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Evaluation of the Effect of *Tenebrio molitor* Frass on the Growth Parameters of Canasta Lettuce (*Lactuca sativa* var. *capitata*) as a Model Plant

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Abstract

The European Commission approval of some insect species for human consumption, starting with *Tenebrio molitor* (TM) in 2021, has drawn attention to the production of insect-derived protein flours and the sustainability of insect-rearing systems, particularly on a large scale. This has also highlighted the importance of utilizing byproducts, such as frass, and obtaining high-value-added products, such as biofertilizers. This study explored the potential for TM frass (TMF) to serve as a natural fertilizer for the cultivation of Canasta lettuce (*Lactuca sativa* var. *capitata*). Specifically, a series of tests was carried out to assess the efficacy of thermal treatment and to verify the trend of certain chemical and growth parameters as a function of the TMF percentage to be added to the potting soil. For this purpose, different percentages of both thermal-treated and untreated TMF and their effects on various growth parameters of Canasta lettuce were evaluated through pot trials. Furthermore, TMF was characterized by using scanning electron microscopy (SEM) to gain insights into its structural features and potential influence on soil–plant interactions. Our results show that heat treatment of TMF is essential to ensure plant survival, and at least in pots, TMF percentages above 5% of soil dry weight are not recommended. In our tests, the most suitable percentage was 4%.

Keywords: insect fertilizers; circular bioeconomy; thermal treatment; SEM analysis

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1. Introduction

The global population reached approximately 8.2 billion in March 2025 [1], marking a substantial increase from 5 billion in 1986 and 7.7 billion in 2019 [2].

According to the latest United Nations World Population Prospect (2024) [2], this growth is expected to peak around 10.3 billion by the 2080s before slightly declining to 10.2 billion by 2100, primarily due to falling fertility rates in major countries such as China [3]. This demographic trend is placing escalating pressure on global food systems, both in terms of production demands and waste management, thereby exacerbating the strain on agricultural resources [3–5].

Global agriculture still largely relies on the intensive use of chemical pesticides and mineral fertilizers [6,7]. While these inputs have historically enhanced crop yields, their widespread and often indiscriminate application has raised serious environmental and public health concerns [8]. Pesticides have been associated with immunotoxicity, respiratory issues, reproductive harm, endocrine disruption, and increased cancer risk [9]. Meanwhile, mineral fertilizer production—supplying essential macronutrients such as nitrogen (N), phosphorus (P), and potassium (K)—is energy-intensive and contributes approximately 2% of total global greenhouse gas emissions (GHGs) [9]. Moreover, excessive or mismanaged fertilizer use causes several environmental problems, including soil acidification, ionic imbalance, inhibition of beneficial soil microorganisms, salinization, accumulation of toxic compounds, and nutrient leaching into groundwater [10]. These cumulative effects contribute to the soil fertility degradation over time [11].

This context underscores the urgency of transitioning toward more sustainable agricultural practices, reducing reliance on non-renewable resources, and adopting nutrient management strategies aligned with circular economy principles. This need is further emphasized by persistent food insecurity, affecting both industrialized and developing nations. In 2018, food shortage rates were 2.3% in the United States, 4.6% in Canada, 8.2% in the United Kingdom, 2.6% in Germany, and 2.9% in Japan. Much higher rates were observed in countries such as Ethiopia (23.4%), Côte d'Ivoire (22.4%), Bangladesh (12.7%), Pakistan (17.2%), Haiti (45.6%), and India (14.3%) [11].

In response to these challenges, natural and organic fertilizers are gaining recognition as viable alternatives to synthetic ones. Derived from biological residues such as compost, manure, or agro-industrial waste, these fertilizers contribute to long-term soil fertility and reduce environmental impact [12].

Within this framework, insects have emerged as a promising dual-purpose resource in sustainable agriculture. In addition to providing alternative protein sources for food and feed, insects generate valuable by-products—particularly frass—that can be reintegrated into agricultural systems through a circular bioeconomy model [13–16]. Frass, the solid excrement of farmed insects, typically mixed with exuviae and undigested substrate, has attracted increasing attention as an organic soil amendment that contributes to nutrient recovery and improved soil health [16].

