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# Plant biostimulants in *Hermetia illucens* frass: insights into fresh and recirculated frass

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**Abstract**

This is the first and extensive study investigating the bioactive composition of black soldier fly larvae (BSFL) frass through liquid chromatography tandem mass spectrometry (LC-MS/MS) with specific focus on phytohormones and their links to frass microbial composition. Fresh frass (Fr-Cycle 1), obtained after a single round of larval bioconversion of food waste, and recirculated frass (Fr-Cycle 2), produced by reintroducing fresh frass into a subsequent bioconversion cycle, were evaluated. Frass recirculation did not affect overall nutrient composition but resulted in marked reductions in humic (80%) and fulvic (65%) acid concentrations, likely due to further decomposition of organic matter during recirculation. In parallel, recirculation significantly altered the bacterial community, decreasing the relative abundance of Actinobacteria while increasing Proteobacteria. Saliently, this is the first mass spectrometric evidence for the presence of cytokinins (both cZ and tZ, as well as their precursors) in BSFL frass, but several other phytohormones were identified in frass at nanomolar levels, including auxins (IAA, PAA, IAM, among others), abscisic acid, salicylates, indoleamines and an ethylene precursor. Interestingly, specific microbial genera were found to be positively correlated with phytohormones, including *Proteus*, *Providencia* and *Morganella*. The potential connections between microorganisms, phytohormones and physico-chemical characteristics of frass might help explain the results reported in the current literature exploring frass as a biofertilizer or soil amendment. The results presented herein open new possibilities for approaching BSFL frass research from a new perspective, paving the way for future studies aiming at a more mechanistic understanding of its interactions with soil, plants and microorganisms.

**Keywords**: phytohormone; microbiome; signalling; auxin; cytokinin; black soldier fly.

## 1. INTRODUCTION

Waste management technologies provide the foundation for recycling organic materials and recovering valuable resources that would otherwise be discarded (Demirbas 2011). Among these approaches, insect-based bioconversion of biowaste - particularly using black soldier fly (BSF) larvae (*Hermetia illucens*, Diptera: Stratiomyidae) - has expanded rapidly in recent years and holds substantial potential to enhance circularity within food production systems (Hamam et al. 2024). In addition to a larval biomass that can be used as an animal feed ingredient, the waste bioconversion process with BSF larvae generates a material that is rich in organic matter and plant nutrients, called frass (Gärttling and Schulz 2022). Depending on the feed substrate and the overall efficiency of the bioconversion process, up to 500 kg of frass can be produced from one tonne of biowaste (Lopes et al. 2022). This underscores the importance of frass within this emerging technology, as it constitutes the most abundant output of the bioconversion process (Lopes et al., 2022).

Frass from BSF larvae has been thoroughly evaluated for its potential use as a fertilizer or soil amendment in recent years, as summarised below. From an agronomic perspective, BSF frass is comparable to compost or vermicompost in relation to its organic matter concentration, particle size and nutrients availability (Lopes et al., 2025). But in addition to that, due to the constant moulting of the developing larvae, frass also contains chitin, a conserved structural polysaccharide recognized by plants' immune system that triggers immune-related responses facing different stressors (Gong et al. 2020). Chitosan (a polysaccharide that derives from chitin) is a widely used molecule in agriculture that when used as a priming agent, enhances plants' resistance to pathogens; *e.g.* frass has been shown to induce synthesis of antifungal compounds and thickening of the plants cell walls when infected by the fungi *Cercospora beticola* (Escobar Rodríguez et al. 2025). Also, chitin functions as an immune elicitor that mediates the crosstalk between chitin signalling pathways and biotic/abiotic stress

signalling (Gong et al. 2020). Both the presence of chitin and nutrients, combined with its varying microbial composition (Gold et al. 2020), rendering frass a unique organic fertilizer.

Despite the demonstrated benefits of frass, it was currently proposed that due to a rapid waste bioconversion process (normally below 14 days), the organic matter in fresh frass is not fully decomposed (Lopes et al., 2025). Post-processing is required to mature and stabilise frass, to reduce the phytotoxicity level and increase the safety for use, *e.g.* through thermophilic composting (Lopes et al., 2025; Song et al., 2021). In a recent study, Lopes et al. (2024) hypothesized that frass could be recirculated back into a new bioconversion step as part of the larval diet, promoting an additional degradation step that would further increase the decomposition degree and maturity of the organic matter. That method was denoted “frass recirculation” and it was shown to result in a higher quality frass fertilizer with increased stability (advanced degree of organic matter decomposition, with resistance to further decomposition) and maturity (readiness for use without harming the plants), assessed by several parameters (*e.g.* pH, respiration, self-heating capacity, seed germination index, among others) (Wichuk and McCartney, 2010).

The impact that BSF frass exerts on the fundamental plant-soil interactions is yet not fully understood. Phytohormones play pivotal roles in regulating plant growth and development, and in responding to biotic and abiotic stresses (Wong et al. 2015; Bhattacharyya et al. 2015a; Egamberdieva et al. 2017; Perera et al. 2025). Thus, the simultaneous quantitative profiling of different groups of phytohormones can be valuable for determining additive, synergistic or antagonistic hormonal activities in any biofertilizer or natural compounds of interest (Tarkowski et al. 2009; Zhang et al. 2015; Vrobel and Tarkowski 2023). Within the phytohormones, cytokinins are also produced by plants and soil organisms, including bacteria, fungi, insects and earthworms. These organisms can release this

hormone to the environment, having positive implications for soil health and plant growth (Wong et al. 2015; Aremu et al. 2015; Jameson 2023). Among the different phytohormones, cytokinins are considered the major hormonal group as they regulate many developmental and physiological processes in plants. Specifically, they have a pivotal role in regulating cell division, proliferation and differentiation, and also the control of various processes in growth and development, including promotion of shoot growth, inhibition of root development, fruit and seed development, delay of senescence, the transduction of nutritional signals, as well as a role in response to both abiotic and biotic stress (Dodd et al. 2010; Yong et al. 2014; Jameson and Song 2016; Jameson 2023)

We have limited knowledge about the effects that BSF frass exerts on the fundamental plant-soil interactions. To the best of our knowledge, no complete characterization of plant biostimulants (especially for the pivotal cytokinins) that can be found in frass has been undertaken, nor how these compounds are related to the microbial composition. A careful search of literature identified one study describing the presence of bioactive compounds in BSF frass, including biogenic amines and a limited selection of phytohormones (*e.g.* indoleacetic acid, abscisic acid, jasmonic acid and gibberellins, but without cytokinins) (Green 2023). Several studies have demonstrated beneficial effects of frass in cultivating varying crops, which has been suggested to be related to either to the presence of plant biostimulants (*e.g.* chitin or exogenous phytohormones in the frass) that triggers beneficial metabolic traits in the organisms, or even to the differential endogenous regulation of hormones in frass-fertilized plants. For instance, Beesigamukama et al. (2020) demonstrated an increased nutrient use efficiency in maize plants fertilized with frass; Barragán-Fonseca et al. (2023) reported a significant shift in the production of volatile compounds in black mustard (*Brassica nigra*) due to the application of frass in pots; Agustiyani et al. (2021) observed plant growth promoting effects of

frass applied to pak choi (*B. rapa*); and Susanto et al. (2021) reported accelerated flowering of corn and pak choi when fertilising with frass. Considering the widespread presence of phytohormones in the environment and in waste streams that can be converted by BSF larvae (Stirk and van Staden 2010; Zhang et al. 2015), it could be assumed that frass also contains these compounds including active hormones, their precursors and catabolites. Additionally, it is likely that the microbial load in frass is somehow associated with its hormonal profile, since microorganisms modulate hormones in the soil-plant continuum (Yong et al. 2014; Egamberdieva et al. 2017) and the effects behind the agricultural input of exogenous phytohormones through organic fertilizers is strongly mediated by the fertilizers' microorganisms and their associated secretions/metabolites (Wong et al. 2015; Aremu et al. 2015; Abbott et al. 2018).

