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Bacterial supplementation during black soldier fly rearing improves frass plant growth-promoting properties

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Abstract

Background

Black soldier fly (BSF, *Hermetia illucens*) frass is increasingly valued as an organic fertilizer, yet how the rearing substrate and its microbial supplementation shape the frass bacterial community and its agronomic quality remains poorly understood.

Aims

We assessed whether microbial supplementation of an olive pomace substrate affects black soldier fly larval performance and the agronomic and microbial quality of the resulting frass.

Methods

The substrate was supplemented with two commercial *Bacillus velezensis* formulations (CAL, LAL) and two gut-derived strains selected for broad enzymatic capabilities (LG, closest match *Paenibacillus cookii*; PD, closest match *Shouchella clausii*), each supplied live or heat-inactivated. Larval performance was recorded, the heat-treated frass (70°C, 60 min) was evaluated on cherry tomato, and the frass bacterial community was characterized by 16S rRNA sequencing.

Results

Effects on larval performance were modest and preparation-dependent: inactivated PD increased larval weight, inactivated LG increased harvest weight, and frass production increased with live CAL, LAL and LG. All supplemented frass types increased root dry weight relative to non-supplemented frass, with LG-F performing best, and frass generally promoted greater plant biomass than mineral and organic fertilizers while maintaining germination. Despite similar chemical profiles, treatment explained 79.1% of the variation in frass community structure; *Bacillus* was enriched in LAL-F and *Oceanobacillus*, a genus with plant growth-promoting traits, in three of the four supplemented treatments.

Conclusions

Although strain viability was not confirmed, microbial supplementation during rearing altered the bacterial composition and agronomic value of BSF frass, supporting its potential as a tailored biofertilizer.

INTRODUCTION

The black soldier fly (BSF) *Hermetia illucens* is increasingly deployed to convert low-value organic by-product into high-value protein and lipids for animal feed (with the sector projected to reach about 4 billion USD by 2033; Meticulous Research, 2024), as well as frass, an insect-derived organic fertiliser with considerable potential in agriculture (Lomonaco et al., 2024). Its high bioconversion efficiency, modest land and water demands, and capacity to upcycle food waste, positioning it as a key element of circular food systems (Amorim et al., 2024). BSF larvae demonstrate remarkable versatility in treating organic by-product streams from diverse food, agricultural, and industrial sources, including fruits and vegetables, brewery and winery by-products, and kitchen waste. Depending on substrate composition, BSF larvae can achieve waste reduction rates ranging from 12% to 85% (Ramzy et al., 2022). BSF larval products provide a relevant protein source for poultry, swine, and companion animals (Bukchin-Peles et al., 2025) and can partially replace fishmeal in aquaculture, reducing pressure on wild fisheries and moderating cost volatility (Hender

et al., 2021; Kobayashi et al., 2015). Compared with conventional livestock, BSF protein production is associated with lower greenhouse gas emissions, and farming can be organized in stacked, climate-controlled facilities with a much smaller land footprint and minimal freshwater inputs, as larvae obtain moisture from the substrate (van Huis & Oonincx, 2017).

However, the successful implementation of BSF bioconversion technology heavily depends on substrate characteristics, with some agricultural residues presenting significant processing challenges. Among the significant agricultural by-product streams generated by the olive oil industry in the Mediterranean region, Olive Pomace (OP) represents a major challenge for sustainable by-product management. According to the International Olive Oil Council, olive oil production generates OP quantities of 330–550 kg per metric ton of processed olives, representing approximately 30% of olive oil manufacturing output (Ramzy et al., 2022). This lignocellulosic waste is characterized by high concentrations of cellulose, hemicellulose, and lignin, along with significant levels of antinutritional factors including phytates and phenolic compounds (Rodriguez-González et al., 2025; Ramzy et al., 2022).

On such substrates, BSF performance can decline, as residues rich in lignocellulose or antinutritional factors limit nutrient access and conversion efficiency (Gold et al., 2018). In OP specifically, BSF larvae showed significantly reduced final body weight and extended development, as its high lignin content remains largely undigested (Ramzy et al., 2022). To mitigate such constraints, dietary supplementation with beneficial microorganisms has been explored in other animal systems to enhance fiber and phytate degradation and to improve host performance (Bandari et al., 2024; Nalla et al., 2022; Santos et al., 2019). Such microorganisms, when live and administered in adequate amounts, may act as probiotics, defined as live microorganisms that confer a health benefit on the host (Hill et al., 2014). In BSF, studies supplementing the substrate with *Bacillus*, *Lactobacillus*, and other gut isolates report substrate-dependent and strain-specific improvements in growth, feed conversion, and enzyme activity, although outcomes remain inconsistent (Gorrens et al., 2023).

In parallel, large-scale rearing of BSF generates substantial quantities of frass, the principal by-product of industrial production. The material flows involved can be considerable; for example, a single commercial facility processing approximately 1,400 kilograms of organic by-product per week generated about 635 kilograms of frass (Wang et al., 2017). Frass is attracting interest as an organic fertiliser because of its nutrient content and agronomic potential (Amorim et al., 2024). In the European Union, it is regulated under Regulation (EU) 2021/1925, which defines frass as a mixture of insect excreta, residual feeding substrate, insect parts, and eggs, with dead insects permitted at no more than 5% by volume and 3% by weight, and which, since November 2021, classifies it as processed animal manure requiring heat treatment at 70°C for 60 min before marketing (European Commission, 2021).

Heat treatment alters the microbiological quality of frass. In black soldier fly frass (BSF-F), treatment at 70°C for 60 min significantly reduces the total cultivable microbial load, microbial activity, and microbial biomass, whereas bacterial endospores remain largely unaffected (Praeg & Klammsteiner, 2024). Although this mandatory pretreatment is intended to ensure pathogen removal, it may also inhibit plant's beneficial microbes, potentially altering the value of frass as a fertiliser. This concern is supported by research on mealworm (*Tenebrio molitor*) frass, where microbial communities play a crucial role in the fertiliser properties of insect excreta: Poveda et al. (2019) reported that sterilized frass produced significantly lower plant growth parameters than non-sterilized frass, including reduced chlorophyll content, fresh weight, and aerial length of plants cultivated under both normal and abiotic stress conditions, and that frass harbored diverse PGP bacteria. Together, these findings suggest that the mandatory heat treatment of BSF-F may inadvertently eliminate beneficial microorganisms that contribute to its agronomic value.

To address these challenges, the present study evaluated the microbial supplementation of the rearing substrate with commercial *Bacillus velezensis* formulations and gut-derived bacterial strains selected for their functional traits, considering both its effect on larval performance and on the resulting frass. We hypothesised that supplementing the

rearing substrate with bacteria selected for enzymatic traits relevant to lignocellulose and antinutritional factor degradation could influence BSF larval performance on olive pomace and, in turn, modify the microbial composition and agronomic properties of the resulting frass. The specific objectives of this study were: (1) to evaluate the effects of microbial supplementation of the substrate (commercial *B. velezensis* formulations and BSF gut isolates) on *H. illucens* larval performance when reared on OP; (2) to assess the PGP properties of the resulting frass through greenhouse trials with tomato plants; and (3) to characterise the bacterial community composition of the heat-treated frass across treatments.

MATERIALS AND METHODS

Insect and gut samples

Young BSF larvae in the fifth stage of development from EntoGreen's industrial production (Santarém, Portugal), reared on olive pomace (OP), were used for gut bacteria isolation. Larvae were surface-disinfected by sequential immersion in sterile distilled water (15 s), 70% ethanol (15 s), and a final rinse in sterile distilled water. Under sterile conditions, the whole digestive tract of 10 randomly selected larvae was dissected and placed in 1 mL of 0.9% NaCl, then homogenized with sterile plastic micro-pestles to release microorganisms. The homogenate was transferred to 9 mL of sterile 0.9% NaCl and vortexed, and tenfold serial dilutions were prepared.

