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Effects of Thermal Sanitization on Insect Frass Nutrient Composition and Biofertilizer Performance in a Lettuce Pot Trial

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Abstract

Insect farming has rapidly expanded in Europe following regulatory approval of insect-derived proteins in aquaculture feed and increasing interest in the valorization of insect by-products. Insect frass, consisting of excreta and exuviae, is a nutrient-rich material with beneficial microorganisms and potential as a sustainable alternative to conventional fertilizers, although its composition varies with insect species and feedstock. EU legislation requires thermal sanitization prior to market release, yet the effects of the thermal treatment on frass nutrient composition and biofertilizer performance remain poorly understood. Insect frass from black soldier flies (BSFFs) fed on a diet based on dairy industry byproducts was sanitized and mixed with sandy soil and used in two lettuce pot trials under greenhouse conditions. The aim of our study was to determine the effects of thermal sanitization on (1) macro- and micronutrient contents and dynamics (plant N and P uptake); and (2) biofertilizer potential, including plant physiology (chlorophyll, anthocyanins, flavonols, Fv/Fm), plant growth (biomass), and soil microbial activity (dehydrogenase and β -glucosaminidase). BSFF showed a clear potential to induce growth of lettuce plants by increasing chlorophyll content, biomass and microbial activity. Furthermore, the sanitization process did not significantly alter the measured agronomic performance of frass under the tested conditions or reduce its benefits on biomass growth, chlorophyll content, microbial enzyme activity and on nutrient uptake by the lettuce plants. These findings suggest that the mandatory sanitization does not compromise its agronomic functionality, supporting its strong potential within circular agricultural systems under the tested conditions. However, the results are valid under greenhouse conditions and for the specific frass, soil and crop combinations; field validation is needed to confirm these results under large-scale high-value crop production conditions.

Keywords: thermal sanitization; EU regulation; nutrient content of frass; circular economy; soil enzyme activity; biofertilizer



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1. Introduction

Growing global demand for food and feed, driven by population growth, is intensifying the pressure on agricultural production systems, raising serious concerns about long-term environmental sustainability [1,2]. Moreover, the increasing population, consumption and production has led to an increase in waste per capita. At the same time, the transition towards a circular bioeconomy is driving an increasing interest in recycling organic by-products into sustainable agricultural inputs, aiming to close nutrient loops and reduce reliance on mineral fertilizers. In this context, insect farming has emerged as

a promising industry capable of converting low-value organic waste streams into insect feed to produce insects as a high-quality protein source for feed and food [3]. Over the last few years, it has gained more attention after EU regulation 2017/893, which allowed the use of insect-processed animal proteins in feed for aquaculture animals [4]. Insects are highlighted as a sustainable food source due to their minimal need for arable land and water, low ecological footprint, and high-quality protein content [5].

As this emerging industry is growing, its byproducts are gaining more attention for their potential uses. The primary byproduct is insect frass, a mixture of excreta and molted skins, making it high in nutrients [6] with potential for a variety of uses in the agricultural sector. Despite its potential uses in agriculture, frass composition is highly variable and depends strongly on insect species and rearing substrate, resulting in wide ranges of carbon and nutrient contents and inconsistent agronomic performance [7–10]. For example, insect frass from black soldier fly (BSFF) was found to exhibit a high variability in nutrients with carbon (C) ranging from 23.5 to 54.3%, nitrogen (N) from 0.6 to 5.1%, phosphorus (P) from 0.5 to 5.2% and potassium (K) from 0.2 to 4.5% depending on the diet they were fed [7]. Furthermore, studies have compared the composition of frass with that of poultry litter, used as manure, and found that insect frass from *Tenebrio molitor* L. has 43% higher C content and 47% higher N content than poultry litter [8]. In another study mealworm frass was evaluated for its microbial metabolic activity and diversity and its results aligned with previous studies showing that the incorporation of insect frass (as an organic amendment) in soil with NPK fertilizer increased soil microbial activity and enhanced microbial functional diversity [9]. Our previous study also showed that the impact of BSF frass on N availability, microbial activity, and plant growth is highly dependent on the original feedstock used to rear the larvae [10]. Despite the variations in its composition, these results show that insect frass can be used as a soil amendment or a biofertilizer to improve soil health and C sequestration.

Beyond its chemical constituents, frass harbors diverse microbial communities that may contribute to nutrient cycling and plant growth promotion through enzymatic processes and rhizosphere interactions. However, because insect-mediated decomposition occurs at low temperatures, the resulting frass may contain some human and plant pathogens. To ensure safety and facilitate the use of frass as an organic fertilizer, EU regulation 2021/1925 mandates thermal treatment at a minimum of 70 °C for an hour before being placed on the market within the EU [11]. Sanitization is essential from a safety perspective, yet the process may alter the quality of insect frass as a biofertilizer by affecting both its chemical and biological composition. Changes in chemical properties can primarily be linked to alterations in carbon fractions and nutrient profiles. A recent study reported that heat treatment increased $\text{NH}_4\text{-N}$ and the electrical conductivity of BSF frass [12], which are crucial for plant growth and development.

Because insect frass naturally contains a substantial community of beneficial microorganisms [9], the compulsory heat treatment can also reduce their abundance, diversity and activity. In a recent study by Praeg & Klammsteiner [12] the sanitization process significantly reduced microbial biomass and eliminated pathogens but had a minor effect on physiochemical properties. Such changes in microbial biomass likely disrupt functional microbial groups involved in key soil processes such as nitrification, denitrification, and enzymatic decomposition. Such possible changes are also expected to directly influence plant physiological responses, particularly in fast-growing leafy vegetables such as lettuce. For example, variations in N availability can affect chlorophyll synthesis, photosynthetic efficiency, and nitrogen use efficiency, while excessive nutrient availability or salinity may induce physiological stress. Despite the potential negative impact of the frass sanitization

process, soil application of the sanitized frass increased the soil microbial respiration [12], indicating that sanitized frass still had potential for boosting soil health.

Despite these emerging insights, knowledge of how thermal treatment affects frass quality, soil microbial activity, nutrient availability, and subsequent plant growth remains severely limited [12,13]. Although some studies have applied sanitization as a pre-experimental step [9], to our knowledge no study has explicitly examined how this mandatory heat treatment influences the nutrient composition and biofertilizer efficacy of insect frass. This knowledge gap is especially critical in vegetable production systems, where crop yield and quality are tightly coupled to both nutrient availability and soil microbial functioning. To address this gap, the present study aimed to evaluate how mandatory thermal sanitization affects the chemical composition and agronomic efficacy of BSFF.