Considering the recently developed knowledge on frass stabilization and improved quality through recirculation, and the need for understanding the potential benefits that this novel, stabilized frass fertilizer could bring to more sustainable agricultural practices (in comparison to fresh, less stabilized frass that is widely being used worldwide), this study was designed with the goals of characterizing frass' bioactive composition. Furthermore, the potential connections between these bioactive compounds and frass' microorganisms was also investigated. It was hypothesized that the microbial diversity in frass would be closely connected to its bioactive composition, which would be then regulated by microorganisms.

## 2. Material and Methods

### *2.1 Materials*

Food waste was collected directly from two restaurants located at the Swedish University of Agricultural Sciences (SLU), campus Ultuna, Uppsala, Sweden. The waste was collected over two weeks on a daily basis, ground to a particle size of 1-2 mm and immediately frozen at -18 °C. Young

larvae (1.46 ± 0.19 mg individual weight) were obtained from a BSF rearing unit located at the same campus of SLU, which has been running continuously since 2015. Before use, the waste was thawed inside a cold storage room (4 °C).

*2.2 Experimental design and frass production*

The frass fertilizers evaluated in this study were obtained from the larval bioconversion of catering waste collected in two restaurants located at the Swedish University of Agricultural Sciences (SLU Uppsala, Sweden). Two treatments with five replicates each were designed, namely Fr-Cycle 1, in which larvae were reared solely on food waste, and Fr-Cycle 2 in which larvae were reared solely on frass derived from a previous food waste bioconversion (Figure 1).

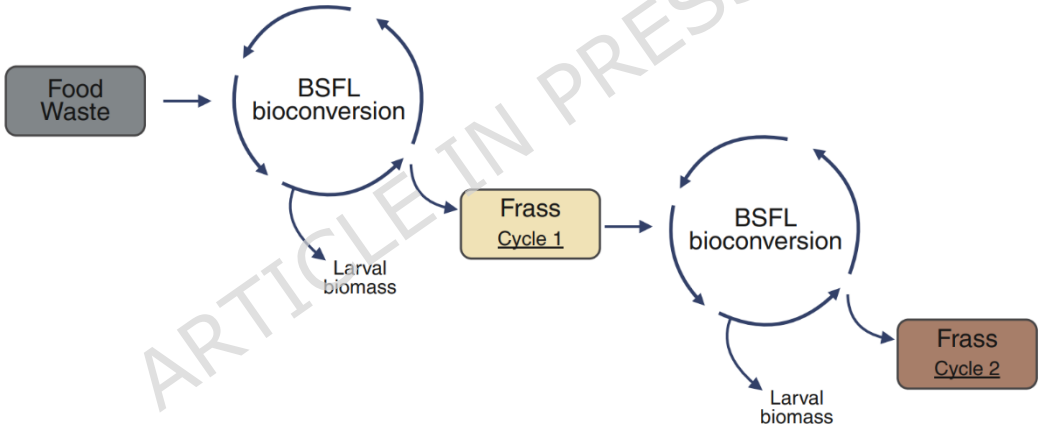


**Figure 1.** Schematic representation of the experimental design used in this study. Fresh frass (Fr-Cycle 1), obtained after one bioconversion step using food waste as a feed substrate, was used as the sole feed substrate for a second step of bioconversion, generating recirculated frass (Fr-Cycle 2).

Details of the bioconversion process and frass production can be found in Lopes et al. (2024). Briefly, the young larvae were reared in plastic boxes ($A$ = 231 $cm^2$) at a density of 5 larvae $cm^{-2}$, receiving a feed dose that ranged from 262 (Fr-Cycle 1) to 376 mg (Fr-Cycle 2) of volatile solids (VS) per larva. Larvae were reared for 12 days and manually sieved out from the frass using a 2 mm mesh sieve (3 mm mesh). After sieving, the frass fertilizers were immediately placed in -20 °C until further analysis.

*2.3 Physico-chemical and bioactive composition of frass*

Three replicates from each treatment were randomly chosen for physico-chemical analysis of frass, while samples from all the five replicates were used for phytohormonal analysis. Dry matter (DM), organic matter, total nitrogen, total organic carbon (TOC), C/N ratio, humic and fulvic acid content, total phosphorus and potassium, in addition to pH and EC were determined as described in Lopes et al. (2024). In addition, the plant hormones existing in the frass samples were analysed according to Vrobel et al. (2024). Briefly, samples were lyophilized and homogenized. A small mass (10 mg) of biological material was extracted using a solvent (7:2:1 acetonitrile:$H_2O$:isopropanol - ACN:$H_2O$:IPA mixture) containing a mixture of stable isotope-labelled internal standards. Extraction was performed in an ice-cold ultrasonic bath for 30 min, followed by centrifugation (20,000 g, 4 °C for 15 min). The supernatants were transferred into 2 mL Eppendorf tubes and evaporated *in vacuo*. Residues were reconstituted in 50 µL of methanol (MeOH) and sonicated for 5 min, followed by the addition of 950 µL of 1M formic acid (FA). Samples were sonicated for an extra 5 min and centrifuged (20,000 g, 4 °C for 20 min), and the supernatants were loaded onto equilibrated Oasis® MCX columns (1 mL, 30 mg) that were activated with 1 mL MeOH and equilibrated with 1 mL of 1M FA. A subsequent washing was performed with 1 mL of Milli-Q water, followed by elution with 1.5 mL of a $NH_4OH$ solution (1.34 M) in 90% methanol, and then evaporated *in vacuo*. Dry residues were reconstituted in 60 µL of sample diluent (a 1:2:10 aq. 30 mM $NH_4FA$ pH 3.0:IPA:ACN mixture), sonicated for 15 min, filtered, transferred to a total recovery vial and then analysed by HILIC-MS/MS.

Chromatographic separation was performed on an analytical column Acquity® BEH Amide 1.7 µm, 2.1×150 mm. Each sample was injected twice in two complementary analytical runs - Method A and Method B. Method A utilized binary gradient at a flow rate of 0.3 mL $min^{-1}$ (30 °C), with mobile

phases A (5 mM acetic acid adjusted by NH4OH to pH 5.5 in 95% ACN) and B (5 mM $NH_4$AcA pH 5.5 in 50% ACN) of a following profile: 0-1 min 0% B; 8 min 37% B, 9.2 min 66% B; 9.3 min 100% B; 11.8 min 100%; 11.9 min 0% B; and 26 min 0% B. Method B employed binary gradient of a flow rate 0.3 mL $min^{-1}$ (30 °C) with mobile phases A (5 mM $NH_4$FA pH 3.0 in 95% ACN) and B (5 mM $NH_4$FA pH 3.0 in 95% ACN) of a following profile: 0-1 min 0% B; 12 min 77% B; 12.1 min 100% B, 15 min 100% B; 15.1 min 0% B; and 26.5 min 0% B. All pH adjustment were performed in aqueous solution before adding organic solvent. The injected sample volume was always 5 µL. All analyses were carried out on a Shimadzu® Nexera X2 modular LC system coupled to a MS-8050 mass spectrometer. Quantification was achieved by multiple reaction monitoring in schedule time windows.

