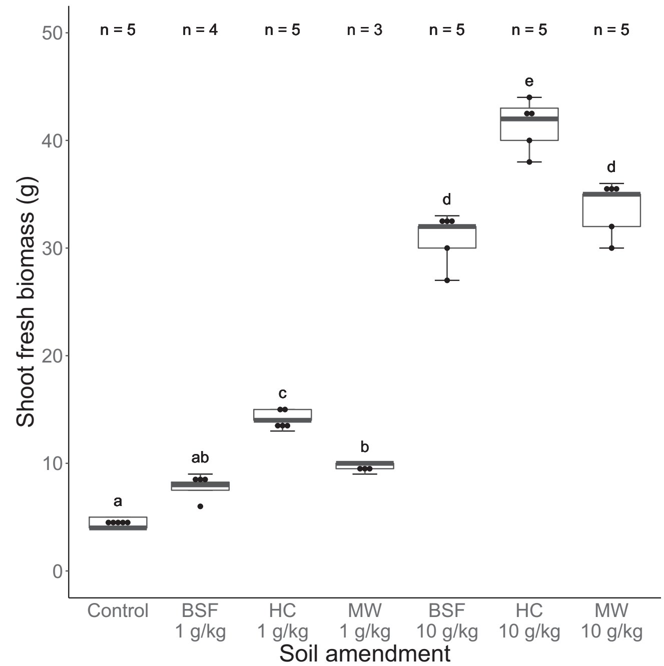

Fig. 1. Shoot fresh biomass of eight-week-old *Brassica oleracea* plants grown in soil amended with black soldier fly (BSF), house cricket (HC) or mealworm (MW) exuviae at a ratio of 1 or 10 g/kg. Treatments denoted with the same letter are not significantly different (EMM, $P > 0.05$). Box plot whiskers represent largest values within 75 % quantiles + 1.5 × interquartile range (IQR) and smallest values within 25 % quantiles − 1.5 × IQR. Numbers of replicate plants per treatment (n) are indicated at the top of the panel.

10 g/kg was significantly higher compared to plants grown in soil amended with 1 g/kg (EMM: $P < 0.001$; Fig. 1).

3.2. Total bacterial abundance

Soil amendment with insect exuviae had a significant effect on bacterial 16S rRNA gene numbers in the *B. oleracea* rhizosphere after two weeks (LM: $F = 18.123$, $df = 6$, $P < 0.001$), four weeks (LM: $F = 4.2247$, $df = 6$, $P = 0.004$) and eight weeks of plant growth (LM: $F = 29.98$, $df = 6$, $P < 0.001$). Compared to the control, 16S rRNA gene numbers were significantly increased by house cricket (EMM: $P = 0.013$; Fig. 2A) and mealworm exuviae (EMM: $P = 0.002$; Fig. 2A) after two weeks and by black soldier fly (EMM: $P = 0.032$; Fig. 2B) and mealworm exuviae (EMM: $P = 0.004$; Fig. 2B) after four weeks when soil was amended with 10 g/kg. After eight weeks, exuviae of all three insect species significantly increased 16S rRNA gene numbers when soil was amended with 10 g/kg (EMM: $P < 0.001$; Fig. 2C).

3.3. Bacterial alpha and beta diversity

The Shannon indices of bacterial communities in the *B. oleracea* rhizosphere were significantly affected by soil amendment with insect exuviae after two weeks (LM: $F = 11.244$, $df = 3$, $P < 0.001$), four weeks (LM: $F = 24.354$, $df = 3$, $P < 0.001$) and eight weeks of plant growth (LM: $F = 21.178$, $df = 3$, $P < 0.001$). Shannon indices were significantly lower than in the control following amendment with house cricket (EMM: $P = 0.001$; Fig. 3A) or mealworm exuviae (EMM: $P = 0.005$; Fig. 3A) after two weeks, four weeks (EMM: $P < 0.001$; Fig. 3C) and eight weeks (EMM: $P < 0.001$; Fig. 3E). Soil amendment with black soldier fly exuviae resulted in a significantly lower Shannon index as compared to the control only after eight weeks (EMM: $P = 0.015$; Fig. 3E). The Shannon index was significantly lower following amendment with mealworm exuviae than when soil was amended with black

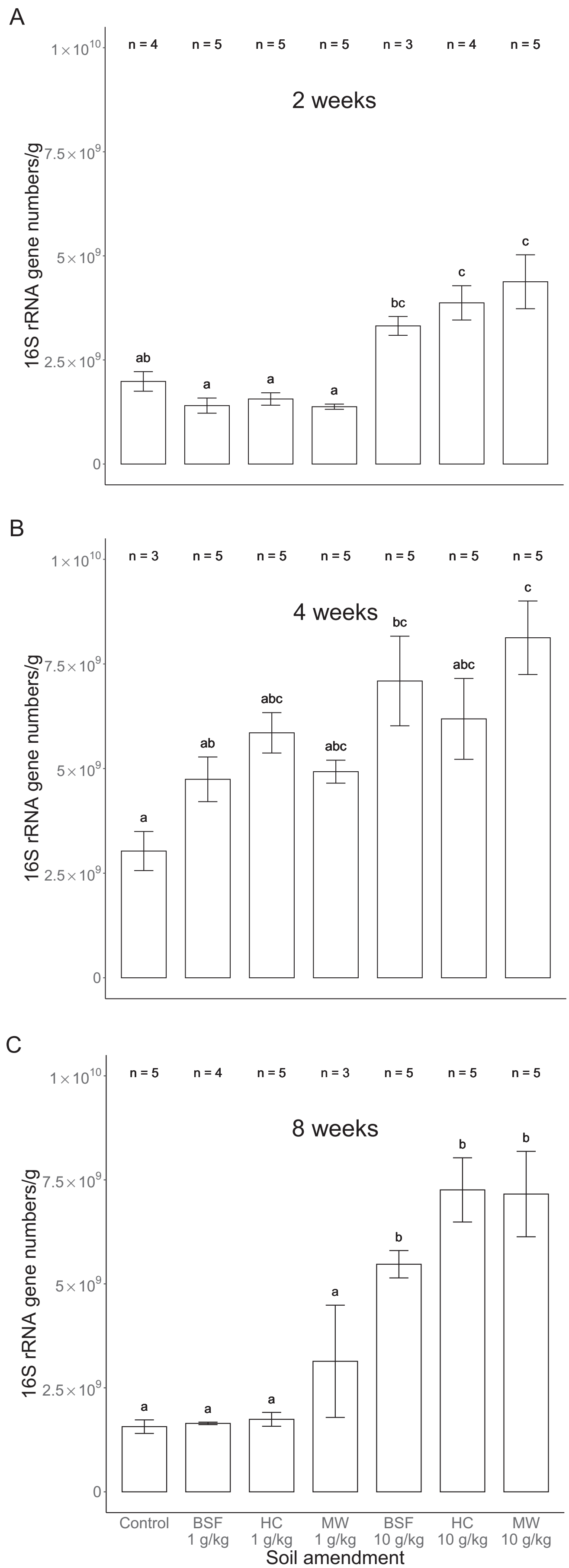

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Fig. 2. Bacterial 16S rRNA gene numbers in the *Brassica oleracea* rhizosphere after two weeks (A), four weeks (B) and eight weeks (C) of plant growth. Plants were grown in soil amended with black soldier fly (BSF), house cricket (HC) or mealworm (MW) exuviae at a ratio of 1 or 10 g/kg. Treatments denoted with the same letter are not significantly different (EMM, $P > 0.05$). Error bars represent standard errors. Numbers of replicate plants per treatment (n) are indicated at the top of the panels.

soldier fly exuviae after four weeks (EMM: $P = 0.002$; Fig. 3C) and was lower following amendment with house cricket exuviae at all three time points (EMM: $P < 0.05$; Figs. 3A, C and E).