We hypothesized that thermal sanitization would: (1) have limited effects on the overall nutrient composition of BSFF but increase its mineral N availability relative to non-sanitized frass; (2) increase lettuce biomass, nitrogen uptake, and physiological response, and (3) reduce soil enzyme activity relative to non-sanitized frass reflecting disruption of frass-born microbial community. To test these hypotheses, we conducted two independent pot experiments using lettuce as a model vegetable crop, and determined the effects of thermal sanitization by analyzing (i) the macro- and micronutrient composition of BSFF; (ii) lettuce biomass, N and P uptake, and physiological response, including chlorophyll content, anthocyanin and flavonol indices, and photosystem II efficiency; and (iii) the post-harvest soil mineral N and dehydrogenase and β -glucosaminidase enzyme activities.

2. Materials and Methods

2.1. Insect Frass, Sanitization Process and N Content

The insect frass was obtained from black soldier fly cultures at Enorm Biofactory A/S (Flemming, Denmark). The larvae were reared and fed on a diet based on dairy industry waste stream (Delactosed permeate). The frass was collected from rearing units dominated by prepupae/pupae, where the residue is more decomposed and stabilized relative to earlier larval stages. A single batch of frass was collected and homogenized prior to division into two subsamples. Half of the homogenized batch was retained as non-sanitized insect frass, while the other half was subjected to thermal sanitization for 1 h at 70 °C using an oven (Memmert (Schwabach, Germany), UNF750plus) according to EU regulations [11]. The frass was spread evenly on a tray as a thin layer to ensure an equal temperature was reached across all of the sample surface. The insect frass samples were stored at −18 °C until use in the pot experiments (one week before the first experiment and 31 weeks before the start of the second experiment). Subsamples were analyzed for dry matter (DM) and total N content [14] before the setup of the pot experiments. Additional details on the exact composition of the larval feeding substrate used by the commercial supplier were a proprietary formulation that could not be fully disclosed, which constrains the reproducibility of the exact frass material and limits the ability to attribute observed agronomic effects to specific dietary components. A complete chemical analysis of the sanitized and non-sanitized insect frass was performed after the setup of the experiments (Table 1). This would allow comparison with BSFF from other substrates reported in the literature.

Table 1. Comparison of sanitized and non-sanitized black soldier fly insect frass (BSFF). Values are means of 3 replicates $\pm$ standard deviation. All total elemental compositions (C, N, P_2O_5 , K_2O , Total S, MgO) and organic matter are reported on a dry matter basis. Soluble/extractable nutrients (Total N, NH_4-N , NO_3-N) were analyzed volumetrically and are reported per liter of fresh frass. No statistical differences were observed (independent samples *t*-test).

	Non-Sanitized BSFF	Sanitized BSFF
<i>Physical Parameters</i>		
Bulk Density ($g\ L^{-1}$)	463.3 ± 15.3	480.0 ± 10
<i>Chemical Properties</i>		
pH	7.1 ± 0.0	7.0 ± 0.0
EC $ms\ cm^{-1}$	6.7 ± 0.2	7.2 ± 0.4
C (%)	39.7 ± 0.3	39.6 ± 0.5
Organic matter (%)	84.7 ± 0.3	84.4 ± 0.6
C:N ratio	14.7 ± 0.7	14.5 ± 0.7
<i>Nutrients</i>		
N (%)	3.4 ± 0.2	3.4 ± 0.2
Total N ($mg\ L^{-1}$)	1223 ± 110	1313 ± 58
NH_4-N ($mg\ L^{-1}$)	1222 ± 110	1312 ± 57.9
NO_3-N ($mg\ L^{-1}$)	0.88 ± 0.2	0.95 ± 0.2
P_2O_5 (%)	4.2 ± 0.1	4.09 ± 0.2
K_2O (%)	3.2 ± 0.1	3.3 ± 0.2
Total S (%)	0.87 ± 0.02	0.89 ± 0.03
MgO (%)	1.1 ± 0.01	1 ± 0.03

2.2. Experimental Design of Pot Trials

Two independent greenhouse pot experiments were conducted to evaluate the effects of the sanitized and non-sanitized frass on plant responses and soil properties. The first experiment focused on plant growth and physiological responses, and based on these findings, the second experiment was expanded to additionally include post-harvest measurements of soil mineral nitrogen availability and microbial enzyme activities (dehydrogenase and β -glucosaminidase), providing a more comprehensive assessment of the effects of thermal sanitization on frass agronomic performance. The first pot trial with lettuce (*Lactuca sativa* cv Alega) in a controlled environment of a greenhouse chamber was conducted from 9 April 2024 to 22 May 2024 at the Department of Food Science, Aarhus University, Denmark. The lettuce plants were grown in seedling trays for 3 weeks before transplanting one seedling per pot. The soil for the pot trials was collected from the experimental station of the Department of Food Science, Aarhus University in Auning, ($56^{\circ}24' N$, $10^{\circ}37' E$) and is characterized by sandy soil texture and $11.1 \pm 1.5\ g\ kg^{-1}$ total organic carbon, $5.5 \pm 0.6\ g\ kg^{-1}$ mineral N, $1.35 \pm 0.27\ mg\ 100\ g^{-1}$ P, $3.25 \pm 0.26\ mg\ 100\ g^{-1}$ K and pH_{CaCl_2} of 6.43. Insect frass was calculated based on the surface area of the 3 L pots (diameter = 0.17 m, depth = 0.15 m) and according to Danish fertilization standards for lettuce. The regulated Danish fertilization standard for lettuce is $165\ kg\ N\ ha^{-1}$ [15]. At the time of planning, the soil contained $13\ kg\ ha^{-1}$ of mineral N. The remaining $152\ kg\ N\ ha^{-1}$ was supplied through insect frass. Sanitized and non-sanitized frass treatments received $0.034\ kg$ of frass per pot, calculated based on the pot surface area ($0.0227\ m^2$) and an application rate of $15\ t\ DM\ ha^{-1}$. This application rate was calculated based on a total N concentration of 3.4% DM and considering that about 30% of the total N would be mineralized and become available for plant uptake during the growing period. For both experiments all pots were filled with 3 L of moist soil. Prior to pot filling, the sanitized and non-sanitized frass was thoroughly mixed with the total volume of soil across all replicates of each treatment using a rotating drum cement mixer to ensure homogeneous distribution of frass throughout the soil. The