One of the most promising insect species for industrial farming is *Tenebrio molitor* (TM), a beetle of the Tenebrionidae family. TM was the first insect approved for human consumption in the European Union, and its larvae (TML) are now mass-reared for food, feed, and other biotechnological uses [17,18]. Frass production scales proportionally with insect farming. Under controlled rearing conditions (27 ± 1 °C, 65 ± 5% RH, continuous darkness, wheat bran diet), TML produce between 200 and 300 g of frass per 100 g of larval biomass, meaning frass output can exceed the harvested larval mass [19–21]. This dry, friable, and nearly odorless by-product is easier to handle, store, and transport than traditional organic fertilizers [22,23].

Chemically, TMF is rich in mineralizable NPK, micronutrients (e.g., Na, Mn, Zn), functional organic compounds (e.g., humic and fulvic acids, chitin, cellulose, lignin, and xylan), and beneficial microbes [22,23]. Comparative studies have validated TMF's fertilizing efficacy. For example, TMF contains up to 93% more total carbon and 60% more nitrogen than poultry litter, with a favorable C/N [24]. Field trials revealed that TMF increases soil N, K, and Mg by 12–35%, achieving yields comparable to poultry litter applied at half the rate [25]. TMF also outperforms or matches compost and hen manure across multiple crops [26]. These findings, alongside the scaling-up of TM industrial production, are boosting the availability of TMF as a residual biomass [18,27]. At the same time, recent geopolitical

instability has disrupted fertilizer supply chains, especially in Europe, accelerating interest in TMF as a viable bio-based alternative [16].

However, regulatory frameworks for insect frass differ widely by state. In the United States, insect frass is classified as a soil improver. In Canada and China, it is managed under organic fertilizer regulations [28,29]. In the European Union, Regulation (EU) 2021/1925 defines frass as "a mixture of excrement derived from farmed insects, the feeding substrate, parts of farmed insects, dead eggs and with a content of dead farmed insects of not more than 5% in volume and not more than 3% in weight" [30]. Regulation (EU) No 142/2011 also mandates thermal treatment at 70 °C for 60 min to minimize microbiological risks, particularly from *Escherichia coli* and *Salmonella* spp. [31,32]. These pathogens, often present in the insect gut, may be excreted in frass and pose risks if untreated. Thus, frass is currently classified as animal manure and permitted only as a soil conditioner after heat treatment, not yet as an organic fertilizer.

It is important to note, however, that such heat treatment can significantly reduce microbial viability, potentially eliminating beneficial microbial communities naturally present in frass. Nonetheless, recent studies have shown that TMF retains a substantial portion of its microbial population even after thermal treatment, unlike Black Soldier Fly (BSF) frass, which undergoes a more pronounced microbial decline [33]. The persistence of heat-resistant microbial taxa—particularly Firmicutes—in TMF suggests that its functional properties, such as organic matter degradation and biocontrol activity, may be better preserved [34–37]. Furthermore, the presence of heat-resistant beneficial taxa—especially *Bacillus* spp., known for their biocontrol properties—adds a relevant dimension. Although no direct studies have yet addressed the antifungal potential of thermally treated TMF, parallel evidence from BSF frass is promising. It has been shown that BSF frass retains in vitro antifungal activity against various phytopathogens, including *Botrytis cinerea*, *Alternaria solani*, *Fusarium oxysporum*, *Sclerotinia sclerotiorum*, and *Rhizoctonia solani*, primarily due to the persistence of microbial communities with antagonistic potential [38–40]. Notably, these effects were observed even after pasteurization, suggesting that thermotolerant strains, such as *Bacillus velezensis*, can survive sanitation processes and maintain their suppressive function [38–40]. These findings provide a useful parallel and support the hypothesis that TMF may also retain plant-protective potential after thermal treatment.