Isolation of thermotolerant gut bacteria displaying functional traits

Tenfold dilutions of the homogenates were incubated at 80°C for 24 h to ensure the elimination of vegetative cells and enrich for heat-resistant spore-forming bacteria, then plated onto Luria-Bertani (LB) medium (1.5% agar) supplemented with nalidixic acid (30 mg/L) at dilutions of 10^{-3} to 10^{-6} and incubated at 28°C for 48 h. Individual colonies were selected based on morphology and colour, subcultured until pure, and preserved in 20% glycerol at -80°C for further experiments and long-term storage.

To screen for plant-polymer degradation, each isolate was spotted onto square LB agar plates (1.5%) supplemented with 0.5% carboxymethyl cellulose (CMC) or 0.5% birchwood xylan for cellulolytic and xylanolytic activity, respectively, or with 1% potato starch or 1% citrus peel pectin for amylolytic and pectinolytic activity, respectively (all substrates from Sigma-Aldrich, Darmstadt, Germany).

After 48 h of incubation, CMC and xylan plates were stained with 1% Congo red for 10 min and washed with 1 M NaCl to visualize hydrolysis halos of cellulolytic and xylanolytic bacteria. Starch and pectin plates were flooded with Lugol's iodine for 20 s; positive isolates were identified by clear halos against the dark blue background, indicating starch and pectin degradation.

To detect phytase activity, isolates were inoculated onto phytase screening medium (20 g/L glucose, 4 g/L sodium phytate, 2 g/L calcium chloride, 5 g/L ammonium nitrate, 0.5 g/L potassium chloride, 0.5 g/L magnesium sulfate, 0.1 g/L iron sulfate, 0.1 g/L manganese sulfate, 15 g/L agar) and incubated at 28°C for 48 h. Phytase activity was identified by the formation of clear halos around isolates indicating the degradation of phytate, an antinutritional factor that restricts nutrient availability.

Identification of thermotolerant gut-derived bacterial displaying functional traits

Only bacterial isolates capable of hydrolysing all four tested plant polymers and phytate were selected for identification. DNA was extracted from these isolates using the DNeasy Blood and Tissue Kit (Qiagen Sciences) following the manufacturer's protocol. Only bacterial isolates capable of hydrolysing all four tested plant polymers and phytate were selected for identification. DNA extraction, 16S rRNA gene amplification with primers 27F and 1492R, purification, and Sanger sequencing were performed as described in Cruz et al. (2025). Sequences were aligned in BioEdit and compared to type strains in the EzTaxon 16S rRNA database (Kim et al., 2012) through the EzBioCloud platform (Chalita et al., 2024).

Preparation of bacterial inoculants.

Two commercial formulations based on *B. velezensis* and two bacterial strains with functional traits, derived from the BSF gut microbiota and isolated in this work, were used as bacterial inoculants in this study. The two commercial formulations were hereafter referred to as CAL and LAL to avoid the use of commercial product names throughout the manuscript. CAL is a spore-based probiotic feed additive based on *B. velezensis* DSM 15544 and LAL is a spore-based biofertiliser formulation based on *B. velezensis* NCIMB 30322.

B. velezensis was selected because it is a thermotolerant, spore-forming bacterium with a demonstrated capacity to degrade plant-derived fibers, has been identified as a member of the *H. illucens* gut microbiome, and has shown positive effects when supplemented to BSF larvae (Tegtmeier et al., 2021; Klüber et al., 2024; Pei et al., 2022; Chen et al., 2021). In addition, this species exhibits well-documented PGP traits and disease-suppressive properties (Figueiredo et al., 2025; Arabzadeh et al., 2023), making it a suitable candidate for the two objectives of this study: improving larval performance and enhancing the agronomic value of frass.

The two remaining strains, isolated from the BSF gut and selected based on their functional traits, exhibited the highest number of enzymatic activities among all screened isolates, including the ability to hydrolyze all four tested plant polymers and phytate.

For inoculum preparation, each strain was cultured in two 2-L Erlenmeyer flasks containing 500 mL of LB broth at 28°C for 48 h. One flask per strain was then autoclaved at 121°C for 15 min to obtain heat-inactivated cells; these heat-inactivated preparations were included to account for any purely nutritional contribution of the bacterial biomass. Cells from both live and inactivated cultures were harvested by centrifugation (10,000 rpm, 7 min), washed three times with sterile 0.9% NaCl, and adjusted to the same cell density (OD_{600} = 1) for both live and heat-inactivated preparations. The viable cell concentration of the live preparation was verified by plate counting, yielding approximately 1×10^8 CFU/mL. This procedure was applied uniformly to all treatments.

Larval performance of BSF fed olive pomace supplemented with commercial bacterial formulations and gut-derived bacterial strains

A feeding experiment was conducted to evaluate the effects of substrate supplementation with commercial bacterial formulations and gut-derived strains on BSF larval performance. For each treatment, 50 mL of sterile 0.9% NaCl containing the corresponding bacterial preparation (OD_{600} = 1) was mixed into the larval diet.

The bacterial treatments comprised the two commercial *B. velezensis* formulations (CAL and LAL) and the two gut-derived strains, LG (closest match *Paenibacillus cookii*, 99.01% 16S similarity) and PD (closest match *Shouchella clausii*, 99.38% 16S similarity). Each was tested as both live (Live) and autoclaved (Aut) preparations at the same cell density (OD_{600} = 1), allowing biological effects of the bacteria to be distinguished from their nutritional contribution. The Control treatment received 50 mL of sterile 0.9% NaCl without bacterial inoculant.

Each experimental unit consisted of a plastic box (60 × 40 × 12 cm) containing 12.5 kg of the EntoGreen standard diet, which comprised 80% OP; the remaining 20% is proprietary and classified by the company. Each treatment was replicated ten times, with approximately 20,000 five-day-old larvae per replicate. All units were maintained under industrial rearing conditions at the EntoGreen facilities (29 ± 5°C, 60% relative humidity, 12:12 h photoperiod).During the twelve days of rearing, the following performance parameters were recorded: mean larval weight, determined from a random sample of ten larvae per box; total harvest weight and frass weight, recorded per box; and pupation rate, calculated as the proportion of pupae among the sampled individuals. Following completion of the bioconversion process by the BSF larvae, the material was sieved to separate the larvae from the resulting organic fertiliser.

Effect of frass derived from bacterial-supplemented BSF larvae on tomato plant growth parameters

Frass collection and sanitization

Frass was collected from the boxes of the larval feeding trial, taking the frass from the live-bacteria treatments (CAL, LAL, LG, and PD) and from the non-supplemented Control boxes. All frass samples were subjected to mandatory sanitation by heat treatment at 70°C for 60 min, in accordance with Regulation (EU) 2021/1925, prior to their use in the tomato cultivation experiment.

Soil and frass characterization

Prior to soil preparation and fertilisation, the chemical properties of the soil and of all frass types were determined. For each treatment, frass recovered from the corresponding boxes was pooled, and a single composite sample per treatment was analysed. Both soil and frass samples were characterised at the Servicio de Análisis e Instrumentación of IRNASA-CSIC (Salamanca, Spain) for pH (H_2O), carbonate content ($CaCO_3$), organic carbon (OC), organic matter (OM), total nitrogen (N), C/N ratio, available phosphorus (P), exchangeable calcium (Ca), potassium (K) and magnesium (Mg), and electrical conductivity (EC). pH was measured potentiometrically in water; $CaCO_3$ by calcimetry; OC and N by combustion; P by the Olsen method; and exchangeable Ca, K and Mg by extraction with ammonium acetate and quantification by ICP-OES. EC was measured conductimetrically at a soil:water ratio of 1:5 (w/v). The chemical composition of the soil and the nutrient profiles of each frass type are presented in Table 2.