soil-frass mixture was then divided equally across the replicate pots of each treatment. The control pots did not receive any further fertilization. The experiment was a completely randomized design with six replicates for the insect frass treatments and three replicates for the control treatment, resulting in a total of 15 pots ($2 \text{ treatments} \times 6 + 1 \text{ treatment} \times 3$). This unequal replication was a deliberate design decision to maximize statistical power for the primary comparison between frass treatments, given the limited volume of field soil available for the experiment. The temperature was set at $20 \text{ }^{\circ}\text{C} \pm 2$ with a 16/8 h day/night photoperiod and a relative humidity of $57\% \pm 5$. The first experiment was conducted under natural daylight with a supplemental LED light of $150 \text{ } \mu\text{mol m}^{-2} \text{ s}^{-1}$ when the natural light was below $250 \text{ } \mu\text{mol m}^{-2} \text{ s}^{-1}$ with a programmed system. The pots were rotated on a weekly basis to avoid potential position effects. Irrigation was performed manually every second day in the first 4 weeks and daily in the last 2 weeks with 100–150 mL provided per pot depending on the plant needs.

The experiment was conducted a second time on 19 November 2024 and the lettuce plants were left to grow for 8 weeks (harvested on 13 January 2025). Five replicates were used per each treatment and control pots, resulting in a total of 15 pots ($3 \text{ treatments} \times 5$). Frass application was calculated and applied in the same way as the first pot trial, and the greenhouse conditions and irrigation were setup as explained above. However, due to lack of natural light in the second experiment supplemental light (a minimum intensity of 150 to $350 \text{ } \mu\text{mol m}^{-2} \text{ s}^{-1}$ in addition to a limited natural light) was provided by fixed LED growing lights (FL300 Grow, Senmatic (San Diego, CA, USA)), which resulted in differences in growth, with the plants of the 2nd pot experiment showing rapid growth. Fresh and dry aboveground biomass were measured at the end of both repetitions, and belowground biomass was measured in the second repetition only. For aboveground biomass, the lettuce heads were collected, weighed and then oven-dried for 48 h at $70 \text{ }^{\circ}\text{C}$ and weighed again. For belowground biomass, roots were cleaned in water and patted down with tissue paper to remove extra moisture before being weighed (fresh biomass) and placed in the oven for 48 h at $70 \text{ }^{\circ}\text{C}$ for dry biomass registration. Due to the inherent differences in environmental conditions of the two trials, specifically in the growing season, duration and lighting, they were treated as two independent experiments rather than technical replicates.

2.3. Chlorophyll, Flavonols and Anthocyanin Content, Fv/Fm and Nitrogen Balance Index

The relative chlorophyll content of all treatments was measured in the 3rd and 5th weeks after transplantation for the first experiment using a Dualex[®] Scientific+ leaf-clip meter (Force-A, model Dx4.5, software 2.4.2). In the second experiment the relative chlorophyll content, relative flavonol content, anthocyanin content and nitrogen balance index (NBI) were measured in the 3rd and 7th weeks after transplantation on second repetition using the portable device Dualex[®] Scientific+ leaf-clip meter (Force-A, model Dx4.5, software 2.4.2). Maximum quantum yield of photosystem II photochemistry (Fv/Fm) was measured on harvest day (6th week after transplantation for the first experiment and 7th week for the second experiment) using a portable chlorophyll fluorometer (FluorPen, FP110, Photon Systems Instruments, Drásov, Czech Republic). The leaves were dark-adapted for 20 min prior to the measurement. Three to four leaves per plant were used for all measurements (3–4 repetitions of readings per plant).

2.4. Nutrient Composition of Frass and Soil Samples

Sanitized and non-sanitized insect frass samples were analyzed for pH, electric conductivity, total carbon [16] (DIN EN 15936), organic matter, total nitrogen [17] (DIN EN 16168), total sulfur, total potassium and total magnesium (S, K, Mg by DIN EN ISO 11885) [18] at the start of the experiment. Soil analysis was carried out only at the harvest of the second

experiment, as the primary focus of the first experiment was the response of the plants to the frass application. Soil samples were collected from each pot, using a 50 mL falcon tube as a soil auger sampler to collect soil from 4 points in the pot, and the soil was then mixed and a subsample was sent for analysis at AgroLab group (Landshut, Germany) for mineral N, phosphorus (P), potassium (K), and magnesium (Mg). Samples were stored at -18°C before all analyses. For mineral N extraction, samples of 100 g from thawed soil were loosened to 1 cm crumbs and analyzed immediately. The soil was extracted for 1 h with 1 M of KCl and the extract was centrifuged. The supernatant was subjected to NH_4^+ and NO_3^- analyses by a colorimeter in an AutoAnalyzer 3 (Bran+Luebbe, Heidelberg, Germany). The potassium content was measured according to the working methods of soil analyses III, method 15: 1994–03 [19]. The magnesium content was calculated based on the working methods of soil analyses III, method 17: 1994–03 [19]. Briefly, an ammonium acetate solution was used for the extraction of exchangeable potassium and magnesium ions from the soil. The phosphorus content was measured according to Rubæk et al. 2017 [20], following the bicarbonate extraction and spectrophotometric approach.