*2.4 Microbial analysis of frass*

Samples from the five replicates of each treatment were analysed for the composition of bacterial and fungal groups. The DNA in the samples was extracted following the protocols of the Earth Microbiome Project (https://earthmicrobiome.org/protocols-and-standards/), using PowerSoil extraction kits (Qiagen, Venlo, Netherlands). The extracted DNA was submitted to two sequencing runs, with the V4 hypervariable region of the 16S rRNA gene being amplified using the primers 515F and 806R (Caporaso et al., 2023), modified with Illumina adaptor overhangs. Subsequently, the amplified products were submitted to a second PCR for indexing, where Illumina Nextera XT indices were added to each sample. Paired-end sequencing was conducted on the Illumina MiSeq platform. A similar protocol was used for sequencing the region ITS1 for fungal groups determination. The raw sequence data were pre-processed in DADA2 v.1.26.0 following the recommended workflows for bacteria and fungi. The databases SILVA v.138 (Callahan, 2024) and UNITE v.10 (Abarenkov et al. 2024) were used for taxonomic assignment of the bacterial and fungal ASVs, respectively. Low-abundance ASVs (< 0.005% total reads), singletons

and doubletons, as well as the ASVs that were not taxonomically assigned at phylum level were filtered from the data. Similarly, sequences of ASVs that were not taxonomically assigned on genus level were extracted and uploaded to NCBI nucleotide BLAST.

*2.5 Statistical analysis*

Physico-chemical parameters and bioactive traits of frass were evaluated for normality of errors (Shapiro-Wilk's test) and homoscedasticity of variances (Levene's test). Once the normality and homogeneity of data were verified, a Student's *t*-test was carried out considering a 5% probability level ($p < 0.05$) for verifying significant differences between the two treatments.

In relation to microbiome data, the Shannon diversity and the Pielou's evenness indexes were calculated using the *alpha()* function in the microbiome v.1.23.1 package (Lahti et al., 2017). The *betadisper()* function from the vegan v.2.6.10 package (Oksanen et al., 2022) was used to check for the homogeneous dispersion of treatment groups. Data with homogeneous group dispersions were subjected to permutational multivariate analysis of variance (PERMANOVA), using the *adonis2()* function, to test for statistical differences between treatment groups. PCoAs were calculated using the *cmdscale()* function, while phytohormone data were fitted using the *envfit()* function of the vegan package v.2.6.10 (Oksanen et al., 2022). Spearman's correlation between bacterial and fungal abundance data and phytohormone data was calculated using the *cor.test()* function and the *p* values were adjusted using the Benjamini-Hochberg correction, in order to control false discovery rates. The genus-level heatmaps were generated using the *amp_heatmap()* function in the ampvis2 v.2.8.9 package, and Venn plots were plotted using the *ggvenn()* function of the ggvenn v.0.1.10 package (Yan, 2025). Other plots were constructed using the ggplot2 v.3.5.1 package (Wickham, 2016). Statistical analysis and plot construction were carried out in R v.4.3.1 (R Core Team, 2023) using the RStudio IDE (R Core Team, 2023).

## 3. Results

### *3.1 Physico-chemical characteristics of frass*

The frass fertilizer resulting from the bioconversion process in which the BSF larvae were fed solely frass (Fr-Cycle 2) resulted in significant changes in its physico-chemical characteristics in comparison to when larvae were fed exclusively food waste (Fr-Cycle 1). Significant reduction in organic matter, TOC and humic substances, as well as an increase in the pH, was demonstrated when feeding the larvae pure frass as compared to feeding them with sole food waste. While the concentrations of phosphorus and potassium changed slightly, no statistical difference was observed for total nitrogen ($p$ = 0.1795) and a 23% reduction in the EC was observed in Fr-Cycle 2. Similarly, a reduction of 67% in the humic extract was observed in Fr-Cycle 2 frass in comparison to Fr-Cycle 1 (Table 1).

**Table 1.** Physico-chemical characteristics of frass from the bioconversion of either food waste (Fr-Cycle 1) or the bioconversion of food waste-derived fresh frass (Fr-Cycle 2). Values are presented as mean ± SD (N = 3) and the $p$ value represents a significant level of 5% according to the Student's t-test.

| | **Fr-Cycle 1** | **Fr-Cycle 2** | ***p* value** |
|---|---|---|---|
| Dry matter (%) | 66.7 ± 1.4 | 50.6 ± 0.8 | < 0.0001 |
| Organic matter ($\%_{DM}$) | 87.5 ± 0.5 | 82.7 ± 0.9 | 0.0012 |
| Total organic carbon (%) | 45.7 ± 0.3 | 40.5 ± 0.5 | 0.0001 |
| Total nitrogen ($\%_{DM}$) | 3.78 ± 0.03 | 3.82 ± 0.04 | 0.1795 |
| Organic nitrogen ($\%_{DM}$) | 2.8 ± 0.1 | 3.1 ± 0.1 | 0.0199 |
| C/N ratio | 12.0 ± 0.1 | 10.7 ± 0.6 | 0.0160 |
| Humic acid (%) | 9.7 ± 0.5 | 1.8 ± 0.2 | < 0.0001 |
| Fulvic acid (%) | 20.0 ± 0.2 | 7.0 ± 0.5 | < 0.0001 |
| Total humic extract | 26.4 ± | 8.8 ± 0.6 | 0.0047 |

| | | | |
|---|---|---|---|
| (%) | 5.3 | | |
| $P_2O_5$ (%$_{DM}$) | 0.43 ± 0.02 | 0.52 ± 0.03 | 0.0150 |
| $K_2O$ (%$_{DM}$) | 2.13 ± 0.07 | 2.70 ± 0.04 | 0.0002 |
| pH | 7.1 ± 0.1 | 8.3 ± 0.1 | < 0.0001 |
| EC (mS cm$^{-1}$) | 25.9 ± 0.9 | 19.9 ± 0.3 | 0.0003 |

*3.2 Phytohormonal profiles of frass*

The method for phytohormone analysis by Vrobel et al. (2024) targeted eight groups of compounds: 1) cytokinins (CKs); 2) auxins (Aux); 3) abscisic acid and its metabolites (ABAs); 4) salicylates (SAs); 5) jasmonates (JAs); 6) gibberellins (GAs); 7) 1-aminocyclopropane-1-carboxylic acid (ACC), and 8) melatonin and its biosynthetic precursors (here referred to as indoleamines). Representatives of six groups were detected in both types of frass samples, only missing JAs and GAs. The concentration of phytohormones in frass was found to be generally lower in the pure frass (Fr-Cycle 2) in comparison to fresh frass derived from a food waste bioconversion (Fr-Cycle 1). While only the *cis* form of zeatin (*c*Z) was found in frass from the different cycles at similar concentrations, *t*Z was observed only in Fr-Cycle 1 at a concentration of 14.4 ± 0.9 pmol g$^{-1}_{DM}$. The zeatin's precursor iP was found in higher concentrations in Fr-Cycle 1 in relation to Fr-Cycle 2, as well as the riboside forms of *c*Z that is *c*ZR (64% higher in Fr-Cycle 1) and iP that is iPR (81% higher in Fr-Cycle 1) (Table 2). The same trend was observed for the hormones belonging to the auxins class, which were measured at nanomolar levels. Indole-3-Acetic Acid (IAA) was found in a much higher concentration in Fr-Cycle 1 (894 nmol g$^{-1}_{DM}$) in relation to Fr-Cycle 2 (17 nmol g$^{-1}_{DM}$), while the auxin-like compound phenylacetic acid (PAA) was found to be 45% higher in Fr-Cycle 1, even though not statistically different ($p$ = 0.2433). Both the precursor (IAM) and catabolite

(OxIAA) of auxins, as well as PEA were also found in higher concentrations in Fr-Cycle 1 in comparison to Fr-Cycle 2 (Table 2).

The interaction between frass physico-chemical characteristics and phytohormones profile was also investigated through a correlation analysis, considering specifically the organic component of the two frass samples, namely in terms of TOC, humic and fulvic acid contents, given the significant reductions in the concentration of several phytohormones in Fr-Cycle 2 compared to Fr-Cycle 1. Most of the profiled hormones correlated positively with the concentration of those parameters, with the exception of the auxin PAA (Pearson's correlation index ranging from 0.57 to 0.73, $p > 0.05$) (Table S1).