16rRNA frass bacteriome characterization

Following completion of the larval experiment and frass collection, DNA was extracted from heat-treated frass using the DNeasy PowerSoil Pro Kit (Qiagen, Hilden, Germany) from three independent biological replicates per treatment. The V3–V4 hypervariable region of the 16S rRNA gene was amplified using primers 341F (5'-CCTAYGGGRBGCASCAG-3') and 806R (5'-GGACTACNNGGGTATCTAAT-3') and sequenced at Novogen (UK) on an Illumina NextSeq platform (250 bp paired-end reads) following library construction with the NEBNext kit (New England Biolabs, USA). Raw paired-end reads were quality-filtered and processed using the DADA2 package (v1.34.0) in R (v4.4.3) (Callahan et al., 2016), including error modelling, denoising, paired-end merging, chimera removal, and inference of amplicon sequence variants (ASVs); low-quality bases were truncated at position 250. Taxonomic assignment was performed using the SILVA v138.2 reference database with species-level refinement via the addSpecies function (Pruesse et al., 2007). Alpha diversity was assessed using the Shannon diversity index and ASV richness (Observed); differences among treatments were evaluated with the Kruskal-Wallis test followed by Dunn's post hoc test with Benjamini-Hochberg correction. Beta diversity was examined by principal coordinates analysis (PCoA) based on Bray-Curtis dissimilarities; group separation was assessed by PERMANOVA (999 permutations) and homogeneity of multivariate dispersion by PERMDISP, both using the vegan package in R (Oksanen et al., 2018). Differential abundance of bacterial genera across treatments was evaluated using ANCOM-BC2 with pairwise comparisons (Lin et al., 2020) and Holm correction for multiple testing. The relative

abundance of genera corresponding to the inoculated taxa (*Bacillus*, *Paenibacillus*, and *Shouchella*) was calculated per sample based on SILVA taxonomic assignments and summarized as treatment means to assess the detectability of supplemented taxa in the heat-treated frass; these values are presented as genus-level proxies, as 16S rRNA sequencing does not provide strain-level resolution.

Soil and amendment preparation, experimental design, and plant measurements

The chemical characterization data (Table 1) were used to standardize application rates across treatments, ensuring an equivalent nitrogen input of 250 kg N ha^{-1} for all frass and fertiliser treatments. Seven treatments were compared: untreated frass from the control boxes of the larval trial that received no bacterial inoculant (C-F); frass from larvae reared on substrate supplemented with the commercial *B. velezensis* formulations CAL and LAL (CAL-F and LAL-F); frass from larvae reared with the gut-derived strains LG and PD (LG-F and PD-F); and two commercial reference fertilisers, a mineral fertiliser (Mineral) and an organic fertiliser (Organic). Each treatment was applied independently; frass and fertilisers were never combined.

For each treatment, the required mass of frass or fertiliser was calculated from its nitrogen content (Table 1) to reach the target input of 250 kg N ha^{-1}, and thoroughly mixed with the soil before sowing. Each pot (8 × 8 cm) was filled with the soil–amendment mixture and sown with three cherry tomato seeds (*Solanum lycopersicum* var. cerasiforme). Germination was recorded seven days after sowing in 48 pots per treatment (three seeds each). Thereafter, one seedling per pot was retained and grown for the remainder of the experiment, giving 48 plants per treatment for growth evaluation. Pots were arranged in a completely randomized design.

All pots were kept in a greenhouse at 25°C, 60% relative humidity, and a 12:12 h photoperiod, and irrigated regularly to avoid moisture stress. Plants were grown for 30 days after seedling selection. At harvest, leaf chlorophyll content was measured with a SPAD meter (Konica Minolta SPAD-502 PLUS) on the third fully expanded leaf, and the following parameters were recorded: aerial fresh and dry weight, aerial length, root fresh and dry weight, and root length.

Statistical analysis

All statistical analyses were performed using R software (version 2026.01.1). Prior to analysis, data distributions and variance structure were examined to inform the choice of statistical approach. Non-parametric statistical methods were applied for all response variables. Statistical significance was evaluated at $\alpha = 0.05$.

Statistical analysis of BSF larval performance

For BSF performance parameters (mean larval weight, total harvest weight, frass weight, and pupation rate, calculated as the proportion of individuals that had reached the pupal stage within the sampled subset), differences among treatments were first assessed using the Kruskal-Wallis rank sum test as an omnibus test. When a significant overall treatment effect was detected ($p < 0.05$), post-hoc pairwise comparisons among all treatments were performed using Dunn's test, with p-values adjusted for multiple comparisons using the Holm method.

Statistical analysis of plant growth parameters

For plant growth and physiological parameters (chlorophyll content, aerial fresh weight, aerial dry weight, aerial length, root fresh weight, root dry weight, and root length), differences among treatments were first tested using the Kruskal-Wallis rank sum test. When significant effects were detected, post-hoc pairwise comparisons among all treatments were performed using Dunn's test, with p-values adjusted using the Holm method. Germination rate, recorded seven days after sowing as the percentage of germinated seeds per pot in the soil–amendment mixture, was assessed descriptively.

RESULTS

Isolation and identification of thermotolerant gut-derived bacterial displaying functional traits

A total of 18 thermotolerant bacterial strains with distinct colony morphologies were isolated from the gut of *H. illucens* larvae. Functional screening showed that 61%, 55%, 61%, 66%, and 27% of the isolates hydrolysed pectin, starch, cellulose, xylan, and phytate, respectively. Five isolates were able to hydrolyse all four plant-derived polymers as well as phytate. One of them, strain PD, was identified by 16S rRNA gene analysis as *Shouchella clausii* (99.38% similarity to the type strain DSM 8716); the remaining four (LE, LG, PA, and PB) were identified as *Paenibacillus cookii*, with sequence similarities of 98.8%, 99.01%, 97.42%, and 99.7% relative to the type strain. Based on their broad enzymatic profiles, one representative of each taxon was selected for the supplementation experiments: strain LG (*P. cookii*, 99.01% similarity) and strain PD (*S. clausii*, 99.38% similarity).

Larval performance of BSF fed olive pomace supplemented with commercial bacterial formulations and gut-derived bacterial strains.

Pupation Rate

The Kruskal-Wallis test revealed significant differences in pupation rate among treatments ($\chi^2 = 15.731$, df = 8, $p = 0.046$). Treatment medians ranged from 20% (LG Live) to 75% (LAL Live). Post-hoc Dunn's test with Holm correction showed that the only significant pairwise difference was between LAL Live and LG Live ($p = 0.003$); no treatment differed significantly from the Control (median = 40%).

Larval Average Weight

Larval weight differed significantly among treatments ($\chi^2 = 18.446$, df = 8, $p = 0.018$). Post-hoc Dunn's test with Holm correction showed that PD Aut had a significantly higher larval weight than the Control (median 0.247 g vs 0.212 g; $p = 0.009$). No other treatment differed significantly from the Control, and no significant differences were found among the bacterial treatments (Table 1).

Harvest Weight

Harvest weight differed significantly among treatments ($\chi^2 = 16.188$, df = 8, $p = 0.040$). Post-hoc Dunn's test with Holm correction showed that LG Aut had a significantly higher harvest weight than the Control (median 1125.5 g vs 936 g; $p = 0.021$). No other treatment differed significantly from the Control (Table 1).