2.5. Beta-Glucosaminidase and Dehydrogenase Activity

The rhizosphere soil was sampled at harvest from the root zone. After gently shaking the roots to remove loosely adhering soil, the soil that remained attached to the roots was carefully collected and designated as rhizosphere soil and used for enzyme analysis. The activity of β -glucosaminidase enzyme was determined following the method described by [21]. Briefly, 1 g of moist soil was incubated with 4 mL of 0.1 M acetate buffer (pH 5.5) and 1 mL of 10 mM p-nitrophenyl-N-acetyl- β -D-glucosaminide (PNNAG) solution at 37°C for 1 h. After incubation, 1 mL of 0.5 M CaCl_2 and 4 mL of 0.1 M Tris buffer (pH 12) were added to terminate the reaction. Blanks were prepared by adding the substrate after incubation. The suspensions were filtered through Whatman No. 5 paper, and the absorbance of the filtrates was measured at 405 nm using a spectrophotometer. Enzyme activity was calculated from a standard curve prepared with p-nitrophenol calibration solutions ($0\text{--}10\text{ }\mu\text{g mL}^{-1}$). Soil dehydrogenase activity was determined following a modified triphenyltetrazolium chloride (TTC) reduction method. Briefly, 5 g of field-moist soil was placed into glass vials and 1 mL of 3% TTC solution and 1 mL of 0.1 M Tris buffer (pH adjusted according to soil pH) were added. Blanks received 2 mL of Tris buffer without TTC. Samples were incubated for 24 h at 37°C in the dark. After incubation, 10 mL of methanol was added to each vial, and samples were shaken for 2 h at 125 rpm in the dark to extract the triphenyl formazan (TPF) produced. The suspensions were filtered through pre-wetted Whatman No. 5 filter paper, and residues were rinsed with additional methanol until clear. The absorbance of the filtrates was measured at 485 nm using a spectrophotometer. Dehydrogenase activity was quantified from a calibration curve prepared with TPF standards ($0\text{--}30\text{ }\mu\text{g mL}^{-1}$). Both β -glucosaminidase and dehydrogenase activity were calculated with 5 biological samples for each treatment from the 2nd experiment. Enzyme analysis was conducted at the Department of Food Science, Aarhus University.

2.6. Data Analysis

Statistical analyses were conducted separately for each trial, and no direct statistical comparison between the two was performed. One-way analysis of variance (ANOVA) was performed with treatment (3 levels, control, non-sanitized insect frass, sanitized insect frass) as the independent variable, followed by post hoc pairwise comparisons using Tukey's test. The assumptions of normality and homogeneity of variance were assessed using the Shapiro–Wilk test and Levene's test, respectively. To meet the assumptions of normality and homogeneity of variance for the ANOVA, data for dehydrogenase and β -glucosaminidase

were transformed using quantile normalization. Each experiment was independent and the results were analyzed separately for each weekly measurement. For comparing the properties of sanitized and non-sanitized insect frass (Table 1) an independent samples *t*-test was performed with $n = 3$. For comparing the nutrient uptake of plants an independent samples *t*-test was performed with $n = 5$. Statistical analyses were conducted for both experiments separately using IBM SPSS Statistics for Windows, version 28.

3. Results

3.1. The Effect of Sanitization on the Frass Nutrient Content and Chemical Properties

There were no statistically significant differences in the nutrient content of the frass or its chemical and physical characteristics (bulk density, pH, EC, C, organic matter) between the sanitized and non-sanitized insect frass (Table 1). However, there were slight increases in the EC ($p = 0.128$) and $\text{NH}_4\text{-N}$ ($p = 0.281$) content in the sanitized compared to non-sanitized frass.

3.2. The Effect of Frass Sanitization on the Physiological Response of Lettuce

Insect frass application significantly increased the relative chlorophyll content of plants compared to the unfertilized control plants in both experiments (Figure 1a–d) except for the 3rd week measurement on the 2nd experiment. There was no statistically significant difference between sanitized and non-sanitized insect frass treatments. The relative anthocyanin contents were significantly higher in control plants compared to both non-sanitized insect frass and sanitized insect frass in the 3rd week of the second experiment (Figure 1e). However, on the 7th week the plants treated with non-sanitized insect frass did not differ from control plants, while the sanitized insect frass-treated plants had significantly lower levels of anthocyanins (Figure 1f). Both insect frass treatments had significantly lower relative flavonol contents compared to control plants in both measurements of the 2nd experiment (Figure 1g–h). The NBI between sanitized and non-sanitized insect frass did not differ significantly in both the 3rd and 7th weeks and both had significantly higher NBI than the control plants (Figure 1i–j). Moreover, the maximum quantum yield of PSII photochemistry (F_v/F_m) was significantly higher in both insect frass treatments compared to the control plants in the first experiment, while there was no statistically significant difference between treatments in the 2nd experiment (Figure 2a,b).

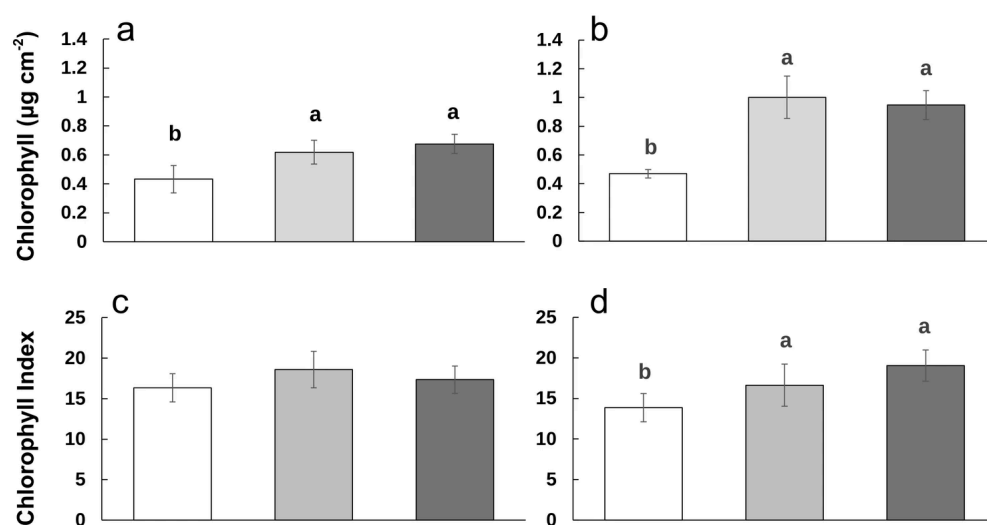


Figure 1. Cont.