Moreover, the International Platform of Insects for Food and Feed (IPIFF) continues to advocate for tailored treatment measures and regulatory recognition of frass as a fertilizer by promoting its inclusion in Component Material Category (CMC) 10 under Regulation (EU) 2019/1009 [41].

Finally, it is worth noting that frass composition is closely influenced by the insect diet, which may affect both its agronomic efficacy and the recommended application rates [16,27,35,42].

This study investigates the potential of TMF as a bio-based soil amendment and sustainable alternative to conventional chemical inputs. The frass was produced under controlled rearing conditions and subjected to thermal treatment following European regulatory requirements. Both untreated (UT-TMF) and thermally treated (TT-TMF) samples were mixed with soil at varying concentrations and tested in greenhouse pot trials using *Lactuca sativa* var. *capitata* (Canasta lettuce) as a model plant. In parallel, a comparative morphological investigation by scanning electron microscopy (SEM) was conducted on UT-TMF and TT-TMF to assess whether heat treatment induces structural changes in the frass matrix.

The primary objective of this work was to assess the agronomic performance of TMF under different application rates and to determine whether thermal processing enhances its efficacy in supporting plant survival and growth. It was hypothesized that TMF, when applied at appropriate concentrations, could act as an effective soil amendment by pro-

moting plant development. Furthermore, it was proposed that thermal treatment might induce morphological changes in the frass matrix—detectable by SEM—which could help interpret differences in plant response, potentially through effects on physical structure.

2. Materials and Methods

A preliminary characterization of TMF was conducted to assess its physicochemical and structural features prior to application in pot trials. This included the determination of pH and electrical conductivity (EC) in frass–soil mixtures, as well as a comparative morphological investigation by scanning electron microscopy (SEM) on both UT-TMF and TT-TMF samples, aimed at identifying the structural changes potentially induced by heat processing.

Subsequently, greenhouse experiments were performed to evaluate the effects of TMF—both UT-TMF and TT-TMF—on the growth and survival of *Lactuca sativa* var. *capitata*, a Batavia-type cultivar widely cultivated in Southern Europe [43]. This model plant was selected due to its short cultivation cycle and relevance for assessing fertilization strategies in horticultural systems [44].

TMF was incorporated into commercial potting soil at various concentrations to simulate different application rates. All experimental activities were carried out under standardized conditions between late August 2024 and February 2025, with particular attention to the reproducibility of treatment protocols and frass dosage effects.

2.1. Frass Producing

The frass used for our tests was produced in the TM laboratory at the ENEA Trisaia Research Centre (Basilicata, southern Italy), where the insect is kept under controlled indoor growing conditions (temperature 28.0 $\pm$ 0.1 °C, relative humidity 65 $\pm$ 3% and photoperiod 0L:24D). The larvae were fed a standard diet consisting of 95% bran, 5% zootechnical yeast (both purchased from local suppliers of zootechnical products), and portions of one-year-old prickly pear cladodes as a wet supplement, collected at the research center, where they grow wild. The standard diet and wet supplement are administered weekly at rates of 250 mg/larva and 100 mg/larva, respectively.

Frass from larvae fed on a standard diet was stored under rearing conditions for three months, from May to July 2024. After this period, it was sieved and one portion was set aside without any treatment (referred to as UT-TMF), while a second portion underwent the legally required heat treatment (referred to as TT-TMF). Some tests were performed using UT-TMF and TT-TMF freshly harvested, without being allowed to mature for three months under rearing conditions, and only sieved. We replicate our tests using commercial TT-TMF (provided by Tarminator s.r.l., Torino, Italy).

2.2. Frass Characterisation

2.2.1. SEM Analysis

Morphological analysis of both samples was carried out using a scanning electron microscope (SEM), model Thermo Scientific™ Phenom Pure, located at the Department of Informatics, Modelling, Electronics and Systems Engineering (DIMES), University of Calabria (Italy). Samples were air-dried and mounted on aluminum stubs using conductive carbon tape. No metallization was applied, as the samples exhibited sufficient surface conductivity and morphological contrast to allow clear imaging under high-vacuum conditions. This approach also preserved the native surface characteristics of the frass, avoiding potential artefacts introduced by coating. Observations were performed in high-vacuum mode at an acceleration voltage of 10 kV, and images were acquired at magnifications ranging from 460× to 2450×, selected to adequately capture both overall morphology and

fine surface details of the untreated and heat-treated frass. For each condition, several representative fields were examined to highlight fibrous fragments, amorphous matrices, and transitional zones. The selected micrographs reflect the intrinsic heterogeneity of the samples and were used for comparative morphological analysis.