PCoAs using Bray-Curtis metrics showed that bacterial communities in the *B. oleracea* rhizosphere separated by soil amendment (Fig. 3B, D and F). After two weeks of plant growth, rhizosphere communities that were exposed to the different amendments separated from the control (Fig. 3B). After four weeks, the rhizosphere communities of control plants and of plants grown in soil amended with black soldier fly exuviae formed distinct clusters; the communities of plants grown in soil amended with house cricket or mealworm exuviae were also distinct from the control and from the black soldier fly treatment; yet they were similar to each other (Fig. 3D). After eight weeks, the rhizosphere communities of control plants and of plants grown in soil amended with house cricket exuviae clustered separately, whereas the communities of plants grown in soil amended with black soldier fly or mealworm exuviae were similar to each other; yet the latter two also separated clearly from the communities of the control and house cricket treatment (Fig. 3F). PERMANOVA based on Bray-Curtis dissimilarity matrices confirmed that soil amendment with insect exuviae had a significant effect on the composition of rhizosphere bacterial communities after two weeks (22.21 % of the variation, $P < 0.001$), four weeks (20.27 % of the variation, $P < 0.001$) and eight weeks of plant growth (18.95 % of the variation, $P < 0.001$).

3.4. Bacterial community composition

Significant differences in the proportions of bacterial taxa in the *B. oleracea* rhizosphere between the control and at least one of the soil amendments were observed for nine phyla (Table 1), 37 families (Table S1) and 34 genera (Table S2). In the heatmap presenting relative abundances of families that were significantly different after two weeks, samples grouped according to soil amendment (Fig. 4A). Samples grouped similarly after four weeks, with the exception of outlying samples BSF6 and MW8 (Fig. 4B), and eight weeks, with the exception of outlier MW14 (Fig. 4C). In the heatmaps presenting relative abundances of genera that were significantly different between the control and at least one of the soil amendments after two weeks and four weeks, samples largely grouped according to soil amendment, with the only outliers being samples MW2 (Fig. 5A) and MW9 (Fig. 5B), respectively. After eight weeks, samples grouped similarly as after two and four weeks, with samples BSF10, MW14 and MW15 as the only outliers (Fig. 5C).

At the phylum level, soil amendment with exuviae of any of the three insect species significantly increased the relative abundance of Bacteroidetes after eight weeks and of Proteobacteria after two and eight weeks of plant growth as compared to the control (EMM: $P < 0.05$; Table 1). Amendment with house cricket exuviae caused a significantly greater increase in relative abundance of Bacteroidetes than either of the other soil amendments (EMM: $P < 0.05$; Table 1). It was the only amendment that significantly increased the relative abundance of Firmicutes after two weeks and caused a significantly greater increase in relative abundance of this phylum than amendment with mealworm exuviae after four weeks (EMM: $P < 0.001$; Table 1). Soil amendment with black soldier fly exuviae caused a significantly greater increase in relative abundance of Proteobacteria than either of the other amendments after two weeks (EMM: $P < 0.05$; Table 1). All amendments significantly reduced the relative abundances of Chloroflexi and Acidobacteria at all time points (EMM: $P < 0.05$; Table 1), whereas the relative abundance of Cyanobacteria was significantly reduced only after four weeks (EMM: $P < 0.001$; Table 1). Soil amendment with house cricket exuviae caused a significantly greater reduction in relative abundance of Chloroflexi and Acidobacteria than either of the other amendments after eight weeks and two weeks, respectively (EMM: $P < 0.05$; Table 1). It was the only amendment that significantly reduced the relative abundance of Armatimonadetes, whereas only amendment with black soldier fly exuviae significantly reduced the relative abundance of Firmicutes (EMM: $P < 0.001$; Table 1). Both of these reductions occurred after eight weeks and were also significant compared to both other soil amendments (EMM: $P < 0.05$; Table 1).

At the family level, soil amendment with any of the insect exuviae significantly increased the relative abundances of *Xanthomonadaceae*, *Sphingomonadaceae*, *Rhizobiaceae*, *Rhodanobacteraceae*, *Pseudomonadaceae*, *Nocardioidaceae*, *Intrasporangiaceae*, *Streptomycetaceae*, *Cellulomonadaceae*, *Isosphaeraceae* and *Sphingobacteriaceae* at one or more time points (EMM: $P < 0.05$; Table S1). Amendment with house cricket exuviae caused a significantly greater increase in relative abundance of *Isosphaeraceae* than either of the other soil amendments after eight weeks (EMM: $P < 0.05$; Table S1) and was the only amendment that significantly increased the relative abundance of this family already after four weeks (EMM: $P < 0.001$; Table S1). Similarly, it caused a significantly greater increase in relative abundance of *Planococcaceae* than amendment with mealworm exuviae after four weeks and was the only soil amendment that significantly increased the relative abundance of this family after two weeks and eight weeks (EMM: $P < 0.001$; Table S1). Only amendment with house cricket exuviae significantly increased the relative abundances of *Caulobacteraceae*, *Nakamurellaceae*, *Microbacteriaceae*, *Alicyclobacillaceae*, *Chitinophagaceae* and *Flavobacteriaceae* at one or more time points (EMM/Dunn's test: $P < 0.05$; Table S1). These increases were also significant compared to both other soil amendments for *Microbacteriaceae* (EMM: $P < 0.05$; Fig. 6A), *Alicyclobacillaceae* (EMM: $P < 0.05$; Fig. 6B), *Chitinophagaceae* (EMM: $P < 0.05$; Fig. 6C) and *Caulobacteraceae* (EMM: $P < 0.001$; Fig. 6D). Soil amendment with black soldier fly exuviae was the only amendment that significantly increased the relative abundance of *Pseudomonadaceae* both after two weeks and four weeks (EMM: $P < 0.001$; Table S1). It was the only amendment that significantly increased the relative abundance of *Burkholderiaceae*, with increases occurring after two weeks and eight weeks (EMM: $P < 0.05$; Table S1). Amendment with mealworm exuviae caused a significantly greater increase in relative abundance of *Nocardiaceae* than amendment with house cricket exuviae after eight weeks (EMM: $P = 0.036$; Table S1) and was the only soil amendment that significantly increased the relative abundance of this family already after two weeks (EMM: $P = 0.014$; Table S1). At each time point, it was the only amendment that significantly increased the relative abundance of *Sandaracinaceae* (EMM: $P < 0.001$; Table S1). This increase was also significant compared to both other soil amendments (EMM: $P < 0.05$; Fig. 6E). All amendments significantly reduced the relative abundances of *Nitrosomonadaceae*, *Hyphomicrobiaceae*, *Gaiellaceae*, *Blastocatellaceae*, *Pyrinomonadaceae*, *Gemmataceae*, *Nostocaceae* and an unclassified family (67–14) belonging to the Actinobacteria at one or more time points (EMM/Dunn's test: $P < 0.05$; Table S1). Only amendment with house cricket exuviae significantly reduced the relative abundances of *Blastocatellaceae* and *Nostocaceae* both after four and eight weeks (EMM/Dunn's test: $P < 0.05$; Table S1). It caused a significantly greater reduction in relative abundance of *Gaillaceae* than either of the other soil amendments after eight weeks (EMM: $P < 0.001$; Table S1) and was the only amendment that significantly reduced the relative abundance of this family already after two weeks (EMM: $P = 0.008$; Table S1) and four weeks (EMM: $P = 0.037$; Table S1). Soil amendment with house