Frass Weight

Frass weight varied significantly among treatments ($\chi^2 = 46.213$, df = 8, $p < 0.001$). Post-hoc Dunn's test with Holm correction showed that CAL Live and LAL Live produced significantly more frass than the Control (median 2775 g and 2923 g, respectively, vs 2253 g; $p = 0.003$ and $p < 0.001$), as did LG Live (2710 g; $p = 0.044$). Notably, the live preparations of CAL and LAL also yielded significantly more frass than several inactivated treatments; in particular, LAL Live exceeded LAL Aut ($p = 0.005$), CAL Live exceeded LAL Aut ($p = 0.049$), and both exceeded PD Aut ($p < 0.001$). PD Aut, which showed the lowest frass production, did not differ from the Control (Table 1).

Table 1

Larval performance of black soldier fly (*H. illucens*) reared on olive pomace supplemented with the commercial *B. velezensis* formulations CAL and LAL and the gut-derived strains LG and PD, each supplied live or heat-inactivated (Aut). Control, non-supplemented substrate. Parameters are pupation rate (%), mean larval weight (g), total harvest weight (g, total live larval biomass per box) and frass weight (g per box). Values are medians (n = 8–10 boxes per treatment). Differences among treatments were tested by Kruskal–Wallis (χ^2 and p shown); within each column, medians not sharing a letter differ significantly according to Dunn's post-hoc test with Holm correction ($\alpha = 0.05$).

Treatment	Pupation (%)	Larval avg. weight (g)	Harvest weight (g)	Frass weight (g)
Control	40 ^{ab}	0.212 ^{b}	936 ^{b}	2253 ^{c}
CAL Live	30 ^{ab}	0.241 ^{ab}	976 ^{b}	2775 ^{ab}
LAL Live	75 ^{a}	0.230 ^{ab}	978 ^{ab}	2923 ^{a}
PD Live	30 ^{ab}	0.233 ^{ab}	1145 ^{ab}	2535 ^{abc}
LG Live	20 ^{b}	0.229 ^{ab}	950 ^{b}	2710 ^{ab}
CAL Aut	40 ^{ab}	0.235 ^{ab}	1092 ^{ab}	2430 ^{bc}
LAL Aut	40 ^{ab}	0.216 ^{ab}	991 ^{b}	2334 ^{bc}
PD Aut	30 ^{ab}	0.247 ^{a}	976 ^{b}	2228 ^{c}
LG Aut	35 ^{ab}	0.224 ^{ab}	1126 ^{a}	2433 ^{bc}
KW χ^2	15.731	18.446	16.188	46.213
p-value	0.046	0.018	0.040	2.17×10^{-7}

Frass chemical characterization

The chemical characterization of the different frass types revealed highly similar nutrient profiles (Table 2). All frass samples showed comparable pH (7–8), organic carbon (39–42%), total nitrogen (3%), and C/N ratios (13–15), and were markedly richer in phosphorus (10,569–12,329 ppm) and potassium (28,723–32,967 ppm) than the reference soil. Among the frass types, available phosphorus was slightly higher in CAL-F, PD-F, and LG-F than in the C-F, and PD-F showed the highest calcium and magnesium contents.

Table 2
Chemical properties of soil, *H. illucens* frass derived from bacterial-supplemented larvae, and control fertilisers used in the study. $CaCO_3$, calcium carbonate; OC, organic carbon; OM, organic matter; N, total nitrogen; C/N, carbon-to-nitrogen ratio; ppm, parts per million; P, phosphorus; Ca, calcium; K, potassium; Mg, magnesium; EC, electrical conductivity.

Sample	pH	% CaCO3	%OC	%OM	%N	C/N	ppm P	ppm Ca	ppm K	ppm Mg	EC
Soil	7	<L.D. (0,058)	1	1	0	8	23	1145	70	134	0.507
C-F	7	0	40	69	3	14	10569	883	30408	2139	0,004
Chemical Fertiliser	-	-	-	-	27	-	-	-	-	-	-
Organic Fertlizer	7	-	40	69	4,2	11,9	-	-	-	-	-
LAL-F	8	<L.D. (0,058)	39	67	3	15	10977	950	28723	1670	0,003
CAL-F	7	0	41	71	3	14	12329	897	32147	1820	0,004
LG-F	8	0	39	68	3	13	11083	984	32360	1848	0,004
PD-F	7	0	42	73	3	14	11567	1075	32967	2249	0,004

Effect of frass derived from bacterial-supplemented BSF larvae on tomato plant growth parameters

Germination rate

Germination rates ranged from 56.94% to 86.81%. The highest values were recorded for LAL-F (86.81%) and CAL-F (86.23%), followed by LG-F (81.94%) and PD-F (79.86%). C-F reached 75.69%, while the Organic and Mineral treatments showed the lowest rates (66.67% and 56.94%, respectively) (Table 3)

Chlorophyll Content

Leaf chlorophyll content differed significantly among treatments ($\chi^2 = 80.296$, df = 6, $p < 0.001$). The Mineral treatment showed the highest values (median = 27.8), significantly exceeding C-F (median = 25.4) and all frass treatments. Conversely, CAL-F (median = 22.2) and LG-F (median = 21.5) showed significantly lower chlorophyll than C-F, whereas LAL-F and PD-F did not differ from C-F. Notably, chlorophyll was the only parameter in which the mineral fertiliser outperformed the frass treatments (Table 3).

Aerial Fresh Weight

Aerial fresh weight differed significantly among treatments ($\chi^2 = 129.04$, df = 6, $p < 0.001$). LG-F showed the highest value (median = 17.82), significantly exceeding C-F (median = 11.71), LAL-F, and both reference fertilisers. CAL-F (median = 15.87) and PD-F (median = 15.86) also exceeded C-F significantly. All frass treatments produced significantly greater aerial fresh weight than the Mineral treatment (median = 7.72), which showed the lowest values, and most exceeded the Organic treatment (median = 11.59) as well (Table 3).

Aerial Dry Weight

Aerial dry weight differed significantly among treatments ($\chi^2 = 135.69$, df = 6, $p < 0.001$). LG-F (median = 1.32) and PD-F (median = 1.305) showed significantly higher aerial dry weight than C-F (median = 1.035), whereas LAL-F and CAL-F did

not differ from C-F. All frass treatments significantly exceeded both the Mineral (median = 0.65) and Organic (median = 0.77) treatments, which showed the lowest values (Table 3).

Aerial Length

Aerial length differed significantly among treatments ($\chi^2 = 138.33$, df = 6, $p < 0.001^6$). LG-F showed the greatest length (median = 65), significantly exceeding C-F (median = 53), LAL-F, and both reference fertilisers. CAL-F (median = 61) also exceeded C-F significantly, while PD-F (median = 58) and LAL-F (median = 56) did not differ from C-F. All frass treatments produced significantly longer aerial parts than the Mineral (median = 38) and Organic (median = 43) treatments (Table 3).

Root Fresh Weight

Root fresh weight differed significantly among treatments ($\chi^2 = 80.615$, df = 6, $p < 0.001$). LG-F showed the highest value (median = 1.88) and was the only treatment significantly exceeding C-F (median = 1.14). LAL-F, CAL-F, and PD-F did not differ significantly from C-F. All frass treatments significantly exceeded the Mineral (median = 0.82) and Organic (median = 0.61) treatments, which showed the lowest values (Table 3).