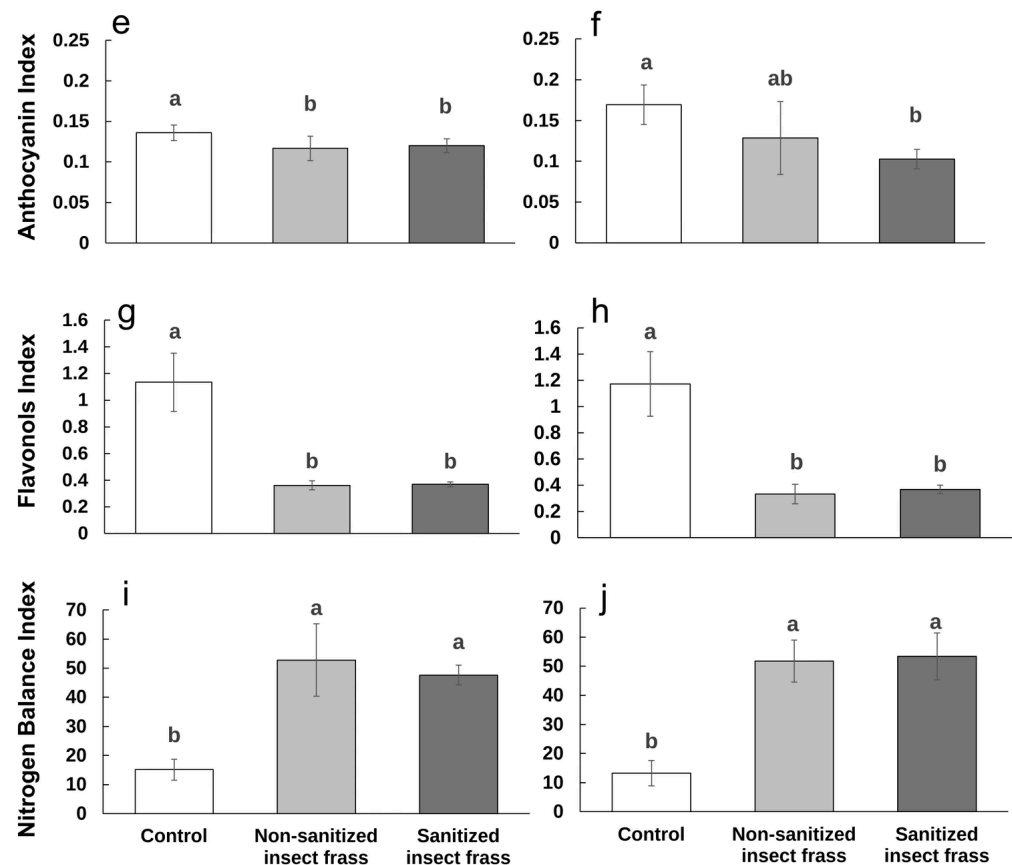


Figure 1. Effects of non-sanitized (light gray) and sanitized insect frass (dark gray) on lettuce pigments over time compared to the unfertilized control (white). Chlorophyll content during the 3rd and 5th of the first experiment ((a,b), respectively) and chlorophyll index during the 3rd and 7th of the second experiment ((c,d), respectively). Relative content of anthocyanins during the 3rd and 7th week ((e,f), respectively), flavonols during the 3rd and 7th week ((g,h), respectively), and nitrogen balance index during the 3rd and 7th week ((i,j), respectively) are provided for the second experiment. Nitrogen Balance Index is provided for the second experiment (i,j). Values for the first experiment represent absolute chlorophyll density ($\mu\text{g cm}^{-2}$), while subsequent measurements are expressed as a relative index. Data are presented as the mean value, with error bars representing the standard deviation ($\pm\text{SD}$) (first experiment: $n = 6$ for sanitized and non-sanitized frass and $n = 3$ for control; second experiment $n = 5$ for all treatments). Different letters above bars indicate statistically significant differences among treatments within each sampling week (Tukey's test; $p < 0.05$). Bars without letters indicate no significant differences between treatments.

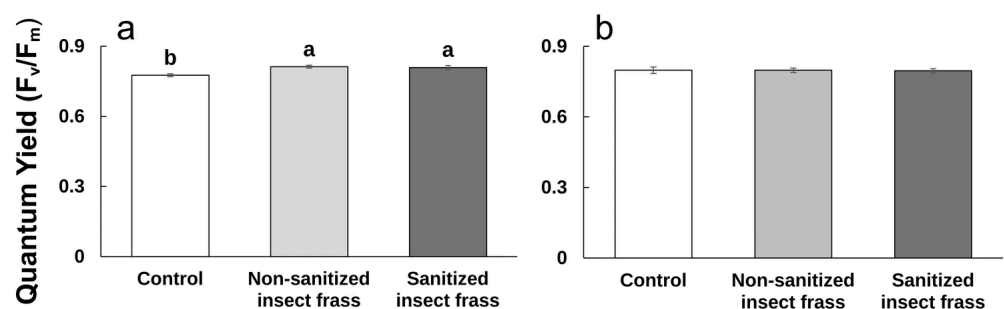


Figure 2. Effects of non-sanitized (light gray) and sanitized (dark gray) insect frass on lettuce maximum quantum yield of photosystem II (F_v/F_m) compared to the unfertilized control (white). Quantum yield is shown for the first experiment (a) and second experiment (b). Data is presented as the mean value, with error bars representing the standard deviation ($\pm\text{SD}$) (first experiment: $n = 6$

for sanitized and non-sanitized frass and 3 for control; second experiment $n = 5$ for all treatments). Different letters above bars indicate statistically significant differences among treatments (Tukey's test; $p < 0.05$). Bars without letters indicate no significant differences between treatments.

3.3. The Effect of Sanitized Frass on Lettuce Plant and Root Biomass

Both insect frass treatments had significantly higher ($p < 0.001$) aboveground lettuce fresh and dry biomass yield compared to the control plants (Figures 3 and 4a–d), but there was no significant difference between the sanitized and non-sanitized insect frass treatments in both experiments (fresh biomass: $p = 0.973$ for the first experiment, $p = 0.952$ for the second experiment, dry biomass: $p = 0.929$ for the first experiment, $p = 0.528$ for the second experiment). Similarly, the fresh and dry root biomass was significantly higher ($p < 0.01$) in frass treatments compared to control plants and there was no statistically significant difference (fresh biomass: $p = 0.494$, dry biomass: $p = 0.173$) between the sanitized and non-sanitized insect frass treatments (Figure 4e–f).



Figure 3. Representative pictures from lettuce plants at the harvest day of the 1st experiment (A) and the 2nd experiment (B).

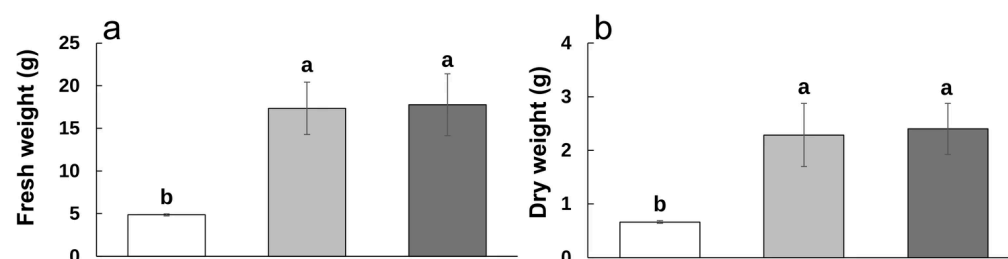


Figure 4. Cont.