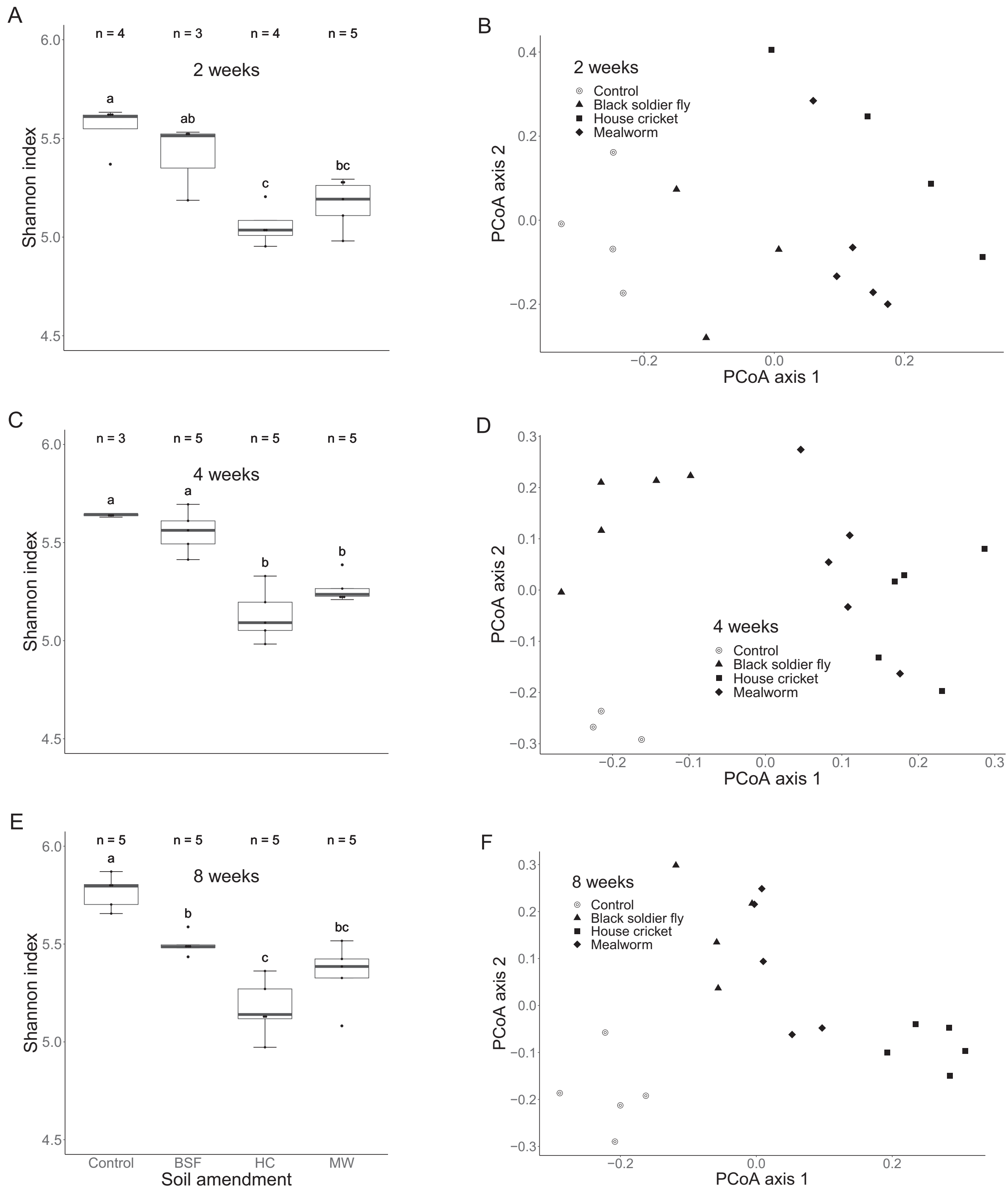

Fig. 3. Shannon indices and PCoAs of bacterial communities in the *Brassica oleracea* rhizosphere after two weeks (A and B), four weeks (C and D) and eight weeks (E and F) of plant growth. Plants were grown in soil amended with black soldier fly (BSF), house cricket (HC) or mealworm (MW) exuviae at a ratio of 10 g/kg. Shannon indices of treatments denoted with the same letter are not significantly different (EMM, $P > 0.05$). Box plot whiskers represent largest values within 75 % quantiles + 1.5 × interquartile range (IQR) and smallest values within 25 % quantiles − 1.5 × IQR. Numbers of replicate plants per treatment (n) are indicated at the top of the panels.

cricket exuviae was the only amendment that significantly reduced the relative abundances of *Sandaracinaceae*, *Solirubrobacteraceae* and *Pirellulaceae*, in all cases after eight weeks (EMM: $P < 0.05$; Table S1). The reduction in relative abundance of *Pirellulaceae* was also significant compared to both other soil amendments (EMM: $P < 0.05$; Fig. 6F). There were no families of which relative abundances were significantly reduced only by amendment with black soldier fly or mealworm exuviae.