2.2.2. Frass and Substrate pH and Electrical Conductivity (EC)

Before the pot trials on canasta, we measured the pH and electrical conductivity (EC) of the substrates containing TMF at various percentages, as well as 100% TMF after heat treatment as required by law, or untreated TMF. EC and pH measurements were performed on suspensions of 10 g of each TMF plus soil sample in 50 mL of distilled water, after stirring for 30 min (pH) or 120 min (EC) and then standing for at least 1 h. A pH-meter (725P, Istek, Seoul, Republic of Korea) and a conductivity-meter (430C, Istek, Seoul, Republic of Korea) were used to take readings directly from the supernatant [45]. Each analysis was repeated three times.

2.3. Canasta Trials

2.3.1. Preparation of Soil with Frass Pots

For all canasta tests, commercial universal potting soil with pH 6, EC 0.75 $dS \cdot m^{-1}$, 25% organic carbon, and 2% nitrogen (values derived from the label) was used. The percentages of TMF added by soil incorporation were calculated on the dry weight of the potting soil. Each percentage tested constitutes a thesis. For each thesis, 7 kg of universal soil with TMF was prepared, mixed repeatedly, and left overnight at room temperature [22]. Subsequently, each mixture was divided into ten portions and put in pots of 12 cm diameter and 10 cm height (700 g of mixture/pot). In each pot, one 5 cm-tall canasta plants were planted. For each TMF percentage, 10 replicas were prepared and placed in a single containing tray, which was filled to the brim at the time of planting (2 cm). Having been tested during the summer, the seedlings were subsequently watered with 75 mL of water/pot every 2 days. All the pots were put in the greenhouse, in controlled conditions, until the canasta reaches marketable size (in summer, one month).

2.3.2. Trials on Canasta with Different Percentages of UT-TMF and TT-TMF

Greenhouse trials were conducted to test the effects of TMF on canasta lettuce. Five different TMF percentages were evaluated: 2.5%, 5%, 7.5%, and 10%, in addition to the control (0% frass, referred to as TMF-0). Ten replicates were prepared for each treatment. Two sets of trials were carried out under identical conditions: one using UT-TMF, and the other using TT-TMF. After one month of cultivation, the control plants (TMF-0) reached the commercial harvest height of 17.5 $\pm$ 2.5 cm, and all trials were terminated. The following parameters were measured and are reported in Figure 1:

- Plant height (PH): distance from the stem cutting point to the apex;
- Number of edible leaves (LN);
- Stem diameter (SD): measured at the base of the plant;
- Fresh weight (FW): weight of all edible leaves, determined using an analytical balance (Gibertini Elettronica, Novate Milanese, Italy, mod. E42S-B; precision $\pm$ 0.1 mg).

2.3.3. Trials on Canasta with Minor Percentages of TT-TMF

As the literature suggests using frass percentages from 2% to 6% [21,46], a second trial was set up with TT-TMF: the 0–5% range was investigated more specifically, testing, in addition to the control, the percentages 1-2-3-4%, defined, respectively, as TMF-0, TMF-1, TMF-2, TMF-3, TMF-4. We kept the 5% as an internal control. All culture conditions and all evaluated parameters remained unchanged.