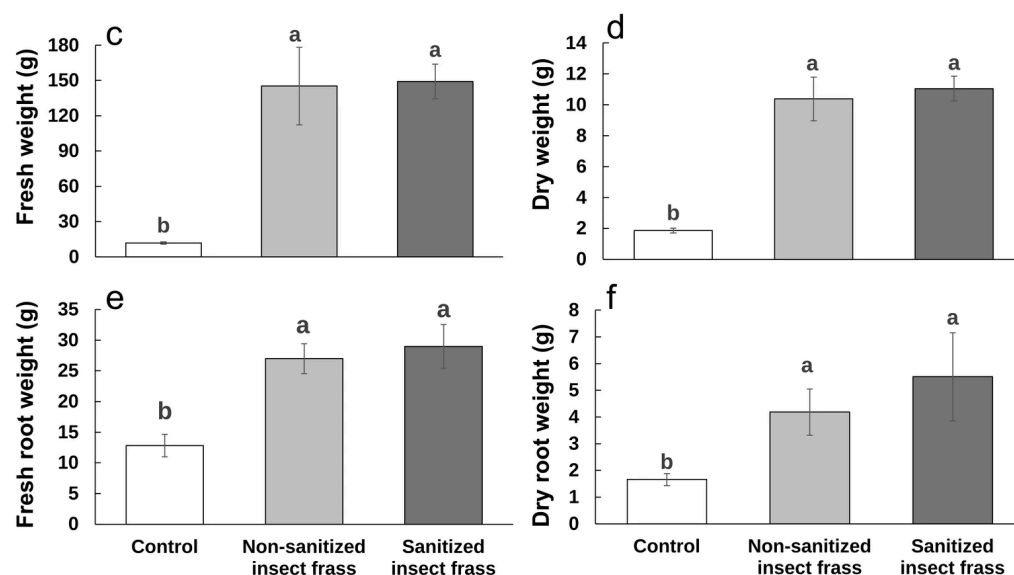


Figure 4. Effects of non-sanitized (light gray) and sanitized (dark gray) insect frass on lettuce fresh and dry biomass compared to the unfertilized control (white). Aboveground fresh and dry biomass is shown for the first experiment ((a,b) respectively) and second experiment ((c,d) respectively). Belowground fresh and dry biomass is shown for the second experiment ((e,f) respectively). Data are presented as the mean value, with error bars representing the standard deviation ($\pm$ SD) (first experiment: $n = 6$ for sanitized and non-sanitized frass and 3 for control; second experiment $n = 5$ for all treatments). Different letters above bars indicate statistically significant differences among treatments (Tukey's test; $p < 0.05$).

3.4. The Effect of Sanitized Frass on Soil Microbial Activity

The addition of both sanitized and non-sanitized frass resulted in a significant increase ($p < 0.01$) in both dehydrogenase and β -glucosaminidase activities compared to the control plants (Figure 5a,b). There was no significant difference ($p = 0.96$) between the sanitized and non-sanitized insect frass treatments in both enzyme activities.

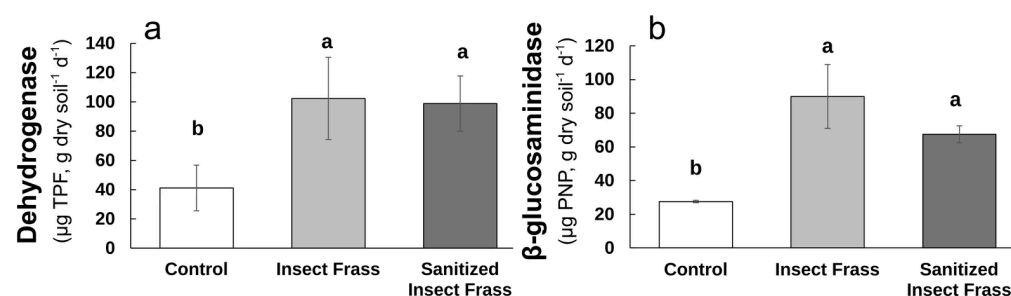


Figure 5. Effects of non-sanitized (light gray) and sanitized (dark gray) insect frass on microbial enzyme activities compared to the unfertilized control (white). Panels show dehydrogenase (a) and β -glucosaminidase (b) activities in the rhizosphere. Data is presented as the mean value, with error bars representing the standard deviation ($\pm$ SD) ($n = 5$). Different letters above bars indicate statistically significant differences among treatments (Tukey's test; $p < 0.05$).

3.5. Nutrient Analysis of Soil Samples and Plant Nutrient Uptake

Both insect frass treatments resulted in higher soil nutrient contents ($p < 0.001$ for pH, P and Mg, $p < 0.05$ for K for the sanitized treatment and 0.141 for the non-sanitized treatment) than the control at harvest. However, there was no significant difference ($p = 0.056$ for pH, $p = 0.935$ for P, $p = 0.385$ for K, $p = 0.575$ for Mg) between the sanitized and non-sanitized treatments in soil available nutrients, except mineral N ($p = 0.0105$) (Table 2).

Table 2. Nutrient analysis of the soil samples treated with non-sanitized and sanitized insect frass compared to the unfertilized control, at the harvest of lettuce from the second experiment. Values are means of 5 replicates $\pm$ st. deviation. Different superscript letters indicate statistically significant differences among treatments (Tukey's test; $p < 0.05$).

Nutrients (mgkg ⁻¹)	Soil Treated with Non-Sanitized Insect Frass	Soil Treated with Sanitized Insect Frass	Soil with No Addition (Control)
Mineral N	150.1 $\pm$ 9.6 ^b	174.7 $\pm$ 15.4 ^a	8.2 $\pm$ 6.5 ^c
P	75.2 $\pm$ 11.7 ^a	77 $\pm$ 7.7 ^a	10.6 $\pm$ 0.5 ^b
K	85 $\pm$ 28.1 ^{ab}	118.6 $\pm$ 48.5 ^a	24.2 $\pm$ 8.3 ^b
Mg	94.8 $\pm$ 13.6 ^a	100.4 $\pm$ 6.1 ^a	36 $\pm$ 0.7 ^b
pH _{CaCl2}	5.7 $\pm$ 0.04 ^b	5.6 $\pm$ 0.04 ^b	6.1 $\pm$ 0 ^a

There was no significant difference in plant N ($p = 0.854$) and P ($p = 0.508$) uptake between the plants treated with sanitized and non-sanitized frass (Table 3). No data was obtained for the control group as the dry aboveground biomass was not sufficient to perform the analysis.