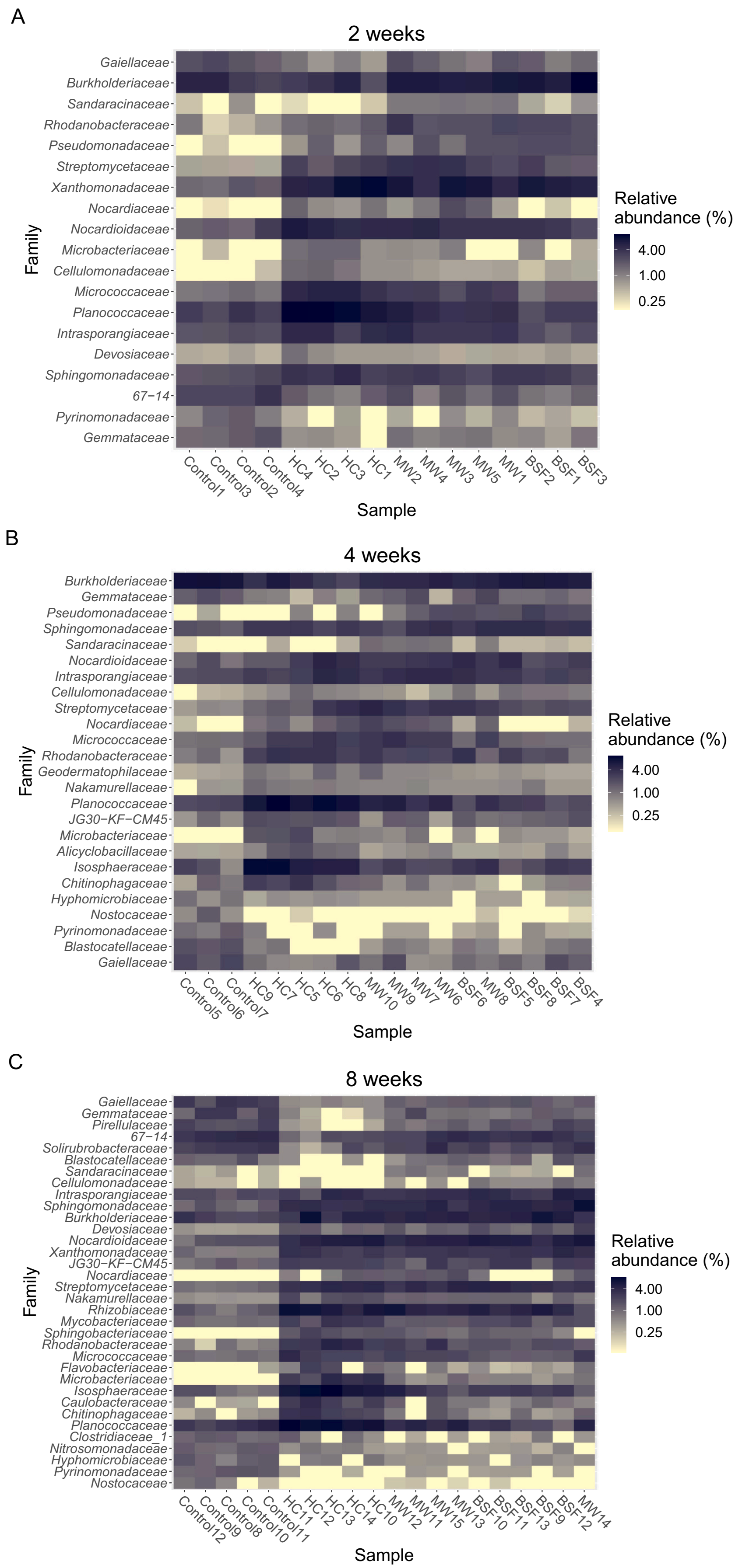

Fig. 4. Relative abundances of bacterial families in the *Brassica oleracea* rhizosphere that were significantly different from the control after two weeks (A), four weeks (B) and eight weeks (C) of plant growth (EMM/Dunn's test: $P < 0.05$). Plants were grown in soil amended with black soldier fly (BSF), house cricket (HC) or mealworm (MW) exuviae at a ratio of 10 g/kg. Families and individual samples are shown in rows and columns, respectively, and are ordered based on PCoA ordination.

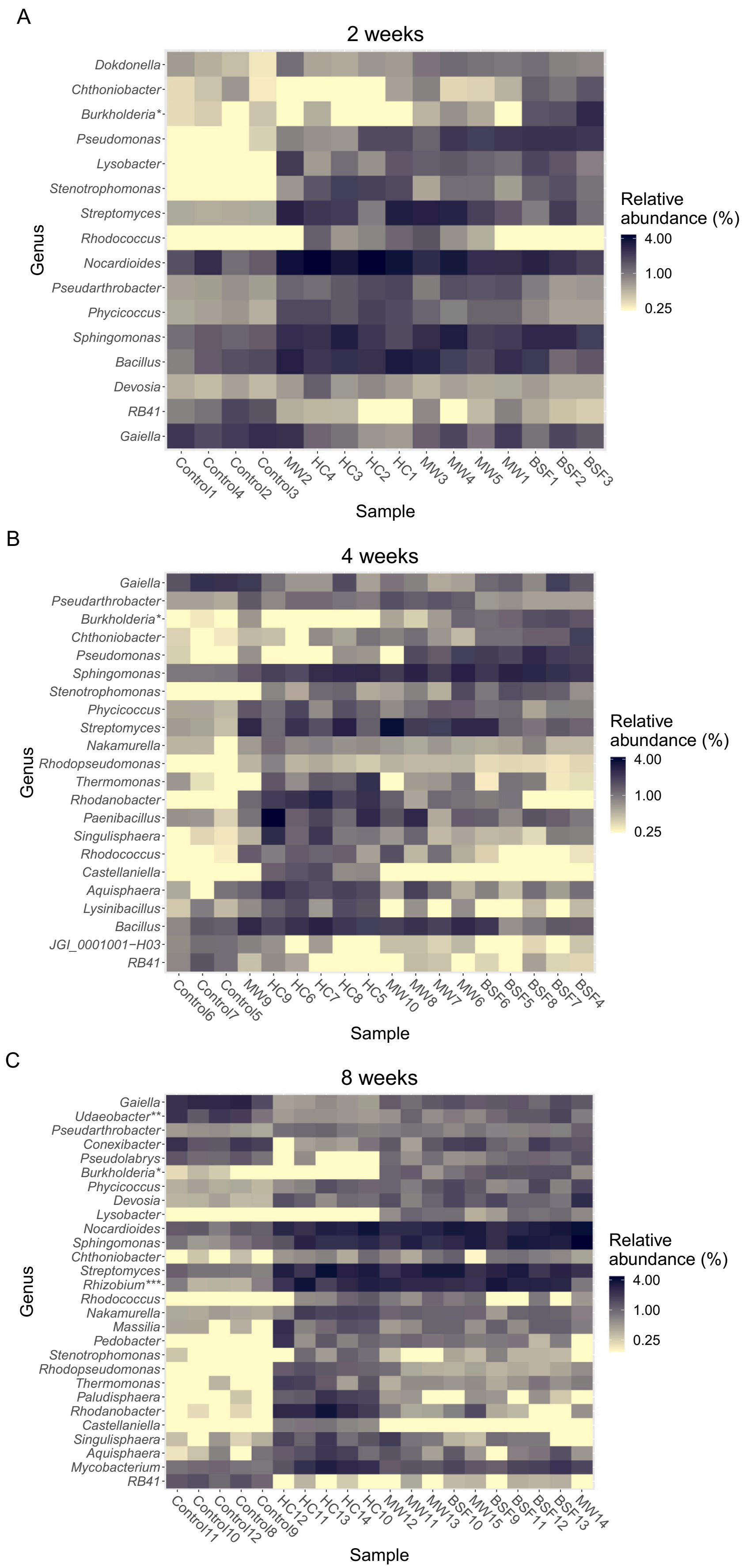

Fig. 5. Relative abundances of bacterial genera in the *Brassica oleracea* rhizosphere that were significantly different from the control after two weeks (A), four weeks (B) and eight weeks (C) of plant growth (EMM/Dunn's test: $P < 0.05$). Plants were grown in soil amended with black soldier fly (BSF), house cricket (HC) or mealworm (MW) exuviae at a ratio of 10 g/kg. Genera and individual samples are shown in rows and columns, respectively, and are ordered based on PCoA ordination.
*Including *Caballeronia* and *Paraburkholderia*.
***Candidatus_Udaeobacter*.
***Including *Allorhizobium*, *Neorhizobium* and *Pararhizobium*.