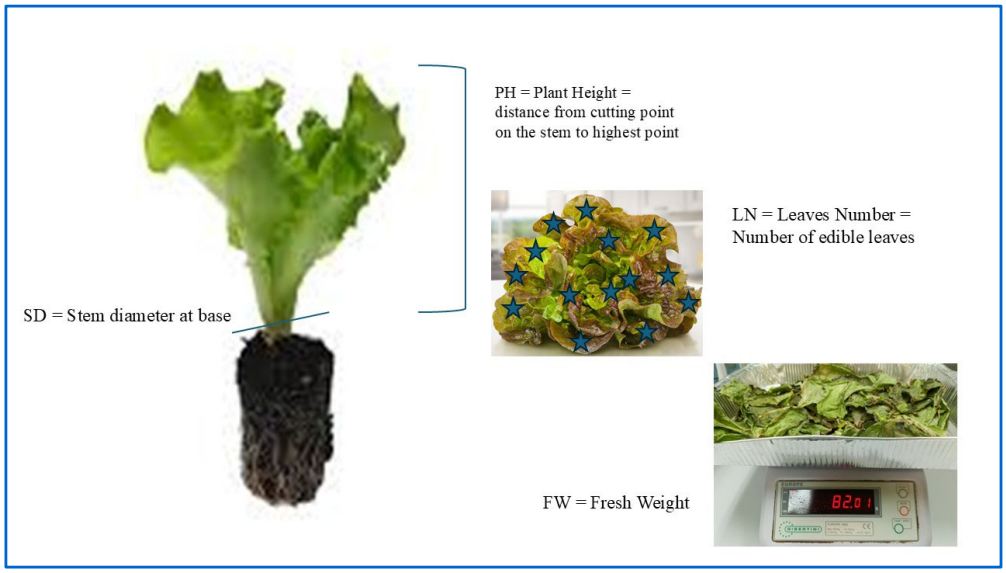

Figure 1. Evaluated parameters: PH = plant height; SD = stem diameter (at base); LN = leaf number; FW = fresh weight.

2.4. Statistical Analyses

For all tests, the data collected were statistically processed using GraphPad Prism version 8 (GraphPad Software, San Diego, CA, USA). Values are shown as means ± standard deviation (SD). Homogeneity of variance and normality were verified before statistical analysis. If this was confirmed, one-way ANOVA was used, followed by Tukey's post hoc test to identify the differences between multiple theses. Alternatively, the Kruskal–Wallis test with pairwise multiple comparisons (Dunn's test) was applied. An unpaired *t*-test was used to compare the means of two groups of independent values with homogeneity of variance and normality. Significance was assumed at $p < 0.05$.

3. Results

3.1. Frass Characterisation

3.1.1. SEM Analysis

Scanning electron microscopy (SEM) analysis was performed on frass samples obtained from TML fed a standard diet, both untreated (UT-TMF) and thermally treated (TT-TMF). Representative images are shown in Figure 2.

In UT-TMF, several micrographs (A1–A4) revealed the presence of elongated and stratified fibrous structures with distinct lamellar organization. These fragments exhibited rough surfaces and clear longitudinal cavities or channels. In many areas, these fibrous components appeared partially embedded in an amorphous matrix with a heterogeneous texture. Additionally, some micrographs (e.g., A2–A3) revealed the presence of small spherical or sub-spherical particles, often partially embedded within the surrounding matrix. Despite localized compaction, the fibrous residues largely retained their morphological integrity and remained distinguishable across most examined areas.

Conversely, TT-TMF (B1–B4) exhibited a markedly altered morphology. The overall structure appeared compact, amorphous, and homogenized, lacking identifiable fibrous features. The surface topology was characterized by dense agglomerates with irregular wrinkling, microfractures, and depressions—features typically associated with thermally induced shrinkage. In some instances, residual lamellar elements were still discernible but appeared embedded and morphologically fused within a consolidated organic matrix.