Root Dry Weight

Root dry weight differed significantly among treatments ($\chi^2 = 109.75$, df = 6, $p < 0.001$). All four supplemented frass treatments showed significantly higher root dry weight than C-F (median = 0.15): LAL-F (median = 0.53), LG-F (median = 0.39), CAL-F (median = 0.30), and PD-F (median = 0.28), with LAL-F showing the highest value overall. C-F, in turn, significantly exceeded the Mineral (median = 0.085) and Organic (median = 0.10) treatments (Table 3).

Root Length

Root length differed significantly among treatments ($\chi^2 = 26.41$, df = 6, $p < 0.001$). PD-F showed the greatest root length (median = 20), significantly exceeding all other treatments, including C-F (median = 12), the other frass treatments, and both reference fertilisers. No other pairwise differences were significant (Table 3).

Table 3

Effect of frass from microbially supplemented BSF larvae on tomato (*S. lycopersicum* var. cerasiforme) plant growth parameters. Values are medians. Different superscript letters within a column indicate significant differences (Dunn's test, Holm correction, $p < 0.05$). C-F = non-supplemented frass (Frass-Control, bold = reference); CAL-F, LAL-F, LG-F, PD-F = frass from supplemented larvae; Mineral, Organic = commercial reference fertilisers. Germination assessed descriptively (%). LG-F bold = best overall agronomic performance.

Treatment	Aer. length (cm)	Chlorophyll (CU)	Aer. FW (g)	Aer. DW (g)	Root FW (g)	Root DW (g)	Root length (cm)	Germination (%)
Mineral	38 ^{d}	27.8 ^{a}	7.72 ^{d}	0.65 ^{d}	0.82 ^{c}	0.085 ^{d}	14 ^{b}	57
Organic	43 ^{cd}	23.0 ^{bc}	11.59 ^{c}	0.77 ^{d}	0.61 ^{c}	0.100 ^{d}	12 ^{b}	67
C-F	**53** ^{bc}	**25.4** ^{ab}	**11.71** ^{c}	**1.035** ^{c}	**1.14** ^{bc}	**0.150** ^{c}	**12** ^{b}	**76**
LAL-F	56 ^{b}	24.4 ^{ab}	15.00 ^{b}	1.12 ^{abc}	1.52 ^{ab}	0.530 ^{a}	14 ^{b}	87
CAL-F	61 ^{ab}	22.2 ^{bc}	15.87 ^{ab}	1.10 ^{bc}	1.44 ^{abc}	0.300 ^{bc}	13 ^{b}	86
PD-F	58 ^{b}	24.7 ^{ab}	15.86 ^{ab}	1.305 ^{ab}	1.37 ^{abc}	0.280 ^{bc}	20 ^{a}	80
LG-F	**65** ^{a}	**21.5** ^{c}	**17.82** ^{a}	**1.32** ^{a}	**1.88** ^{a}	**0.390** ^{b}	**14** ^{b}	**82**
KW χ^2	138.33	80.30	129.04	135.69	80.62	109.75	26.41	—
p-value	$< 2.2\times10^{-16}$	3.1×10^{-15}	$< 2.2\times10^{-16}$	$< 2.2\times10^{-16}$	2.7×10^{-15}	$< 2.2\times10^{-16}$	1.9×10^{-4}	—

Frass bacteriome composition

The bacteriome of heat-treated frass was characterized to evaluate the effects of microbial substrate supplementation on bacterial community composition, diversity, and richness.

At the genus level, the frass bacterial community was dominated by *Rhodococcus* (15.0–17.7%), *Pseudomonas* (6.2–14.6%), *Sphingobacterium* (6.4–8.1%), and *Timonella* (2.5–7.1%) across all treatments (Fig. 1A). Among the inoculated taxa, *Bacillus* was enriched in the LAL-F treatment (6.7%) compared to all other treatments (< 0.3%), *Paenibacillus* was detected across all treatments including the non-supplemented Control (0.7–1.4%), and no sequences assigned to *Shouchella* were recovered in any treatment (Fig. 1B).

ASV richness (Observed) did not differ significantly among treatments (Kruskal-Wallis: $\chi^2 = 11.033$, df = 4, $p = 0.026$; all pairwise adjusted $p > 0.05$) (Fig. 2A). Shannon diversity differed significantly among treatments ($\chi^2 = 11.433$, df = 4, $p = 0.022$), with pairwise Dunn's test with Benjamini- Hochberg correction showing that only C-F and LG-F differed significantly (adjusted $p = 0.010$), with LG-F exhibiting higher Shannon diversity (Fig. 2B).

Beta diversity based on Bray-Curtis dissimilarities revealed significant differences in community composition among treatments (PERMANOVA: $F_{4,10} = 9.487$, $R^2 = 0.791$, $p = 0.001$), with treatment explaining 79.1% of the variation. PERMDISP confirmed homogeneity of within-group dispersion ($p > 0.05$). PCoA ordination showed clear separation among treatments along the first two axes, which together explained 55.9% of the total variation (Fig. 2C).

Differential abundance analysis (ANCOM-BC2, pairwise comparisons with Holm correction) identified 29 genera that differed significantly between at least one pair of treatments (Fig. 3), including *Bacillus*, *Oceanobacillus*, *Sphingobacterium*, and *Pseudoxanthomonas*.

DISCUSSION

This study evaluated whether microbial supplementation of an OP substrate with commercial *B. velezensis* formulations (CAL and LAL) and two gut-derived strains selected for their enzymatic traits (LG, closest match *Paenibacillus cookii*; PD, closest match *Shouchella clausii*) could improve BSF larval performance and, in turn, modify the bacterial community and enhance the agronomic value of the resulting frass. Supplementation affected larval performance in a strain- and preparation-dependent manner, with significant but modest effects on larval weight, harvest weight, and frass production. More consistently, frass from supplemented larvae improved several tomato growth parameters relative to frass from non-supplemented larvae (C-F), and all frass treatments promoted greater plant biomass than the mineral and organic reference fertilisers, although the mineral fertiliser yielded the highest leaf chlorophyll.

The comparison between live and heat-inactivated (Aut) preparations offers a first insight into whether the larval responses depended on bacterial viability. Both PD Live and PD Aut increased mean larval weight to a comparable extent, and LG Aut increased harvest weight despite consisting of non-viable cells, indicating that these particular effects did not require viable bacteria.

A possible explanation is a nutritional contribution of the bacterial biomass itself. The fact that the significant increase in larval weight was observed for the inactivated preparation PD Aut, rather than for its live counterpart, is consistent with a nutritional rather than a strictly probiotic effect, since inactivated biomass represents an additional source of protein, amino acids, and lipids available to the larvae. This interpretation aligns with Gorrens et al. (2023), who stressed the need for inactivated controls precisely to separate the nutritional value of the added biomass from a genuine probiotic effect. Beyond a purely nutritional role, heat-stable cell components retained after inactivation, such as peptidoglycan, teichoic acids, and surface-layer proteins, may also exert regulatory activity on the host, a possibility formalized by the concept of postbiotics, defined by ISAPP as preparations of inanimate microorganisms and/or their components that confer a benefit on the host (Zhu et al., 2025).