Table 1
Relative abundances of bacterial phyla in the *Brassica oleracea* rhizosphere that were significantly different from the control after two, four or eight weeks of plant growth following soil amendment with black soldier fly (BSF), house cricket (HC) or mealworm (MW) exuviae at 10 g/kg (% ± standard error).

Phylum	2 weeks				4 weeks				8 weeks			
	Control (n = 4)	BSF (n = 3)	HC (n = 4)	MW (n = 5)	Control (n = 3)	BSF (n = 5)	HC (n = 5)	MW (n = 5)	Control (n = 5)	BSF (n = 5)	HC (n = 5)	MW (n = 5)
Actinobacteria	24.31 ±1.92^{a}	22.44 ±1.20^{a}	**30.04 ±0.92^{b}**	**31.50 ±0.89^{b}**	21.58 ±0.96^{a}	25.59 ±1.30^{a}	26.06 ±2.49^{a}	29.35 ±1.48^{a}	26.26 ±1.12^{a}	32.07 ±1.29^{ab}	29.04 ±2.36^{ab}	**32.65 ±1.18^{b}**
Firmicutes	10.56 ±0.65^{a}	8.72 ±0.63^{a}	**17.64 ±0.58^{b}**	11.02 ±0.85^{a}	8.82 ±0.87^{a}	8.93 ±0.42^{a}	**18.58 ±0.62^{c}**	**12.72 ±0.98^{b}**	13.28 ±0.45^{ab}	<u>7.36 ±0.48^{c}</u>	15.76 ±0.77^{a}	10.81 ±1.02^{b}
Proteobacteria	24.81 ±0.74^{a}	**38.35 ±0.31^{c}**	**30.46 ±0.61^{b}**	**32.46 ±0.96^{b}**	32.30 ±1.60^{ab}	37.37 ±1.55^{b}	28.74 ±1.29^{a}	32.03 ±2.06^{ab}	22.48 ±0.94^{a}	**31.22 ±1.38^{b}**	**32.30 ±1.88^{b}**	**30.24 ±1.59^{b}**
Verrucomicrobia	3.53 ±0.18^{ab}	4.19 ±0.48^{a}	2.59 ±0.20^{bc}	<u>2.34 ±0.25^{c}</u>	3.47 ±0.71^{a}	3.65 ±0.44^{a}	2.55 ±0.23^{a}	2.81 ±0.20^{a}	3.58 ±0.35^{a}	2.98 ±0.21^{ab}	<u>2.06 ±0.13^{b}</u>	2.79 ±0.21^{ab}
Chloroflexi	11.00 ±0.87^{a}	<u>7.41 ±0.45^{b}</u>	<u>5.04 ±0.29^{b}</u>	<u>6.61 ±0.37^{b}</u>	9.67 ±0.62^{a}	<u>6.86 ±0.48^{b}</u>	<u>5.71 ±0.40^{b}</u>	<u>5.90 ±0.72^{b}</u>	11.98 ±0.39^{a}	<u>8.39 ±0.27^{b}</u>	<u>4.14 ±0.34^{c}</u>	<u>6.55 ±0.79^{b}</u>
Acidobacteria	8.90 ±0.28^{a}	<u>5.52 ±0.45^{b}</u>	<u>3.05 ±0.40^{c}</u>	<u>5.85 ±0.62^{b}</u>	8.41 ±0.06^{a}	<u>4.46 ±0.57^{b}</u>	<u>2.72 ±0.57^{b}</u>	<u>4.56 ±0.21^{b}</u>	8.12 ±0.74^{a}	<u>4.33 ±0.35^{b}</u>	<u>1.27 ±0.37^{c}</u>	<u>3.31 ±0.73^{bc}</u>
Bacteroidetes	2.61 ±0.31^{ab}	4.14 ±0.28^{a}	3.73 ±0.62^{a}	1.88 ±0.32^{b}	3.02 ±0.43^{a}	2.65 ±0.38^{a}	4.20 ±0.77^{a}	2.81± 0.46^{a}	1.11 ±0.17^{a}	**2.28 ±0.19^{b}**	**6.00 ±0.95^{c}**	**2.77 ±0.42^{b}**
Cyanobacteria	4.65 ±2.45^{a}	0.26 ±0.14^{a}	0.43 ±0.16^{a}	0.20 ±0.10^{a}	2.14 ±0.25^{a}	<u>0.33 ±0.07^{b}</u>	<u>0.53 ±0.10^{b}</u>	<u>0.38 ±0.15^{b}</u>	0.89 ±0.29^{a}	0.52 ±0.11^{a}	0.64 ±0.18^{a}	0.52 ±0.10^{a}
Armatimonadetes	0.73 ±0.27^{a}	0.37 ±0.05^{a}	0.51 ±0.18^{a}	0.52 ±0.26^{a}	0.92 ±0.33^{a}	0.66 ±0.13^{a}	0.30 ±0.04^{a}	0.81 ±0.17^{a}	1.09 ±0.23^{a}	0.75 ±0.15^{a}	<u>0.06 ±0.04^{b}</u>	0.83 ±0.05^{a}

Note. Treatments denoted with the same letter are not significantly different (EMM/Dunn's test, $P > 0.05$). Bold relative abundances are significantly higher than in the control. Underlined relative abundances are significantly lower than in the control.