**Table 2.** Concentration of phytohormones from distinct classes (cytokinins, auxins, abscisic acid, salicylates, indoleamines and ethylene) in frass from either the bioconversion of post-consumer food waste (Fr-Cycle 2) or from the bioconversion of frass that derived from the same waste stream (Fr-Cycle 2). Values are presented as mean ± SD (N = 5) and the *p* value represents a significant level of 5% according to the Student's t-test. ND: not detected.

| | **Fr-Cycle 1** | **Fr-Cycle 2** | ***p* value** |
|---|---|---|---|
| *Cytokinins* | | | |
| tZ (pmol $g^{-1}_{DM}$) | 14.4 ± 0.9 | ND | - |
| cZ (pmol $g^{-1}_{DM}$) | 58.1 ± 16.4 | 40.4 ± 11.3 | 0.0818 |
| iP (pmol $g^{-1}_{DM}$) | 1308.9 ± 332.7 | 276.9 ± 74.9 | 0.0001 |
| cZR (pmol $g^{-1}_{DM}$) | 15.1 ± 4.4 | 5.6 ± 0.8 | 0.0039 |
| iPR (pmol $g^{-1}_{DM}$) | 48.7 ± 15.3 | 8.8 ± 2.5 | 0.0004 |
| *Auxins* | | | |
| IAA (nmol $g^{-1}_{DM}$) | 894.2 ± 147.8 | 17.2 ± 7.1 | < 0.0001 |
| PAA (nmol $g^{-1}_{DM}$) | 173.4 ± 131.6 | 95.5 ± 11.1 | 0.2433 |
| IAM (nmol $g^{-1}_{DM}$) | 1.1 ± 0.1 | 0.4 ± 0.0 | < 0.0001 |
| OxIAA (nmol $g^{-1}_{DM}$) | 10.9 ± 3.3 | 0.2 ± 0.0 | < 0.0001 |

| | | | |
|---|---|---|---|
| *Abscisic acid and metabolites* | | | |
| ABA (nmol $g^{-1}_{DM}$) | 2.1 ± 0.2 | 0.3 ± 0.1 | < 0.0001 |
| PA (nmol $g^{-1}_{DM}$) | 8.5 ± 1.6 | 0.7 ± 0.4 | < 0.0001 |
| DPA (nmol $g^{-1}_{DM}$) | 80.7 ± 4.7 | 5.6 ± 4.3 | < 0.0001 |
| NeoPA (nmol $g^{-1}_{DM}$) | 0.3 ± 0.1 | 0.6 ± 0.2 | 0.0158 |
| *Salicylates* | | | |
| SA (nmol $g^{-1}_{DM}$) | 27.5 ± 1.5 | 2.85 ± 0.6 | < 0.0001 |
| BA (nmol $g^{-1}_{DM}$) | 1630.8 ± 157.2 | 463.8 ± 54.4 | < 0.0001 |
| *Indoleamines* | | | |
| TRA (nmol $g^{-1}_{DM}$) | 705.1 ± 106.5 | 21.6 ± 9.5 | < 0.0001 |
| *Ethylene* | | | |
| ACC (nmol $g^{-1}_{DM}$) | ND | 7.25 ± 0.8 | - |

Regarding the concentrations of abscisic acid (ABA) and its catabolites phaseic acid (PA) and dihydrophaseic acid (DPA), these were all statistically higher in Fr-Cycle 1 in comparison to Fr-Cycle 2 ($p < 0.0001$), while the isomer form of PA (NeoPA)was the single hormone that was found in higher concentration in cycle 2 frass than in Fr-Cycle 1 (Table 2). The concentrations of salicylic acid (SA) and its precursor benzoic acid (BA) were 9-fold and 3-fold higher in Fr-Cycle 1 in comparison to Fr-Cycle 2, as also observed for the indoleamine TRA. The only indoleamine found in the frass samples was tryptamine, which was found in much higher concentration in Fr-Cycle 1 in comparison to Fr-Cycle 2, while the precursor of ethylene ACC (1-Aminocyclopropane-1-Carboxylic Acid) was found solely in the Fr-Cycle 2 (Table 2).

*3.3 Frass bacterio- and mycobiota and their relation to phytohormones*

The diversity of bacterial communities was significantly different in the Fr-Cycle 2 samples in comparison to Fr-Cycle 1, while fungal communities were found to be similar between treatments, as observed by the Shannon diversity (Figure 2a) and the Pielou's evenness (Figure 2b) indices. Actinobacteria and Firmicutes were the most abundant bacterial phyla in both frass types, but a significantly higher presence of Proteobacteria was observed in the Fr-Cycle 2 frass. As for fungal phyla, primarily Ascomycota and Basidiomycota were observed in both treatments, with a stronger prevalence of the first (Figure 2c). As observed in the constructed Venn diagrams, a big overlap between Fr-Cycle 1 (green) and Fr-Cycle 2 (yellow) was observed for fungal ASVs (Figure 2e), while a less pronounced overlap was found for bacteria (Figure 2d).

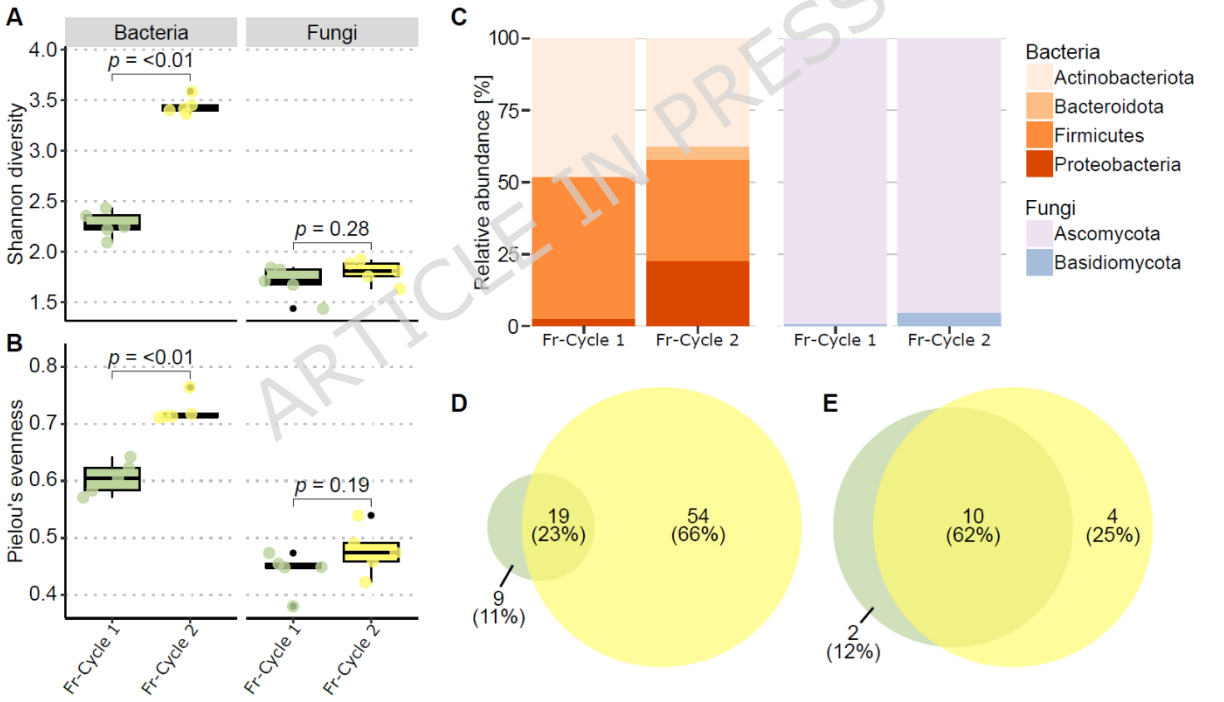


**Figure 2.** Indexes related to the microbial composition of fresh (Fr-Cycle 1) and recirculated (Fr-Cycle 2) frass. Alpha diversity in bacterial and fungal communities as described by the Shannon diversity (A) and the Pielou's evenness (B) indices; stacked bar charts showing the phylum-level composition of bacterial and fungal communities (C); Venn diagrams showing the distinct and shared number of core ASVs in bacterial (D) and fungal (E) communities in Fr-Cycle 1 (green) and Fr-Cycle 2 (yellow) frass fertilizers.