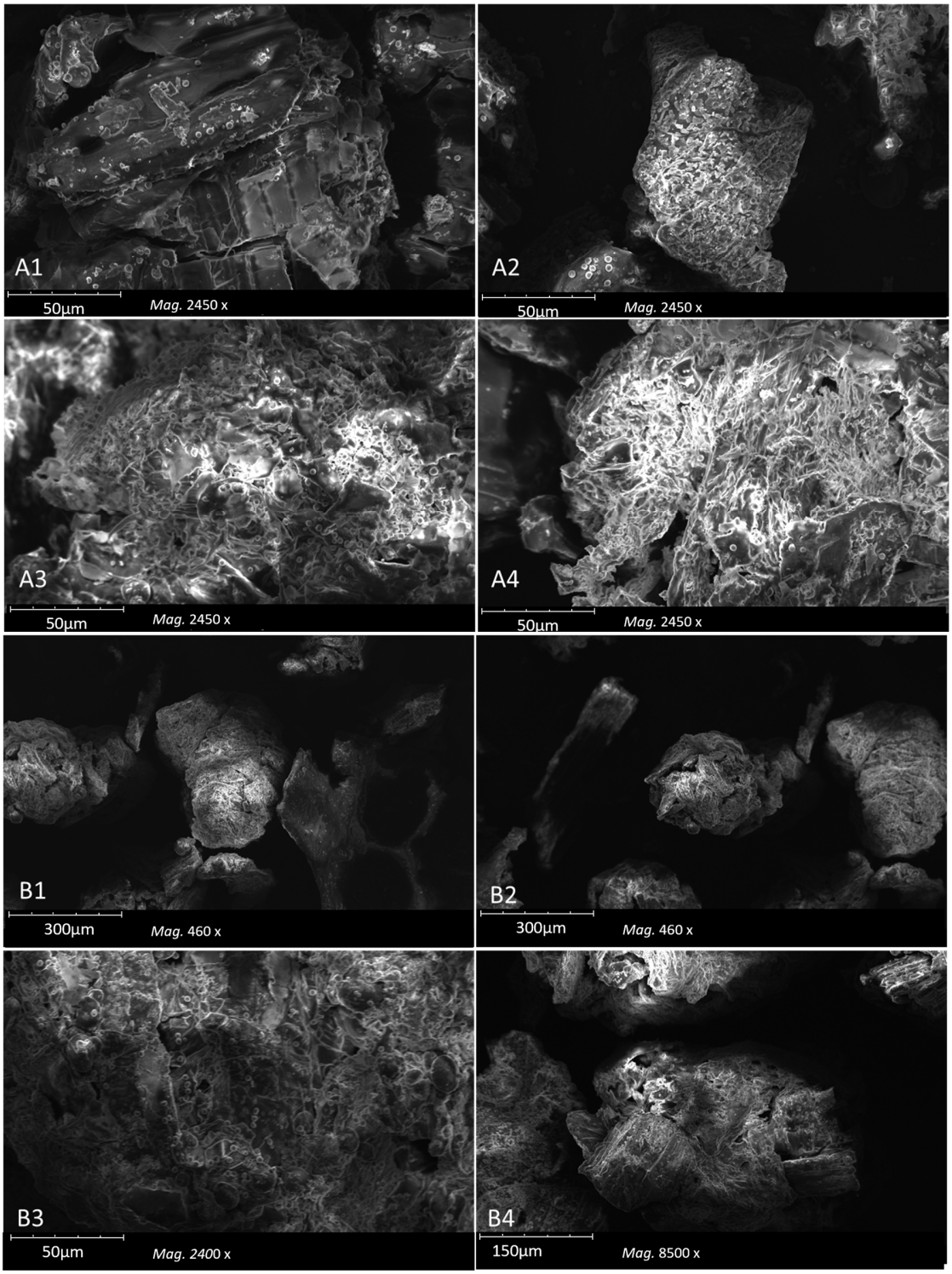

Figure 2. SEM micrographs of UT-TMF samples (**A1**–**A4**) and TT-TMF samples (**B1**–**B4**), acquired at magnifications ranging from 460× to 2450×. (**A1**–**A4**): Fibrous fragments with visible lamellar structures, rough surfaces, and longitudinal channels, typical of partially degraded plant residues from the feeding substrate. In some cases, fibrous elements are embedded within an amorphous matrix. (**B1**–**B4**): Compact and amorphous morphology resulting from thermal treatment at 70 °C for 60 min. Micrographs show dense agglomerates, surface wrinkling, and, occasionally, residual lamellar structures partially fused within the organic matrix.