Notably, effects of heat-killed bacteria have already been documented in dipterans: in *Drosophila*, oral administration of heat-killed *Lactobacillus fermentum* enhanced larval locomotion and antioxidant activity to an extent comparable to, or greater than, its live counterpart (Kumar et al., 2023), supporting the notion that non-viable bacterial preparations can elicit measurable phenotypic responses in insects. However, because these and most other postbiotic effects concern behavioural, immune, or physiological outcomes rather than biomass gain, and because we did not measure larval body composition or physiological markers, this pathway remains speculative in our system and cannot be disentangled from the nutritional contribution. A particularly informative case is that of LG, whose inactivated preparation (LG Aut) significantly increased harvest weight relative to the Control, while its live counterpart did not. If the effect were solely nutritional, the live preparation, containing the same biomass plus viable cells, would be expected to produce a comparable or greater response. We hypothesize that live *Paenibacillus* cells may release antagonistic compounds, such as cyclic lipopeptides, bacteriocins, and antibiotics, which are well documented for this genus (Tariq et al., 2025), and that in the rearing substrate these could interfere with the resident gut microbiota of the larvae, which is central to digestion and nutrient assimilation, thereby offsetting the nutritional benefit provided by the biomass. Upon inactivation this antagonistic activity would be abolished, leaving only the nutritional contribution; this remains a hypothesis, however, as antagonistic activity and its effect on the larval microbiota were not directly assessed. In contrast, higher frass production was observed specifically in the live preparations: CAL Live, LAL Live, and LG Live all produced significantly more frass than the Control, and LAL Live significantly exceeded its own inactivated counterpart (LAL Aut). This viability-dependent pattern could point to a biologically mediated response of the live bacteria rather than a nutritional effect of the biomass.

These findings can be contextualized within the growing but still limited body of work on bacterial supplementation in insect rearing. The two commercial formulations used here are based on *B. velezensis*, a species previously tested in BSF by Pei et al. (2022), who reported that the strain EEAM 10B increased the substrate conversion rate, average dry weight, and protein content of the larvae by 5%, 0.13 g, and 8%, respectively, through modulation of the gut microbiota

and amino acid metabolism. Notably, in that study *B. velezensis* EEAM 10B significantly altered the microbial community of the larval gut but not that of the frass, an observation that resonates with our own difficulty in tracing the supplied strains within the frass bacteriome and reinforces the idea that the frass microbial community may not faithfully reflect events occurring in the gut during rearing. The comparatively weaker and more restricted effects observed in our study, limited, in the case of *B. velezensis*, to increased frass production rather than larval weight or composition, may stem from differences in strain identity, substrate (nutrient-poor, lignocellulosic olive pomace versus more balanced substrates), inoculation dose, or rearing conditions. The choice of spore-forming *Bacillus* and related genera as candidates is nonetheless well grounded: their defining advantage as feed probiotics is the heat stability of their endospores and their ability to survive harsh gastrointestinal and environmental conditions, together with the secretion of extracellular enzymes such as amylases, proteases, phytases, cellulases, and xylanases that assist substrate digestion (Mingmongkolchai & Panbangred, 2018). Likewise, *S. clausii*, the closest relative of strain PD, is a well-characterized spore-forming probiotic with documented antioxidant and enzymatic activity (Corona et al., 2025). The outcome of such supplementation, however, appears strongly system-dependent: using the same host-derived selection strategy, we previously found that live *P. agglomerans* improved cricket (*G. bimaculatus*) performance in a viability-dependent manner only on a high-fiber diet, whereas its heat-inactivated form had no effect (Cruz et al., 2026), a contrast with the largely viability-independent, nutritional responses observed here that highlights how the same conceptual approach can yield different mechanisms across host–diet systems.

On the other hand, frass generally promoted greater tomato aerial and root biomass than the mineral and organic reference fertilisers, despite all treatments being normalised to an equivalent nitrogen input (250 kg N ha^{-1}); this was most consistent for the supplemented frass and for biomass-related parameters, whereas the mineral fertiliser yielded the highest leaf chlorophyll. This superiority of frass over conventional fertilisers is consistent with the growing body of evidence positioning BSF frass as a high-value organic amendment (Zhang et al., 2026; Lomonaco et al., 2026). A meta-analysis of BSF frass effects on plant growth reported average increases of 23.7% in shoot biomass and 5.1% in root biomass following frass application, together with higher leaf soluble protein and chlorophyll content (Zhang et al., 2026), a pattern that mirrors the aerial and root improvements observed here. The comparatively poor performance of the mineral fertiliser, despite delivering the same amount of nitrogen, likely reflects fundamental differences in nutrient dynamics between soluble mineral and organic amendments. Whereas mineral fertiliser supplies nitrogen in a readily available but rapidly leached and osmotically active form, the nitrogen in frass is predominantly organic and must be gradually mineralised by soil microorganisms into plant-available ammonium and nitrate (Zhang & Suseela, 2021; Beesigamukama et al., 2020). This microbially mediated, slow-release dynamic provides a more sustained nutrient supply and avoids the transient salt stress that high doses of soluble mineral fertiliser can impose on developing plants. Moreover, the organic carbon supplied by frass stimulates soil microbial proliferation and extracellular enzyme secretion, further enhancing nutrient turnover and mineral solubilisation (Beesigamukama et al., 2020), which may explain why frass sustained higher plant biomass than both reference fertilisers. Notably, germination in the frass treatments was maintained at or above the level of the controls, contrasting with the average reduction in germination reported for BSF frass at the meta-analytical level (Zhang et al., 2026); this suggests that, under our conditions and application rate, the frass did not reach the phytotoxic thresholds sometimes associated with immature or nitrogen-rich organic amendments.

The most relevant outcome of the greenhouse trial was that frass from bacterially supplemented substrate improved several tomato growth parameters relative to frass from non-supplemented larvae (C-F). All four supplemented frass types significantly increased root dry weight compared with C-F. In addition, LG-F, CAL-F, and PD-F increased aerial fresh weight; LG-F and PD-F increased aerial dry weight; LG-F and CAL-F increased aerial length; LG-F was the only treatment to increase root fresh weight; and PD-F was the only treatment to increase root length, exceeding all other treatments. Overall, LG-F showed the most consistent improvement across parameters. Since all frass types shared the same olive pomace base and displayed highly comparable chemical profiles (Table 1), these differences are unlikely to be explained

by bulk nutrient content alone and could instead be linked to changes in the microbial or bioactive fraction of the frass induced during rearing. Consistent with this interpretation, beta diversity analysis showed that treatment explained 79.1% of the variation in frass community structure, indicating that substrate supplementation did modify the resulting bacterial communities even though the dominant genera were shared across treatments. A comparable phenomenon has been described by Reyer et al. (2025), who reported that supplementing the BSF larvae substrate enriched frass microorganisms with phosphate-solubilising properties, such as *Pseudomonas*, thereby improving phosphorus bioavailability. More broadly, feed composition has been identified as the primary driver of frass microbial community structure (Kreft et al., 2026), and phytohormone concentrations in BSF frass have been found to correlate with the abundance of specific bacterial genera (Lopes et al., 2026). Together, these observations suggest that modifying the rearing substrate could represent a viable strategy to tailor the agronomic quality of frass, although the specific mechanism operating in our system was not identified.

Notably, the dominant genera detected across all frass treatments are recognised PGP taxa. *Rhodococcus*, the most abundant genus in all treatments (15.0–17.7%), produces auxins and cytokinins and possesses ACC deaminase activity, phytohormones that stimulate shoot proliferation and cell expansion (Oubohssaine et al., 2024; Francis et al., 2016). *Pseudomonas* (6.2–14.6%) is among the best-characterised PGP genera, producing indole-3-acetic acid (IAA), solubilising phosphate, and synthesising siderophores and ACC deaminase (Khoshru et al., 2025; Oteino et al., 2015), while *Sphingobacterium* (6.4–8.1%) has been reported to solubilise phosphate and produce organic acids and IAA (Gupta et al., 2018). Importantly, both *Pseudomonas* and *Sphingobacterium* have been specifically identified in BSF frass as taxa with phosphate-solubilising and pathogen-antagonistic potential (Reyer et al., 2025), reinforcing their plausible contribution to the agronomic value of the frass produced here.