The most abundant bacterial genera in Fr-Cycle 1 assessed were *Corynebacterium* and *Pseudogracilibacillus*, while a more even distribution

of genera was observed in Fr-Cycle 2, including *Corynebacterium*, *Pseudomonas*, *Halomonas*, *Atopostipes* and others (Figure 3a). The fungal community observed was dominated by *Pichia* and *Candida* in both frass fertilizers, with a minor appearance of *Trichosporon* in Fr-Cycle 2 (Figure 3b). Additional genera of bacteria and fungi that were abundantly identified in the frass samples are presented in Figure S1.

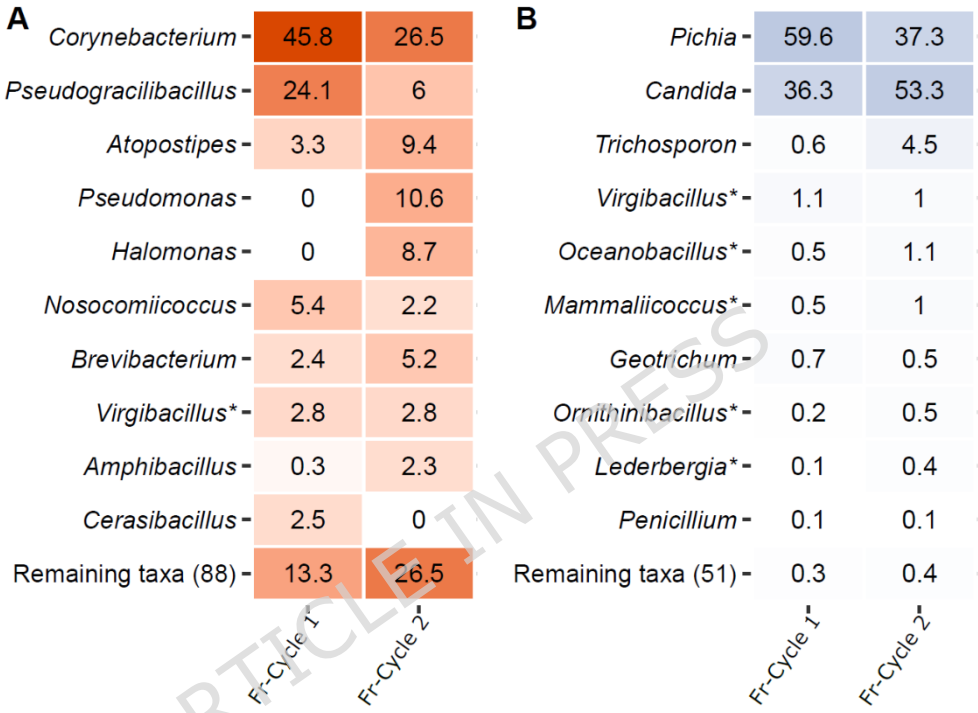


**Figure 3.** Top 10 bacterial (A) and fungal (B) genera found in fresh and recirculated frass based on relative abundance. Asterisks (*) next to taxon names denote that genus-level taxonomy was assigned through a nucleotide BLAST® search for sequences that were not previously classified using the SILVA database.

The interactions between hormones and bacterial communities of frass fertilizers derived from treatments Fr-Cycle 1 were significantly different from those found in Fr-Cycle 2, demonstrating that the phytohormonal profile of the two materials were different (Figure 4a). Over 90% of the total variation of the model was explained within PCoA1 and PCoA2. While Fr-Cycle 2 was more closely associated solely with NeoPA, the community found in Fr-Cycle 1 displayed higher complexity, being strongly associated with *c*Z and PAA, and associated to a lower extent to auxins (OxIAA, IAA and IAM) and ABA. As for fungal communities, around

90% of the total variation was also explained by the two PCoA (Figure 4b). However, a greater overlap between Fr-Cycle 1 and Fr-Cycle 2 was observed in comparison to bacterial communities, again with NeoPA being one of the major contributors to rightward variations in PCoA1, but now more associated with Fr-Cycle 1. Individual PCoAs were built with every major group of phytohormones for both bacterial (Figure S2) and fungal (Figure S3) communities and are presented in the Supplementary Material.

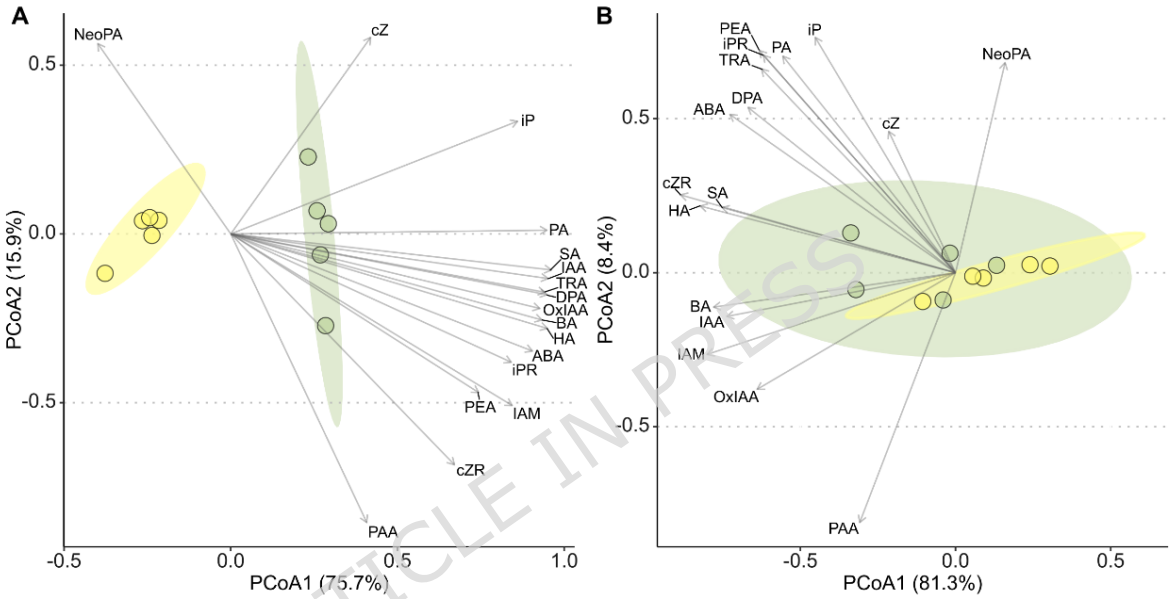


**Figure 4.** Principle coordinate analysis (PCoA) of bacterial (A) and fungal (B) communities in Fr-Cycle 1 (green) and Fr-Cycle 2 (yellow) frass fertilizers with phytohormone concentrations fitted as environmental variables (arrows), indicating their contribution to the spread of sample groups.