Soil amendment with the exuviae of any insect species significantly increased the relative abundances of the genera *Stenotrophomonas*, *Lysobacter*, *Sphingomonas*, *Pseudomonas*, *Devosia*, *Nocardioides*, *Phycicoccus*, *Streptomyces* and a group of different *Rhizobiaceae* (*Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*) at one or more time points (EMM: $P < 0.05$; Table S2). Only amendment with house cricket exuviae significantly increased the relative abundances of the genera *Stenotrophomonas*, *Rhodopseudomonas* and *Rhodanobacter* not only after two weeks or four weeks but also after eight weeks and of the genus *Rhodococcus* already after two weeks (EMM/Dunn's test: $P < 0.05$; Table S2). It caused a significantly greater increase in relative abundance of the genus *Rhodanobacter* than amendment with mealworm exuviae after four weeks (EMM: $P = 0.002$; Table S2). Soil amendment with house cricket exuviae was the only amendment that significantly increased the relative abundances of the genera *Castellaniella*, *Thermomonas*, *Mycobacterium*, *Nakamurella*, *Lysinibacillus*, *Paenibacillus*, *Aquisphaera*, *Singulisphaera*, *Paludisphaera* and *Pedobacter* at one or more time points (EMM/Dunn's test: $P < 0.05$; Table S2). These increases were also significant compared to both other soil amendments for the genera *Thermomonas* (EMM: $P < 0.05$; Fig. 7A), *Lysinibacillus* (EMM: $P < 0.05$; Fig. 7B), *Aquisphaera* (EMM: $P < 0.05$; Fig. 7C), *Singulisphaera* (EMM: $P < 0.05$; Fig. 7D), *Castellaniella* (Dunn's test: $P < 0.05$; Fig. 7E) and *Paludisphaera* (EMM: $P < 0.001$; Fig. 7F). Only amendment with black soldier fly exuviae significantly increased the relative abundance of the genus *Pseudomonas* after two and after four weeks (EMM: $P < 0.001$; Table S2). It caused a significantly greater increase in relative abundance of a group of different *Burkholderiaceae* (*Burkholderia-Caballeronia-Paraburkholderia*) than amendment with mealworm exuviae after four weeks (EMM: $P < 0.001$; Table S2) and eight weeks (EMM: $P = 0.046$; Table S2) and was the only soil amendment that significantly increased the relative abundance of these genera already after two weeks (EMM: $P < 0.001$; Table S2). Amendment with mealworm exuviae caused a significantly greater increase in relative abundance of the genus *Pseudarthrobacter* than amendment with house cricket exuviae after four weeks (EMM: $P < 0.001$; Table S2). There were no genera of which relative abundances were significantly increased only by amendment with black soldier fly or mealworm exuviae. All soil amendments significantly reduced the relative abundances of the genus *Gaiella* after eight weeks (EMM: $P < 0.001$; Table S2) and of an unclassified genus belonging to the *Pyrinomonadaceae* (RB41) at all time points (EMM: $P < 0.05$; Table S2). Amendment with house cricket exuviae caused a significantly greater reduction in relative abundance of the genus *Gaiella* than either of the other soil amendments (EMM: $P < 0.001$; Table S2) and was the only amendment that significantly reduced the relative abundance of this genus already after two weeks (EMM: $P = 0.008$; Table S2) and four weeks (EMM: $P = 0.037$; Table S2). It was the only soil amendment that significantly reduced the relative abundances of the genera *Pseudolabrys* and *Conexibacter*, in both cases after eight weeks (EMM: $P < 0.05$; Table S2). The reduction in relative abundance of the genus *Pseudolabrys* was also significant compared to both other soil amendments (EMM: $P = 0.001$; Table S2). There were no genera whose relative abundances were significantly reduced only by amendment with black soldier fly or mealworm exuviae.

4. Discussion

The exuviae of all three insect species tested here positively affected *B. oleracea* growth in amended soil. Shoot fresh biomass was increased by a factor of 1.7–2.2 when soil was amended with 1 g/kg and by a factor of 7–9.5 when soil was amended with 10 g/kg. A previous study that used the same insect exuviae and soil showed that the effect of exuviae on *B. oleracea* biomass is comparable to that of synthetic fertilizer (Wantulla et al., 2022). As control plants did not receive any fertilizer in the present study, nutrient shortage in the soil might have limited plant growth and thus resulted in more pronounced effects of the soil amendments. Insect exuviae are rich in plant nutrients and may also stimulate plant growth-promoting bacteria (Barragán-Fonseca et al., 2022). It remains unknown to which extent the enhanced plant growth observed here can be attributed to bacterial plant growth-promotion or whether it might have been the result of nutrient addition alone. Compared with shrimp-derived chitin, black soldier fly exuviae have been reported to contain more phosphate and potassium but less nitrogen (Postma et al., 2022). This nitrogen is partially present in the form of chitin and so only available to the plant upon bacterial degradation of the chitin. Soil amendment with chitin, a major component of exuviae, has previously been shown to increase the biomass of lettuce plants (Debode et al., 2016). Interestingly, differences in effects on plant growth between soil amendments with exuviae from different insect species do not seem to be related to their chitin content. House cricket exuviae, which by far had the strongest effect on plant biomass, have an intermediate chitin content between that of mealworm exuviae and the exuviae of black soldier fly larvae (Nurfikari and de Boer, 2021).