Table 3. Nutrient uptake in plant tissue of plants treated with non-sanitized and sanitized insect frass. Values are means of 5 replicates $\pm$ st. deviation. No statistical differences were observed (independent samples t -test $p < 0.05$). N uptake data for the control plants were unavailable due to insufficient plant biomass for tissue analysis, which precluded the calculation of nutrient use efficiency indices such as agronomic efficiency and apparent nutrient recovery.

Nutrients	Plants Treated with Non-Sanitized Insect Frass	Plants Treated with Sanitized Insect Frass
Total N (g kg ⁻¹ dry biomass)	26.5 $\pm$ 3.5	24.6 $\pm$ 1.4
Total P (g kg ⁻¹ dry biomass)	5.2 $\pm$ 0.6	5.2 $\pm$ 0.6
N uptake (g N per pot)	0.275 $\pm$ 0.03	0.271 $\pm$ 0.01
P uptake (g P per pot)	0.054 $\pm$ 0.00	0.056 $\pm$ 0.00

4. Discussion

Our results showcase that the agronomic benefits of frass as a biofertilizer persist after the mandatory sanitization process required under EU Regulation 2021/1925 [11] under greenhouse conditions using lettuce as a test crop. In contrast to our hypotheses, we did not observe significant differences in plant growth metrics (aboveground biomass, chlorophyll content, and maximum quantum yield of photosystem II), soil microbial enzyme activity, or nutrient uptake by lettuce plants between sanitized and non-sanitized treatments. While our hypotheses predicted that sanitization would increase NH₄⁺-N availability, reduce soil enzyme activity, and produce measurable differences in plant responses, the results consistently showed that thermal treatment at 70 °C for 1 h did not compromise the agronomic performance of BSF frass. This finding carries direct practical relevance for producers operating under EU regulatory requirements. Both sanitized and non-sanitized frass increased lettuce above- and belowground biomass, plant physiological parameters, and soil β -glucosaminidase and dehydrogenase activities relative to the unfertilized control, further indicating the potential of BSF frass as a biofertilizer under greenhouse conditions.

4.1. Effect of Thermal Sanitization on the Nutrient Composition of BSFF

The nutrient composition of BSF frass is in accordance with previous studies, with the exception of a higher Mg content, ranging from 1.0 to 1.1% in our study compared to 0.13–0.8% reported for black soldier fly frass in earlier studies [22–25]. This higher Mg content may reflect the larval diet, which was primarily based on a co-product of cheese

production known to be rich in minerals [26]. The C:N ratio of the BSFF in the current study (14.5–14.7) falls within the range reported in the literature (13–16) [27]. C:N ratios in this range are generally associated with net N mineralization upon soil incorporation of organic amendments, suggesting that frass application is unlikely to cause net N immobilization.

In line with our first hypothesis that sanitized frass would exhibit higher $\text{NH}_4^+\text{-N}$ concentrations, a small but statistically non-significant increase in both EC ($p = 0.128$) and $\text{NH}_4\text{-N}$ ($p = 0.281$) was observed in sanitized frass. This suggests that while the thermal process may promote partial transformation of organic nitrogen into mineral forms, the magnitude of this effect was insufficient to produce a statistically detectable difference at the application rate used. Previous studies have reported that heating stimulates the transformation of organic nitrogen into more mineralizable forms—for example, heating air-dried sewage sludge has been shown to accelerate nitrogen mineralization upon soil application [28], and microwave treatment has been shown to significantly increase both nitrate and ammonium concentrations [29]. These increases, while non-significant here, may nonetheless contribute to subtle variations in early N availability or salt exposure that could be relevant for salt-sensitive vegetable crops.

4.2. Microbial Community and Nutrient-Mediated Mechanisms

Previous studies have shown that heat treatment at 70 °C for 1 h reduces total viable counts and strongly decreases Enterobacteriaceae and other potential pathogens in insect frass, while heat-tolerant and spore-forming taxa persist [30]. This indicates that the microbial community in sanitized frass is not identical to that of non-sanitized frass. When evaluating the biofertilizer performance of sanitized and non-sanitized frass, it is therefore essential to distinguish between nutrient-mediated effects, organic-matter-mediated effects, and direct microbial effects. In the current study, nutrient-mediated effects are clearly evidenced by the substantial increases in plant biomass and N/P uptake for both sanitized and non-sanitized frass relative to the unfertilized control, attributable to the rapid mineralization and release of macro- and micronutrients from the frass.

Despite our third hypothesis that sanitized frass would reduce soil enzyme activity than sanitized frass, both dehydrogenase and β -glucosaminidase activities were stimulated by both frass types relative to the control, irrespective of sanitization. This suggests that either heat-resistant microorganisms, such as *Bacillus* spp., survived the thermal treatment and remained functionally active, or that native soil microbial communities were rapidly stimulated by the organic matter input from frass regardless of its sanitization status.

Given these results, the enhanced soil microbial activity observed in both treatments is most likely driven by C and nutrient inputs rather than by frass-borne microorganisms. This is further supported by the consistently higher DHA and BGA activities in frass-amended soils relative to the unamended control across both treatments, indicating that frass organic matter is the primary driver of soil microbial activity and that thermal sanitization does not negatively affect this response [10]. These findings are consistent with previous studies reporting that frass promotes plant growth and soil health through a combination of nutrient supply and microbial effects, including alterations in rhizosphere microbial communities and enhancement of plant responses to biotic and abiotic stresses [9,31–35]. However, disentangling the specific contributions of surviving frass microorganisms and native soil microbes to the observed functional responses is beyond the scope of the current study, as direct microbial effects can only be assessed through explicit microbial community analyses. It should also be noted that frozen storage may have reduced the viability of non-spore-forming microorganisms in both frass types. This could have narrowed the microbiological differences between sanitized and non-sanitized frass prior to soil application, potentially contributing to the absence of treatment differences in soil enzyme responses.

Future research should therefore characterize active microbial communities in frass before and after sanitization and across storage durations to determine the relative contributions of frass-borne and native soil microorganisms to the observed functional responses.