Specific bacterial and fungal genera were found to correlate significantly with the analysed phytohormones (Figure 5). In the heatmap, constructed with all the frass samples analysed, using the relative abundance of microbial genera and the concentration of phytohormones, genera with at least one significant and very strong ($\rho \geq 0.81$) positive correlation with some phytohormone were included. All the bacterial genera belonging to the phyla Actinobacteria, Bacteroidota and Firmicutes were correlated positively solely with NeoPA (an isomeric form of phaseic acid) and the ethylene precursor ACC. However, many representants of the families

Bacillaceae and Lactobacillaceae belonging to the Firmicutes phylum were positively correlated with several of the phytohormones, with stronger correlations observed for instance between *Oceanobacillus* and BA, and between *Cerabacillus*, tZ and DPA. Regarding the phylum Proteobacteria, some genera also correlated positively with selected phytohormones, highlighting *Providencia* with tZ and ABA, *Morganella* with IAA, OxIAA and ABA (Figure 5; Table S2). In terms of fungi, no significant positive correlations were found between genera and the phytohormones analysed (Table S3).

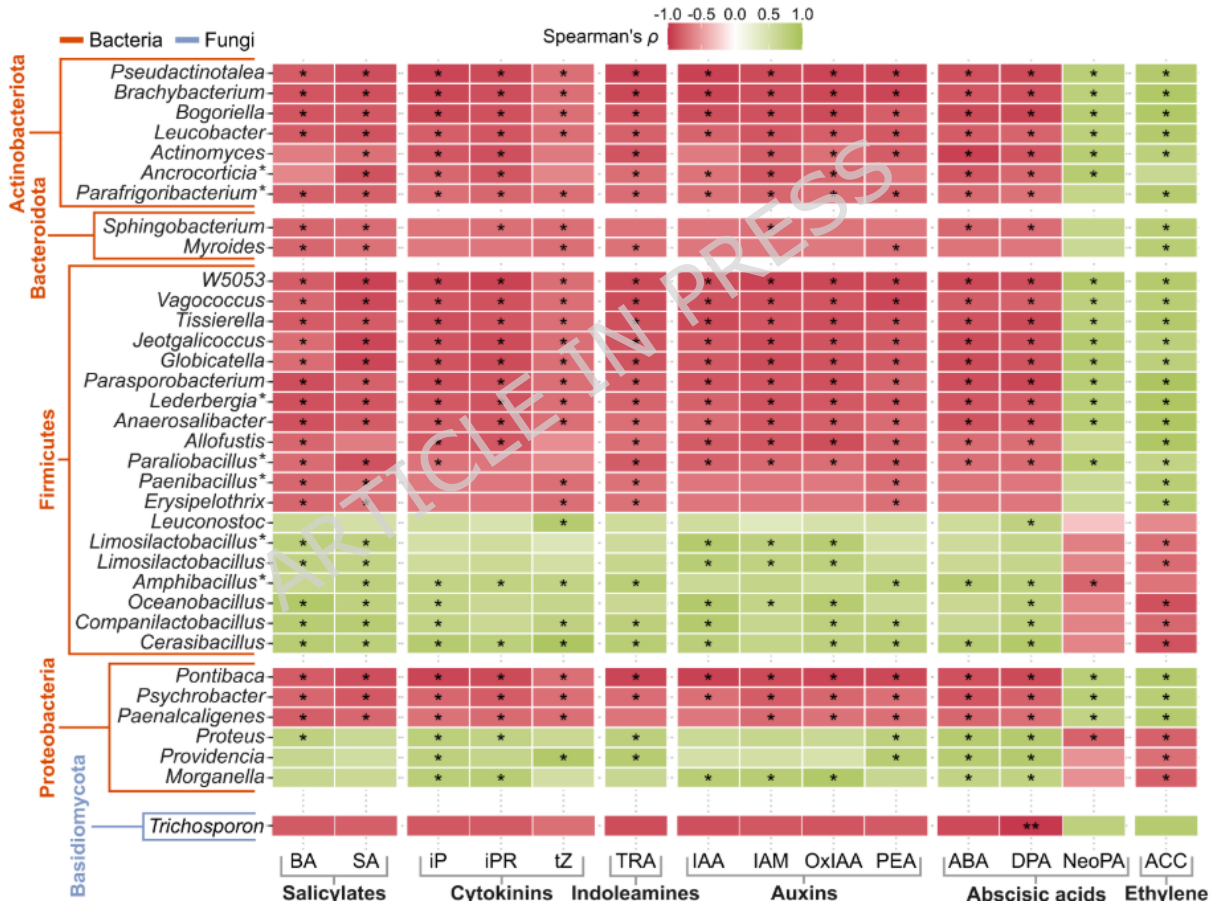


**Figure 5.** Spearman rank correlation calculated between phytohormones and bacterial and fungal genera. All genera with at least one very strong (Spearman's ρ >= 0.81) positive correlation with a phytohormone are shown. *p*-values were adjusted using Bonferroni-Holm correction. Asterisks (*) next to taxon names denote that genus-level taxonomy was assigned through a nucleotide BLAST® search for sequences that were not previously classified using the SILVA database. Significance levels are illustrated by asterisks within the tiles: * = 0.05 > *p* >= 0.01, ** = 0.01 > *p* >= 0.001.

## 4. Discussion

*4.1 Frass recirculation strongly affected frass physico-chemical traits*

Recirculating frass back in another bioconversion step as a dietary component for young BSF larvae resulted in similar results to the ones reported by Lopes et al. (2024) in relation to the physico-chemical quality of frass. The significant reductions observed in the organic matter, TOC and C/N ratio of the recirculated frass' resembles the same changes observed in mature compost in comparison to fresh compost, including the pH increase and EC reduction (Bernai et al. 1998). With an increased degree of organic matter decomposition, it was expected that humic substances would be found at similar or higher concentrations in Fr-Cycle 2 in comparison to Fr-Cycle 1. However, as pointed out by Guo et al. (2019), humic acids are highly complex molecules that are formed through the action of microbial groups in succession over time, using the precursors originating from the raw material breakdown. It was plausible to infer that even after a second round of bioconversion (with 10-12 days, similar to the first biowaste transformation), there is not enough time or conditions for the formation of humic substances in frass, which would in fact require a composting process or other long-term organic matter stabilization.

*4.2. Frass contains substantial concentrations of phytohormones*

Plant hormones and related compounds in BSF frass has been reported only by Green (2023), with only a few compounds being reported, and with a different methodology Consequently, meaningful comparisons can be made only with various plant tissues and other organic fertilizers. We presume that the phytohormones detected in the frass originate primarily from the pool of phytohormones present in the food waste used to rear the larvae. The substantial decrease in their contents from cycle 1 to cycle 2 suggests that potential microbial synthesis plays only a marginal role in producing the phytohormones compared with the predominant process of phytohormone catabolism. Nevertheless, the concentrations measured in both Fr-Cycle 1 and in some cases in Fr-Cycle 2 remain orders

of magnitude higher than those typically reported in plant tissues such as leaves, roots, or tubers (Ordaz-Ortiz et al. 2015; Široká et al. 2022; Vrobel et al. 2024). For some compounds, such as iP, levels in frass samples differ by up to three orders of magnitude, making these tissues an unlikely source of these compounds. In contrast, plant seeds (grains) are known to accumulate high endogenous hormone levels, as for example documented in wheat and maize (Matsuura et al. 2019; Ciarkowska et al. 2022; Jameson 2023). They may therefore represent a more plausible initial reservoir of hormones within the food waste, ultimately leading to a formation of a frass containing levels of Aux, CKs and ABAs much higher than those found in other organic fertilizers such as compost (Table 2).