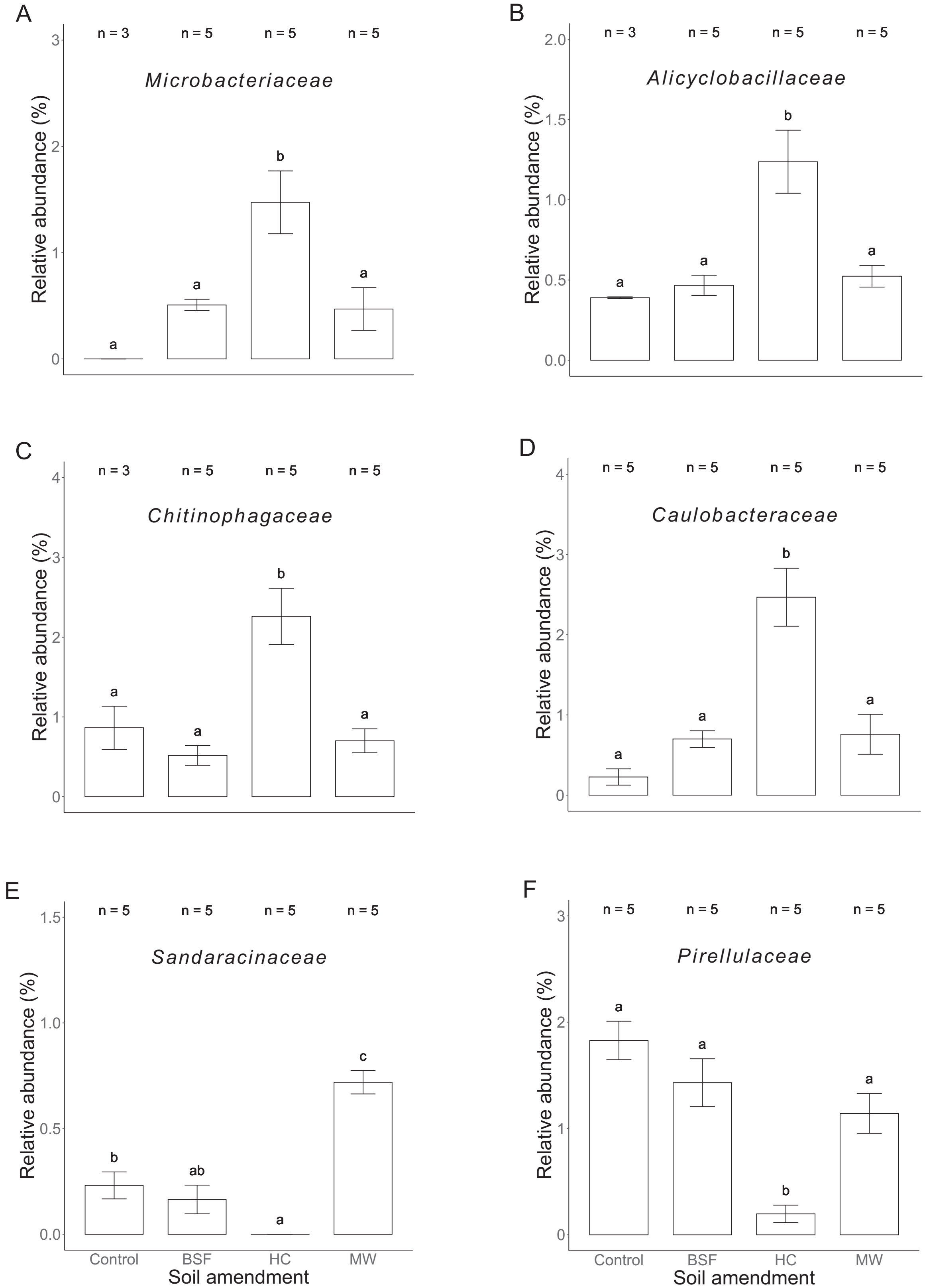

Fig. 6. Relative abundances of bacterial families in the *Brassica oleracea* rhizosphere that were differentially enriched or depleted by soil amendment with black soldier fly (BSF), house cricket (HC) or mealworm (MW) exuviae after four weeks (A, B and C) or eight weeks (D, E and F) of plant growth. Soil was amended with exuviae at a ratio of 10 g/kg. Treatments denoted with the same letter are not significantly different (EMM, $P > 0.05$). Error bars represent standard errors. Numbers of replicate plants per treatment (n) are indicated at the top of the panels.

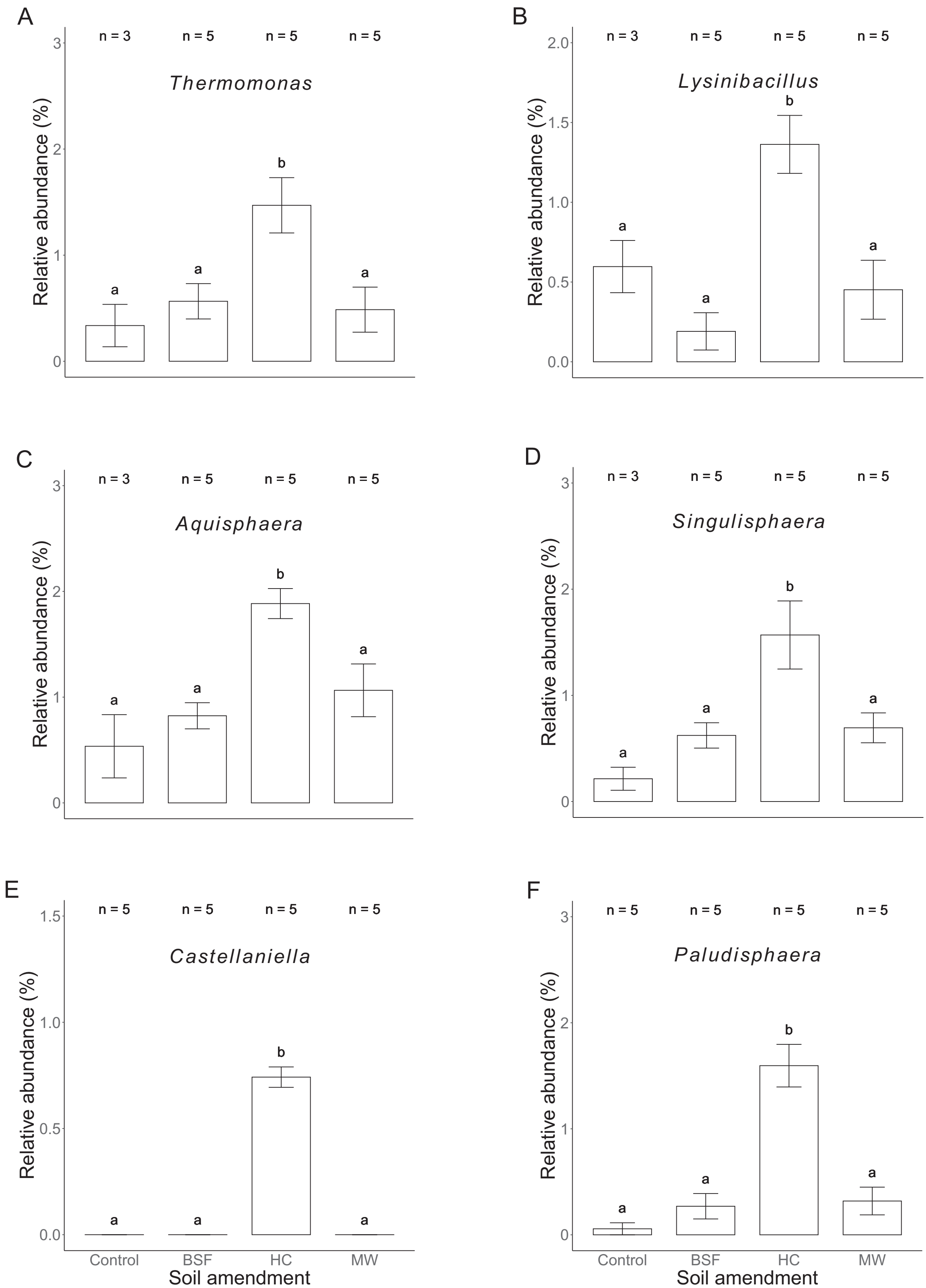

Fig. 7. Relative abundances of bacterial genera in the *Brassica oleracea* rhizosphere that were differentially enriched by soil amendment with black soldier fly (BSF), house cricket (HC) or mealworm (MW) exuviae after four (A, B, C and D) or eight weeks of plant growth (E and F). Soil was amended with exuviae at a ratio of 10 g/kg. Treatments denoted with the same letter are not significantly different (EMM/Dunn's test, $P > 0.05$). Error bars represent standard errors. Numbers of replicate plants per treatment (n) are indicated at the top of the panels.

Therefore, it is unlikely that the observed differences in plant growth are solely due to different chitin contents. Nonetheless, the contents of other components, such as proteins and lipids, might contribute to the nutrient content of insect exuviae. Compounds other than chitin may also improve plant growth, either directly via nutrient supply or indirectly by stimulating plant-beneficial bacteria.