4.3. Residual Mineral N and Nutrient Uptake

The frass generally resulted in relatively higher residual mineral N after harvest, which is related to the initial high N content of the BSFF (3.4% N) and the high application rate (15 t ha⁻¹). Importantly, residual soil mineral N at harvest was significantly higher in the sanitized frass treatment (174.7 mg kg⁻¹) compared to the non-sanitized treatment (150.1 mg kg⁻¹), despite the absence of significant differences in NH₄⁺-N between the two frass materials prior to application (Table 1). This suggests that the thermal treatment promoted greater net nitrogen mineralization during the pot experiment, rather than simply altering the initial mineral N pool in the frass. This finding is partly consistent with our first hypothesis, which predicted that sanitization would increase mineral N availability, and aligns with observations from other organic amendments where heat treatment has been associated with accelerated nitrogen mineralization [28,29]. The specific organic fractions driving this response were not characterized in the current study and warrant further investigation. In contrast, no significant differences between sanitized and non-sanitized treatments were observed for residual soil P, K, or Mg, suggesting that the effect of thermal sanitization on nutrient dynamics was specific to nitrogen cycling.

However, despite the significantly higher residual mineral N in the sanitized treatment, nutrient uptake by plants did not differ between treatments for either N or P (Table 3). This decoupling between soil mineral N availability and plant N uptake is consistent with the strong control of mineral N release from organic sources by soil conditions [36] and the physiological regulation of N assimilation in crops, which is primarily driven by growth demand and dry matter accumulation rather than being proportional to soil N supply [37]. Under the specific environmental conditions of this study (pot size and light intensity), lettuce likely reached a physiological threshold for nitrogen assimilation as biomass expanded [38]. Because non-sanitized frass already supplied sufficient nitrogen to meet the maximum physiological requirements for lettuce growth, the surplus mineral N released from sanitized frass exceeded crop uptake capacity and remained in the soil. This luxury N supply scenario is consistent with the absence of significant differences in aboveground biomass between treatments and the equivalent N and P uptake observed (Table 3) and highlights the importance of application rate optimization in future studies. Furthermore, the rapid transformation of organic waste by black soldier fly larvae within approximately five weeks compared to 24 weeks for conventional composting [39] may produce a biochemically stable material whose intrinsic properties may explain why the sanitization process had limited effects on overall nutrient availability and plant uptake, despite the observed difference in residual mineral N.

4.4. Plant Growth and Physiological Responses

Several studies have reported the positive impacts of insect frass on plant growth [6,9,32], showing that frass addition can achieve effects comparable to synthetic fertilizers, though results vary depending on insect species, application rate, and crop species [32]. Our results are aligned with these findings: both sanitized and non-sanitized frass substantially increased aboveground biomass across both experiments (Figure 4), with no significant effect of sanitization on this response. The consistent increase in belowground biomass observed in the second experiment further supports the positive effect of frass on overall plant development, suggesting that root growth was also stimulated by the nutrient inputs from both frass types.

This result contradicts our second hypothesis, which predicted that thermal sanitization would reduce lettuce biomass, nitrogen uptake, and its physiological status. The absence of such differences suggests that the nutrient pools supplied by both frass types were functionally equivalent from the plant's perspective and that the modest increases in NH_4^+ -N and EC in sanitized frass were below the threshold required to produce a detectable agronomic response under the tested conditions.

We also observed increases in chlorophyll content and maximum quantum yield of photosystem II following frass application, indicating that frass application boosts photosynthetic efficiency. Plants treated with frass (both sanitized and non-sanitized) exhibited lower flavonol and anthocyanin contents in both experiments. Flavonoids (including flavonols) and anthocyanins are secondary metabolites with roles in plant stress responses [40]. The elevated values of both compounds in control plants likely reflect responses to nutrient deficiency, particularly N deficiency [41–43], whereas frass application provided sufficient nutrients to prevent these stress-associated accumulations. The nitrogen balance index, which is a ratio reflecting the balance between chlorophyll and flavonol content indicative of plant nitrogen status, was significantly higher in both frass treatments compared to the control at both timepoints (Figure 1i–j), further confirming that frass application, irrespective of sanitization, adequately met the nitrogen demands of the lettuce crop.

Despite differences in growth conditions between the two greenhouse experiments, including supplemental lighting and a longer duration in the second pot experiment, which limits direct comparisons, plant responses were consistent across both trials. The absence of significant differences in plant growth response between sanitized and non-sanitized frass under both lighting conditions reinforces the robustness of the material's agronomic performance and suggests that the mandatory EU thermal treatment (70 °C for 1 h) does not compromise the functional value of BSF frass as a biofertilizer across varying environmental conditions.

5. Conclusions

Under the tested greenhouse pot conditions, BSF insect frass demonstrated biofertilizer potential for lettuce cultivation, consistently increasing above- and belowground biomass, chlorophyll content, nitrogen balance index, photosynthetic efficiency, and soil enzyme activity relative to unfertilized control. Thermal sanitization at 70 °C for 1 h, as mandated under EU Regulation 2021/1925, did not significantly alter the macro- or micronutrient composition of the frass or diminish its agronomic performance. Partially consistent with our first hypothesis, sanitized frass resulted in significantly higher residual soil mineral N at harvest compared to non-sanitized frass, suggesting that thermal treatment promoted greater net nitrogen mineralization during the experiment. However, this did not translate into differences in plant N uptake, consistent with luxury N dynamics at the high application rate used. Contrary to our second and third hypotheses, neither soil enzyme activity, plant biomass, nutrient uptake, nor physiological status differed significantly between sanitized and non-sanitized treatments. The enhancement of soil microbial activity regardless of the thermal sanitization suggests a nutrient- and organic-matter-driven mechanism, though explicit microbial community analyses are needed to disentangle the contributions of surviving frass microorganisms and native soil communities.

The comparable performance of both frass types on plant growth across two independent greenhouse experiments under varying environmental conditions indicates the robustness of these findings and supports the integration of thermally sanitized BSF frass into circular horticultural production systems. However, the study was conducted in pots with one frass type, one soil type, and a single crop species without positive fertilizer control.

Future studies should assess frass performance under field conditions across diverse soils and cropping systems, optimize application rates to reduce post-harvest mineral N accumulation and characterize microbial diversity and functional activity across frass sources before and after sanitization to elucidate the biological mechanisms underpinning frass biofertilizer function.

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