In addition to the high concentration of phytohormones in the frass, their profiles are distinct from profiles typically found in plant material and further undergoes changes between bioconversion cycles (Tarkowski et al. 2009; Hošek et al. 2020; Vrobel et al. 2024). The analytical method employed in this study targets not only active hormones but also their precursors, transport forms, and catabolites, granting deeper insight into the processes occurring within the material. Somewhat unexpectedly, the Aux and CK pools in the frass consisted almost entirely of active hormones, in stark contrast to the profiles commonly found in living plant tissues, whereas the ABA pool showed no such deviation. For CKs, the bioactive free bases (iP, tZ, and cZ) accounted for 95.5% and 95.7% of the total CK pool in Fr-Cycle 1 and Fr-Cycle 2, respectively. Likewise, the Aux pool was dominated by free IAA, detected at several orders of magnitude higher concentrations (tens to hundreds of nmol/g DW) in Fr-Cycle 1, although substantially reduced in Fr-Cycle 2. In contrast, the ABA pool consisted predominantly of inactive catabolites (PA, DPA in Table 2). The predominance of inactive Aux and CKs forms in plant tissues is well established, reflecting their strict regulation (Fukui and Hayashi, 2018; Zhao et al. 2024): once perceived by target cells, bioactive compounds are

rapidly inactivated, and inactive forms often represent most of the total pool (Fukui and Hayashi 2018; Zhao et al. 2024). Catabolic pathways of Aux and CKs commonly involve conjugation to amino acids (Aux) or monosaccharides, mainly glucose (Aux and CKs) (Hošek et al. 2020; Hayashi et al. 2021; Brunoni et al. 2023) forming reversible storage forms. ABA catabolism, however, proceeds through one of three (species-specific) pathways based on hydroxylation followed by non-enzymatic rearrangement, forming irreversible catabolites (Nambara and Marion-Poll 2005).

The unusually high abundance of active Aux and CKs in the frass raises the question of whether these compounds contribute to its plant growth-promoting effects. The origin of these bioactive hormones remains unclear, but one plausible explanation is their release from the abundant conjugated forms present in plant tissues, particularly seeds, which were likely present in the food waste used for rearing the larvae in the first cycle of bioconversion. However, it is not yet known at which stage this release occurs, during food processing or during larval digestion of the waste, as no studies have examined how these processes affect phytohormone stability. Based on the obtained results, it is plausible to assume that, even if larval digestion of the food waste containing the hormones is the reason behind hormones release in the frass, the lower concentration observed in Fr-Cycle 2 leads to the conclusion that a repeated digestion of the material containing the compounds results in a further reduction of its concentration. Future studies should investigate the presence of phytohormones in all stages of the bioconversion, including non-processed and processed food waste.

BSF frass remains a rich source of bioactive phytohormones even when compared with other organic fertilizers. Sienkiewicz et al. (2024) reported phytohormone contents across several organic fertilizers, including five types of compost and a biohumus extract, and found either an

absence or only low abundance of phytohormones, with the exception of brassinosteroids in the biohumus extract. Similarly, mature compost derived from the water fern *Salvinia molesta* contained at least tenfold lower concentrations of cZ, tZ, and IAA (Arthur et al. 2007). Additional studies on liquid fertilizers such as vermicompost leachates also reported markedly lower hormone levels. For instance, Aremu et al. (2015) measured CKs and IAA at concentrations four to five orders of magnitude lower, while Zhang et al. (2014) measured CK pools roughly three orders of magnitude lower; yet iP constituted the majority of the cytokinin pool.

More recently, Green (2023) quantified a few phytohormones in BSF frass and catering leachate and reported extremely low concentrations; however, these stark discrepancies are likely attributable to methodological differences. Overall, our findings diverged substantially from previous reports examining phytohormone content in biological fertilizers. We do not aim to discuss each phytohormonal concentrations in detail, in order to avoid overinterpretation. In addition, because the chemical composition of food waste, including their associated microorganisms can vary significantly between batches (Denafas et al. 2014), the measured phytohormonal profiles of food waste-derived frass would likely always be variable to some extent. Nevertheless, we show that both Fr-Cycle 1 and Fr-Cycle 2 contain exceptionally high concentrations of bioactive plant hormones, in particular iP and IAA, compared with other organic fertilizers, strongly suggesting that these compounds contribute to reported plant growth-promoting effects of BSF frass.

Frass recirculation resulted in a significant decrease in the concentration of humic substances, as observed in the total humic extract in Fr-Cycle 2 ($8.8 \pm 0.6\%_{DM}$) that was 67% lower in comparison to Fr-Cycle 1 ($26.4 \pm 5.3\%_{DM}$), similarly to the analysed concentration of phytohormones. Liu et al. (2020) observed around a 50% reduction in fulvic acids in BSFL-bioconverted manures but an increase in humic acid (up to 3-fold) after

bioconversion. Wang et al. (2021), on the other hand, reported structural changes in organic matter following bioconversion, highlighting a higher aromatic degree and increased molecular weight of organic molecules. Nevertheless, as discussed by Lopes et al. (2024), the complexity of humic substance formation and the variability in feedstock types (*e.g.* manures vs. food waste) may explain why no increase - but rather a decrease - in humic substances was observed in Fr-Cycle 2. Despite the lower concentration of humic substances in Fr-Cycle 2, certain microbial taxa were proportionally more abundant in this treatment than in Fr-Cycle 1, indicating a shift in community composition, likely due to the repeated larval digestion of the frass. This suggests that the phytohormone profile of frass might not be directly linked to the overall microbial abundance, but rather to specific microbial taxa and its organic matter profile. Additionally, given the well-known role of microorganisms in modulating phytohormones within the soil-plant continuum (Orozco-Mosqueda et al. 2023; Liu et al. 2024), it is feasible to consider frass subjected to more than one cycle of bioconversion as a source of exogenous biostimulants, including microorganisms and phytohormones. Fresh frass (Fr-Cycle 1) can also be considered a source of exogenous phytohormones, but it should be noted that food waste-derived fresh frass can have phytotoxic traits that overcome the potential benefits of having hormones in its composition, as reported by Sani et al. (2025).

As reported previously, fresh frass is often biologically and chemically unstable, frequently causing stunted plant growth due to its phytotoxic properties (Song et al. 2021). In contrast, frass recirculation has been shown to enhance the stability of the material, allowing for the application of higher doses and promoting plant growth (Lopes et al. 2024). Still, in the present study, increasing the number of BSFL bioconversion cycles of food waste led to a notable reduction in both the humic extract content and phytohormone concentrations. Phytohormones affect plants in a dose-dependent manner, ranging from growth stimulation when applied in low

concentrations, to growth inhibition at high concentrations (Weyers and Paterson 2001).

Considering the complexity of BSF frass composition in terms of nutrients, bioactive compounds and microorganisms, simple explanations for the potential biostimulant action of frass are unlikely and would be considered extensively speculative. Thus, it is plausible that even with lower concentrations of humic substances and phytohormones in Fr-Cycle 2 in comparison to Fr-Cycle 1, its effects on plant growth might still be positive; albeit this has to be further investigated. Without the combination of plant bioassays with general omics approaches (*e.g.* acquiring relevant transcriptomics, proteomics, metabolomics and phenomics data), it will be difficult to unravel the mechanisms behind frass' bioactive traits in plant growth. Nevertheless, as recently discussed by Li et al. (2025), the application of exogenous sources of plant biostimulants, including phytohormones and microbial inoculants have an enormous potential to increase crops resistance to biotic and abiotic stress, increasing agriculture's sustainability and resilience. Therefore, future studies should evaluate the effects of different types of frass derived from various waste streams, on plant growth, considering the interplay between phytotoxicity and phytohormone levels, and preferably combining omics tools. It is noteworthy that in this study, the additional bioconversion cycle that generated Fr-Cycle 2 was carried out using only frass as a feed substrate, with the aim of investigating the effects of such approach in the composition of the obtained fertilizer. However, it is likely that this will not be practical under real industrial conditions, while frass inclusion as a part of the feed substrate - such as the inclusion levels evaluated by Lopes et al. (2024) - is expected to occur in real settings. Thus, frass recirculation should be considered as an inoculation of frass, similarly to what is done in composting, when mature compost is added to the process, increasing the retention time of the material in the cycle.