These observations are consistent with the growing recognition of BSF frass as a reservoir of PGP microorganisms and bioactive compounds. BSF frass has been associated with functional profiles enriched in nitrogen fixation and phytohormone biosynthesis relative to other insect species (Amorim et al., 2024), and its bacterial communities harbour diverse taxa capable of producing phytohormones, fixing nitrogen, and solubilising nutrients (Lomonaco et al., 2026; Lopes et al., 2026). However, the extent to which the individual frass treatments could be distinguished on the basis of their bacterial communities was limited. Although beta diversity differed significantly among treatments, the dominant genera were shared across all frass types, and among the inoculated taxa only *Bacillus* showed a clear treatment-specific enrichment (in LAL-F). This pattern aligns with the observation that, in BSF frass, feed composition is the primary driver of microbial community structure, whereas thermal hygienisation exerts comparatively minor effects on the amplicon-inferred community despite reducing viable counts (Kreft et al., 2026). Since all treatments in our study shared the same olive pomace base, the resulting frass communities were broadly similar, and the PGP effects are therefore better attributed to the frass microbial consortium as a whole than to any specific supplemented strain.

Differential abundance analysis provided further insight into which taxa distinguished the supplemented frass from the C-F. Consistent with the identity of the commercial formulation, *Bacillus* was strongly enriched in LAL-F (log-fold change = 4.64), the only inoculated genus showing a clear treatment-specific increase. More notably, *Oceanobacillus* was significantly enriched in three of the four supplemented treatments relative to the C-F (log-fold changes of 3.87, 3.05 and 2.89 in LAL-F, LG-F and PD-F, respectively), which were among the treatments that improved the largest number of tomato growth parameters. *Oceanobacillus* is a spore-forming, halotolerant genus with well-documented PGP traits: genomic analysis of *O. jordanicus* revealed genes involved in auxin biosynthesis, nitrogen, phosphate and iron acquisition, and chitin utilisation (Alhindi & Albdaiwi, 2022), while characterised strains produce indole-3-acetic acid, degrade ACC, and display antifungal activity against *Fusarium culmorum* (Alhindi & Albdaiwi, 2022). Isolates of *O. oncorhynchi* have been evaluated as biofertiliser candidates for their phosphate-solubilising capacity and effects on seed germination and seedling growth (Deepalaxmi & Gayathri, 2018), and inoculation with *O. picturae* has been shown to improve plant height, root length and fresh weight, with a particularly marked effect on root development (Yang et al.,

2024; Li et al., 2026). This last observation is of interest here, since root dry weight was the only parameter improved by all four supplemented frass types relative to the C-F. Although the enrichment of *Oceanobacillus* cannot be causally linked to the plant responses with the present design, its consistent increase across supplemented treatments and its documented root growth-promoting activity make it a plausible contributor to the agronomic value of the frass and a candidate worth targeting in future work.

The relative abundance of the genera corresponding to the inoculated taxa provides a descriptive, although limited, picture of what happened to the supplemented bacteria. In contrast to *Bacillus*, *Paenibacillus* sequences were recovered at comparable levels across all treatments, including the non-supplemented C-F (0.7–1.4%), indicating that this genus is a resident member of the frass bacteriome and that any contribution of strain LG cannot be distinguished from the native population. Finally, no sequences assigned to *Shouchella* were recovered in any sample, which could mean that strain PD did not persist in the frass, its abundance remained below the detection threshold of the assay or technical biases in DNA extraction or PCR amplification hindered its recovery.

CONCLUSIONS

This study evaluated the microbial supplementation of an OP substrate with commercial *B. velezensis* formulations and gut-derived bacterial strains as a strategy to improve BSF larval performance and the agronomic value of the resulting frass. Supplementation produced modest, strain- and preparation-dependent effects on larval performance: the occurrence of significant effects with heat-inactivated preparations points to a predominantly nutritional or postbiotic contribution of the bacterial biomass, whereas the increased frass production associated specifically with live *B. velezensis* suggests that viable-cell-associated mechanisms may contribute to this effect. The most consistent finding was agronomic: frass from supplemented larvae improved several tomato growth parameters relative to non-supplemented frass, and generally promoted greater aerial and root biomass than the mineral and organic reference fertilisers while maintaining germination, although the mineral fertiliser yielded the highest leaf chlorophyll. Among the treatments, LG-F produced the best overall agronomic performance, significantly improving aerial fresh weight, aerial dry weight, aerial length, and root fresh weight relative to the C-F, followed by PD-F and CAL-F, whereas the differential enrichment of *Bacillus* in LAL-F and of *Oceanobacillus* in three of the four supplemented treatments points to the microbial fraction as a plausible contributor to these effects. Although the viability and persistence of the supplied strains were not confirmed, and the PGP effects cannot be causally attributed to the inoculated bacteria, these results demonstrate that microbial supplementation during rearing can modify both the bacterial community and the agronomic quality of BSF frass, supporting its potential as a tailored, value-added biofertiliser.

Future work should combine strain-specific approaches, such as quantitative PCR with strain-targeted primers or selective re-isolation applied before and after heat treatment, with a broader screening of dual-function strains selected for both larval performance and PGP traits. As an alternative to relying on the persistence of bacteria supplied during rearing, direct bioaugmentation of the heat-treated frass could be explored, where chitinolytic PGP bacteria are particularly promising, since chitinase-producing species can suppress chitin-containing phytopathogens and induce systemic resistance (Tariq et al., 2025), an activity especially relevant in a chitin-rich matrix such as frass. Nonetheless, because *Paenibacillus* includes entomopathogenic species such as *P. larvae* (Genersch, 2010) and *P. thiaminolyticus*, the latter recently identified as a pathogen of *H. illucens* (She et al., 2023), screening and sequencing are essential before supplying strains of this genus, in order to ensure the safety of mass-reared insects (Savio et al., 2022).

Declarations

Conflict of interest

No potential conflict of interest was reported by the authors.

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Data availability statement

The 16S rRNA gene amplicon sequences generated in this study have been deposited in the NCBI Sequence Read Archive (SRA) under BioProject accession PRJNA1524916 and are publicly available.

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Figures

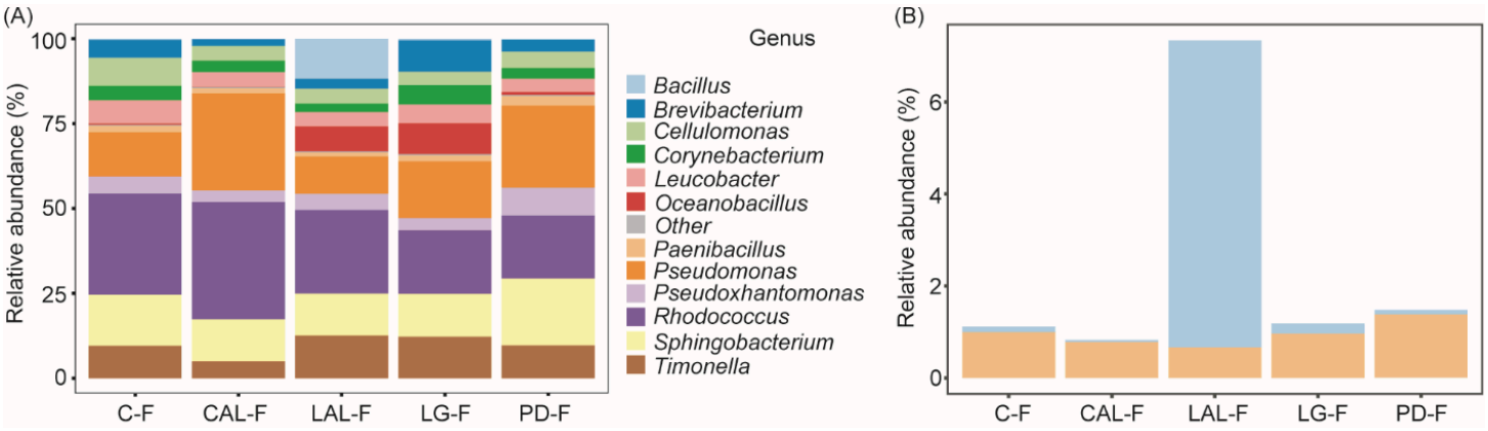

Figure 1

Frass bacterial community composition. (A) Relative abundance of the ten most abundant genera across treatments, with *Bacillus* and *Paenibacillus*shown regardless of overall rank. (B) Mean relative abundance (%) of *Bacillus*spp., *Paenibacillus* spp., and *Shouchella* spp. as genus-level proxies for the inoculated taxa.