Across all observed samples, no structures resembling unhatched or dead eggs were identified. Ellipsoidal, smooth-shelled, or regularly shaped elements were absent, sug-

gesting complete morphological degradation or absence of egg structures at the point of sampling.

3.1.2. Frass and Substrate pH and Electrical Conductivity (EC)

Both untreated and treated TMF exhibited similar pH values: 5.75 ± 0.04 for UT-TMF and 5.83 ± 0.07 for TT-TMF. In contrast, electrical conductivity (EC) values were significantly different, with untreated frass showing a higher EC of 15.6 ± 0.53 mS/cm compared to 12.68 ± 0.31 mS/cm for treated frass.

The pH of both treated and untreated frass shows significantly lower values than the pH of the TMF/soil mixtures with different percentages of frass. The increasing percentages of both UT-TMF and TT-TMF initially led to an increase in the pH of the mixtures, reaching a maximum of around pH 7.75 for the TMF-2.5, and then to a decrease in values approaching those of 100% frass. Figure 3 illustrates this trend for the TT-TMF mixtures.

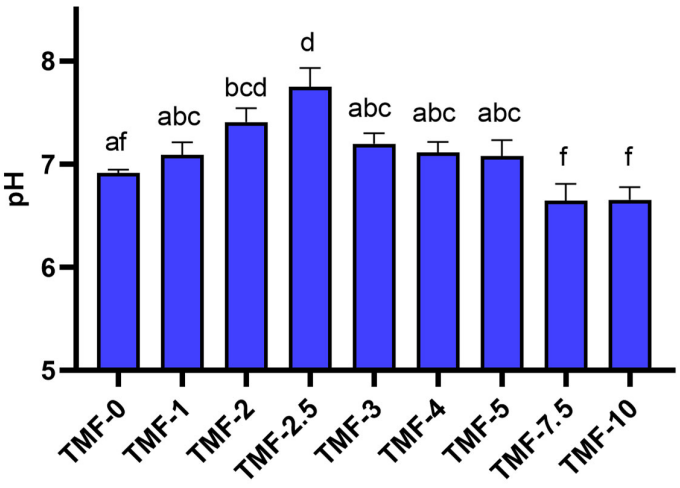

Figure 3. pH of the TMF/soil mixtures with different percentages of TT-TMF. Bars represent mean values ± standard deviation. Different letters above the bars indicate statistically significant differences between treatments ($p < 0.05$), as determined by Kruskal–Wallis's test followed by Dunn's multiple comparison test.

Regarding EC, all the tested substrates (soil and TMF at rates up to 10%) show growing values from 0.75 dS m^{-1} for 100% soil (from the label) to values of 1.93 ± 0.05 dS m^{-1} and 2.44 ± 0.09 dS m^{-1} for the UT-TMF-10 (in orange) and TT-TMF-10 (in blue), respectively (Figure 4). For TMF percentages below 2.5%, the resulting substrate exhibits an EC similar to that of soil alone, for both UT-TMF and TT-TMF. A significant increase in EC is observed at higher percentages.

3.2. Canasta Trials

In preliminary trials, TMF, including both UT-TMF and TT-TMF, was tested without a maturation period of 3 months under rearing conditions. The same percentages (0%, 2.5%, 5%, 7.5%, and 10%) used later were applied. No growth was observed; the plants completely dried up and died.

Trials on Canasta with Different Percentages of UT-TMF and TT-TMF

Figure 5 shows the results obtained on canasta plants grown with the addition of UT-TMF (in orange). In the case of TMF-10, no plants survived. Therefore, in addition to the PH, LN, SD, and FW parameters, we also included a parameter indicating live plants to show that, at TMF-10, all ten plants died; at TMF-7.5, three plants died; and at TMF-5, one plant died.