*4.3 Frass recirculation changes the dynamics of bacterial groups*

Frass recirculation clearly changed the fertilizer's microbial composition in relation to frass from the first cycle of bioconversion. Frass from the second cycle (Fr-Cycle 2) displayed a significantly higher richness and evenness of bacterial communities in relation to Fr-Cycle 1. This might indicate that with an additional bioconversion step, there is an accumulation of microorganisms (likely deriving from the larval gut) in the resulting frass. However, the contribution of gut microorganisms' accumulation over several cycles of bioconversion needs to be further investigated. Interestingly, the observed increase in Proteobacteria together with a reduction in the proportion of Firmicutes and Actinobacteria resembled the microbial dynamics seen during thermophilic composting of biowaste, as demonstrated by Tian et al. (2012) throughout a 112 day-long composting process of dairy manure and rice chaff. Similar results were found by Wei et al. (2018) when composting rice and maize straw and by Meng et al. (2019) during the composting of cow manure and corn straw. This indicates that recirculation has the potential of increasing frass maturity as a proxy for a microbial composition shift towards the predominance of certain groups. Following the microbial dynamics of composting, it is likely that fungal communities were not statistically distinct between Fr-Cycle 1 and Fr-Cycle 2 because fungi play their major roles during the long-term curing composting phase, which was possibly not achieved with two cycles of BSFL bioconversion. Future studies should investigate if the adoption of several cycles of bioconversion would affect also the fungal community in frass.

While the most dominant fungal genera in Fr-Cycle 1 and Fr-Cycle 2 were *Pichia* and *Candida*, with a slightly increase in *Trichosporon* being observed in Fr-Cycle 2, the bacterial communities changed significantly after a second cycle of bioconversion (Figure 1d; Figure 2). *Corynebacterium* dominated Fr-Cycle 1 together with *Pseudogracilibacillus*, while Fr-Cycle 2 had a community with higher richness of groups (Figure 2a). Interestingly,

several of the genera associated with high relative abundances in frass samples have been demonstrated to have a biostimulatory effect in plants, both *in vitro* and *in vivo*. For instance, El-Tarabily (2004) demonstrated that *Trichosporon asahii* inhibited the growth of *Rhizoctonia solani in vitro*, through biostimulant-mediated enzymatic and volatile compounds metabolism that inhibited mycelial growth. Similarly, it was demonstrated by Dodd et al. (2010) that isolates from the rhizosphere of several plant species containing *Corynebacterium* spp. had phytohormone-induced growth stimulation and protection mechanisms, both *in vitro* and *in vivo*.

Several genera belonging to the family Bacillaceae and Lactobacillaceae were found in both frass types Fr-Cycle 1 and Fr-Cycle 2 (Figure 2a). Several members of those families are known to exert plant growth-promoting effects, especially boosting plants metabolism and growth under abiotic stress conditions (Tsotetsi et al. 2022; Sun and Shahrajabian 2025). For instance, *Bacillus* spp. is a genus known to produce cytokinins (Poveda and González-Andrés 2021). These microorganisms coordinate plant growth during drought and salt stress conditions by regulating endogenous IAA levels in plants through differential gene expression, stimulating the root meristem initiation, thus promoting root branching (Iqbal et al. 2014). Not only for auxins, but the genera *Bacillus* also regulates other hormones in plants, including cytokinins, gibberellins, and abscisic acid, by either producing the hormone (including its precursors) or mediating its production within a plant's metabolism (Etesami et al. 2023). Several of the bacteria found in the frass samples in this study (*e.g. Pseudomonas*, *Morganella*, *Providencia*, *Cerabacillus*, among others) have been isolated from the BSF larval gut (Tanga et al. 2021; Gorrens et al. 2021). Thus, the results presented herein corroborate those studies and showcase the contribution of the BSF larvae core gut microbiome to the microbial composition in frass (Klammsteiner et al. 2020).

Interestingly, several positive correlations were found between specific microbial taxa and phytohormones, both in relation to the main active forms of the hormones (*e.g.* IAA and tZ) and to their precursors (Figure 4). While the catabolite of abscisic acid NeoPA and ethylene were found to correlate positively with several taxa, the majority of taxa related to other hormones belongs to the families Bacillaceae (*Amphibacillus*, *Cerasibacillus*), Amphibacillaceae (*Oceanobacillus*) and Lactobacillaceae (*Limosilactobacillus* and *Companilactobacillus*), as well as some Proteobacteria of the families Morganellaceae (*Morganella* and *Providencia*) and Enterobacteriaceae (Proteus). Several species belonging to those genera have known biostimulatory effects in plants, including abiotic stress resistance, phytohormone production and others.

Karadeniz et al. (2006) demonstrated, by means of high performance liquid chromatography (HPLC), that *Proteus vulgaris* (Enterobacteriaceae), *Bacillus cereus* and *Bacillus megaterium* (Bacillaceae) produced several plant growth regulators (auxins, gibberellins, abscisic acid and cytokinins) *in vitro*. Similarly, *P. vulgaris* was showcased as a plant growth promoting rhizobacteria in the cultivation of *Arabidopsis thaliana*, by triggering phytohormones' production pathways, as verified by Bhattacharyya et al. (2015). Orhan and Demirci (2020) demonstrated that a strain of *Oceanobacillus* containing plant growth-promoting traits (including siderophore production and IAA, phosphate solubilization and nitrogen fixation) increased the salt stress tolerance of barley. Similarly, *Morganella* spp. was identified as contribution to plant growth promoting traits, with special regard to biocontrol mechanisms, identified by their potential in inhibiting the growth of plant pathogens (Das et al. 2022).

It is noteworthy that some of the most abundant genera found in the present study that also correlated with selected phytohormones - such as *Providencia*, *Morganella*, *Proteus* and some *Bacillaceae* - has been previously reported in the BSF larvae gut and frass, in several distinct

studies, rearing the larvae in various waste streams (Gold et al. 2020; Tanga et al. 2021; Gorrens et al. 2021; Klammsteiner et al. 2025). This, together with the results presented in this study, supports the hypothesis that frass microbial and bioactive composition is directly dependent on the contribution of larval gut's microorganisms.

## 5. Conclusions

Frass from black soldier fly larvae contains a significant concentration of phytohormones as part of its bioactive composition, and especially cytokinins and auxins. When frass is recirculated back as a dietary component for the larvae, the additional decomposition process lowered the concentration of humic substances and phytohormones, concomitantly reducing the fertilizer's phytotoxic effect. Even though this effect was verified in food waste-derived frass, future studies should investigate if that is the case for other biowaste streams. The recirculation process increases stability of frass and its microbial richness, especially with bacterial groups, and specific groups found in frass (including *Proteus*, *Providencia*, *Morganella* and species of the families *Bacillaceae* and *Lactobacillaceae*) displayed positive correlations with several phytohormones, demonstrating the importance of the larval gut's microbiome to the bioactive composition of frass.



## 7. Declarations

Author contributions: I.G.L, T.M. and O.V. conceived the idea, conducted experiments, analysed the data and wrote the original manuscript; P.T., C.L. and J.W.H.Y. provided insights, gathered funding, assisted the analysis, supervised and revised the manuscript. All authors revised and approved the final version of the manuscript.

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

Data availability: data will be made available upon reasonable request. The raw sequences used in the microbial profiling and the corresponding metadata will be made available at the European Bioinformatics Institute (EBI).

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