Bacterial growth in the *B. oleracea* rhizosphere was stimulated by the exuviae of each insect species when soil was amended with 10 g/kg. At a ratio of 1 g/kg, however, no significant changes in bacterial abundance occurred. Positive effects on the abundance of bacteria are consistent with research on chitin amendments, which have been shown to increase bacterial population densities in soil (Cretoiu et al., 2013; Kielak et al., 2013). While total abundance increased, bacterial diversity in the rhizosphere was diminished by soil amendment with any of the insect exuviae. This indicates that only specific groups of bacteria were able to use exuviae as a substrate and to proliferate in response to the soil amendments. Each amendment clearly shifted the structure of the bacterial community, which differed from the control in all cases. These findings are largely in line with what has been observed in chitin-enriched soil. Although amendment with chitin was not found to affect bacterial diversity in the rhizosphere of lettuce (Debode et al., 2016), it has been reported to reduce the diversity of bacteria in bulk soil (Andreo-Jimenez et al., 2021; Ootsuka et al., 2021). Furthermore, chitin amendment has been shown to alter microbial community structures in soil and in the plant rhizosphere (Andreo-Jimenez et al., 2021; Cretoiu et al., 2013; Debode et al., 2016). Nonetheless, it seems that the effects of insect exuviae on rhizosphere bacterial communities cannot be explained by chitin alone. Soil amendment with house cricket or mealworm exuviae resulted in lower community diversity than amendment with the exuviae of black soldier fly larvae, which contain a larger proportion of chitin. Among the exuviae tested here, those of mealworms and black soldier fly larvae reportedly have the lowest and highest chitin content, respectively (Nurfikari and de Boer, 2021). However, their addition to soil resulted in rhizosphere communities that clustered close to each other after eight weeks of plant growth, while those in soil amended with house cricket exuviae formed a distinct cluster. The influence of chitin-containing substrates on soil bacterial communities has been shown to depend on the nature of the chitin resource and is thought to be determined by co-occurring structural compounds (Bai, 2015). While so far only arthropod and fungal chitin resources had been described to affect bacterial communities differently, such differences also appear to exist between the exuviae of different insect species. Although soil amendment with exuviae affected bacterial community structure at all measured time points, separation of the communities by amendment became clearer for individual treatments after four and eight weeks. Interestingly, the proportion of variation between communities that is attributed to soil amendment decreased over time. On the other hand, the number of bacterial families and genera that were enriched or depleted by at least one amendment increased with each time point. This indicates that rhizosphere communities in soil amended with exuviae continued to differentiate from those in unamended soil over a period of at least eight weeks.

Most bacterial taxa that were enriched in the *B. oleracea* rhizosphere following soil amendment with any of the insect exuviae are known to be stimulated by chitin. Among them are the phyla Bacteroidetes and Proteobacteria (Cretoiu et al., 2014; Debode et al., 2016) as well as the families *Xanthomonadaceae* (Crocker et al., 2019; Ootsuka et al., 2021), *Pseudomonadaceae* (Crocker et al., 2019), *Streptomycetaceae* (Ootsuka et al., 2021; Shimoi et al., 2020), *Cellulomonadaceae* (Crocker et al., 2019) and *Sphingobacteriaceae* (Cretoiu et al., 2014). Genera that were enriched by each of the amendments and that have also been reported to respond positively to chitin are *Stenotrophomonas* (Jacquiod et al., 2013), *Lysobacter* (Iwasaki et al., 2020; Jacquiod et al., 2013), *Pseudomonas* (Andreo-Jimenez et al., 2021), *Devosia* (Andreo-Jimenez et al., 2021), *Nocardioides* (Jacquiod et al., 2013), *Streptomyces* (Debode et al., 2016; Iwasaki et al., 2020) and *Rhizobium* (Andreo-Jimenez et al., 2021). Indeed, all of these genera include species with plant growth-promoting or biocontrol properties (Chhetri et al., 2022; Gopalakrishnan et al., 2015; Hayward et al., 2010; Mercado-Blanco and Bakker, 2007; Nafis et al., 2019; Rey and Dumas, 2017; Ryan et al., 2009). This suggests that improved plant growth as a result of soil amendment with insect exuviae is, at least partly, mediated by beneficial soil bacteria that are involved in chitin degradation. The enrichment of several bacterial families and genera only following amendment with house cricket exuviae is particularly interesting, given the outstanding effect of this amendment on *B. oleracea* growth. Notable examples of genera that were stimulated by house cricket exuviae but that did not respond to black soldier fly or mealworm exuviae are *Lysinibacillus* and *Paenibacillus*, both of which are well-known for comprising various plant-beneficial species (Ahsan and Shimizu, 2021; Grady et al., 2016). Other genera that were only enriched by soil amendment with house cricket exuviae such as *Singulisphaera* or *Pedobacter* have sporadically been associated with enhanced plant growth (Gu et al., 2020; Morais et al., 2019). Moreover, some genera that only responded to house cricket exuviae, such as *Castellaniella* or *Thermomonas*, have been reported to play important roles in denitrification processes (McIlroy et al., 2016; Wu et al., 2022). Thus, soil nitrate levels might have been considerably higher after amendment with house cricket exuviae than after amendment with black soldier fly or mealworm exuviae. Among the taxa that were enriched by more than one soil amendment, certain bacterial families and genera were stimulated earlier or longer by house cricket exuviae than by the exuviae of either of the other insect species. This was the case for the genus *Stenotrophomonas* as well as for other genera that reportedly contain plant growth-promoting species such as *Rhodopseudomonas* or *Rhodococcus* (Francis and Vereecke, 2019; Hsu et al., 2021). In the same way, soil amendment with black soldier fly exuviae resulted in longer-lasting enrichment of the genus *Pseudomonas* and earlier as well as stronger enrichment of different *Burkholderiaceae* than either of the other amendments. Several *Pseudomonas* species do not only promote plant growth, but are also able to control insect pests or suppress plant diseases (Kupferschmied et al., 2013). Similarly, many members of the genus *Burkholderia* exhibit biocontrol activity against plant pathogens, while others have been found to display insecticidal activity (Cordova-Kreylos et al., 2013; Eberl and Vandamme, 2016). More effective stimulation of these genera in the soil might explain the negative effects of black soldier fly exuviae on cabbage root maggots that were observed in a previous study (Wantulla et al., 2022).

In view of their effects on the composition of rhizosphere bacterial communities, insect exuviae seem to have an enormous potential for the promotion of plant growth and health. When added to soil, the exuviae of black soldier fly larvae, house crickets or mealworms all stimulated bacteria that have been demonstrated to improve plant growth and are likely to benefit plant health. Although various potentially beneficial bacteria were commonly enriched by these soil amendments, there were striking differences regarding the enrichment of important genera such as *Lysinibacillus*, *Paenibacillus* or *Pseudomonas*. The exuviae of each insect species might thus have a potential in their own right to be used in crop production. Depending on the crop, its pathogens and pests as well as soil quality, exuviae of particular species might be more or less useful. It is therefore essential to study the effects of insect exuviae on other plant species and their rhizosphere communities but also to compare them in different agricultural soils. In addition, more extensive research on the impact of soil amendment with exuviae on plant pathogens and pests is needed. While the promotion of naturally occurring beneficial bacteria is promising, an integrated application of insect exuviae with commercial microbial agents should also be considered. As introduced agents often suffer from poor field persistence, the use of organic amendments to aid their establishment in the soil has often been suggested. The results presented here provide a basis for testing the combined application of exuviae from certain insect species and selected beneficial bacteria.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.apsoil.2023.104854.

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 statement

The raw sequencing data for this study have been deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under accession number PRJEB58190 (https://www.ebi.ac.uk/ena/browser/view/PRJEB58190).

Acknowledgements

This work was supported by the Dutch Research Council, NWO (grant number ALWGK.2016.010). We are grateful to Azkia Nurfikari for providing the *Bacillus circulans* DNA and her advice on processing and analyzing the amplicon sequencing data.

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