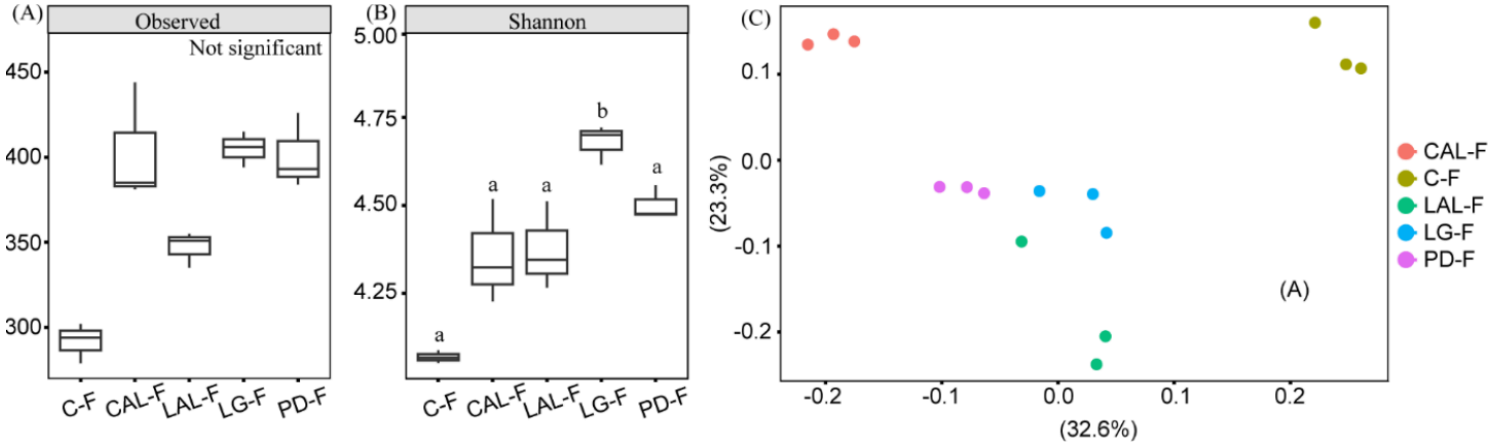

Figure 2

Alpha and beta diversity of frass bacterial communities. (A) ASV richness (Observed) and (B) Shannon diversity index across treatments; letters indicate significant differences. (C) Principal Coordinates Analysis (PCoA) based on Bray–Curtis dissimilarities.

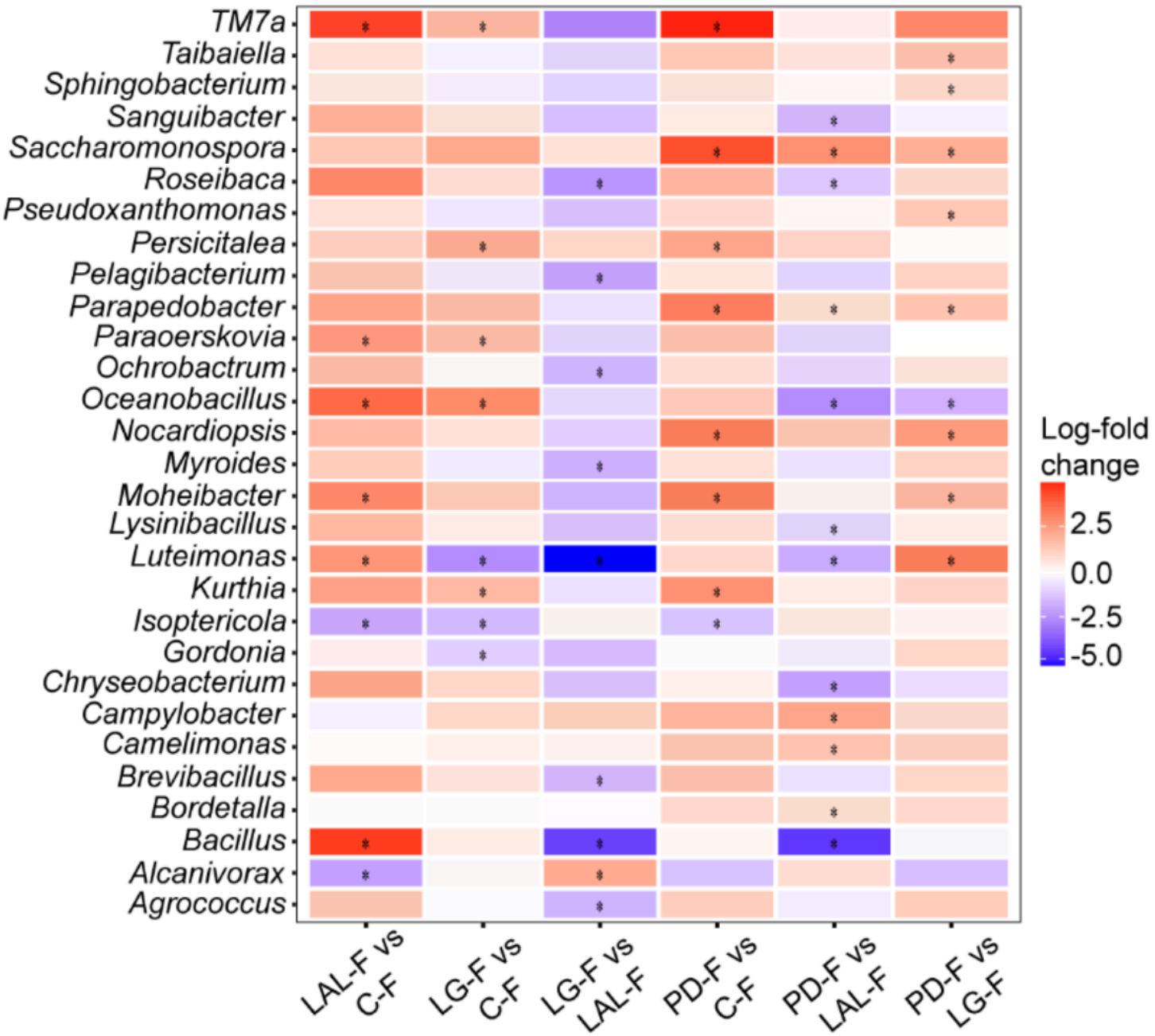

Figure 3

Heatmap of log-fold changes from ANCOM-BC2 pairwise comparisons across bacterial genera. Asterisks indicate significant differences; red indicates higher and blue lower abundance in the first treatment of each pair.