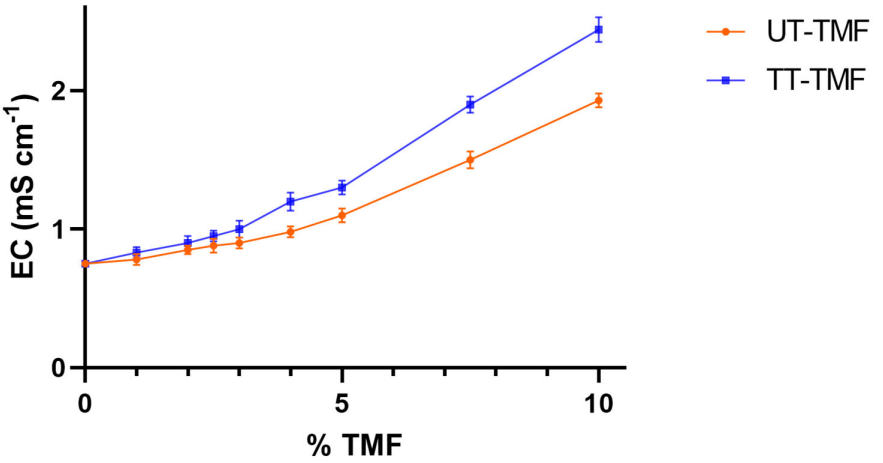

Figure 4. EC of the TMF/soil mixtures with different percentages of TT-TMF. Bars represent mean values ± standard deviation.

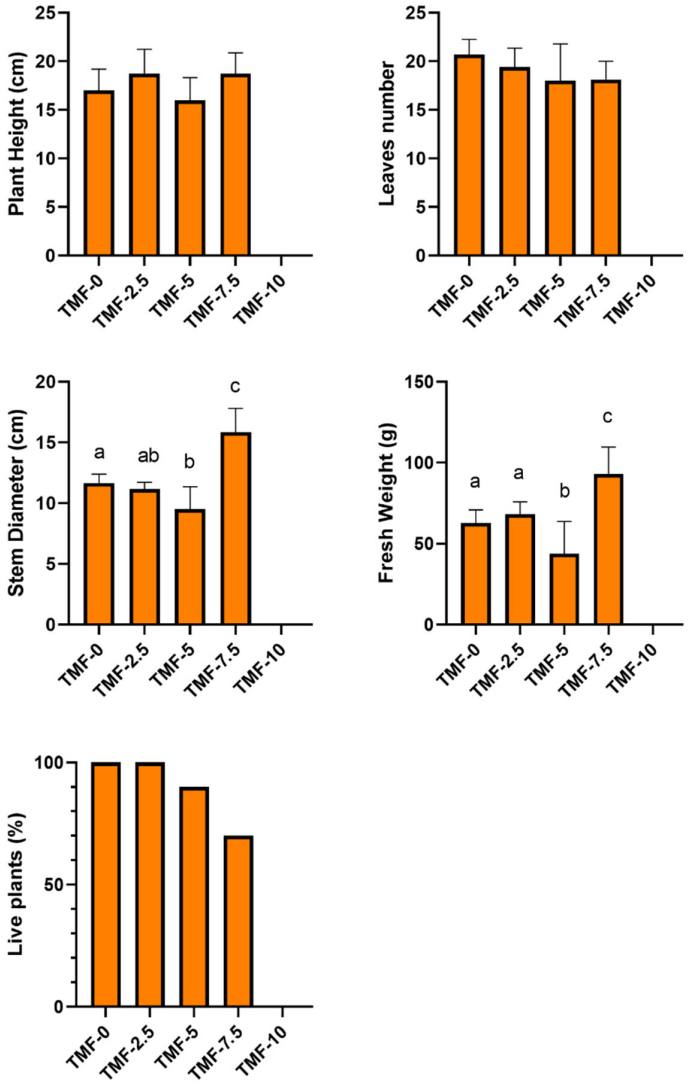

Figure 5. Growth and survival parameters of *Lactuca sativa* plants cultivated with different percentages of UT-TMF. Bars represent mean values ± standard deviation. Different letters above the bars indicate statistically significant differences between treatments ($p < 0.05$), as determined by one-way ANOVA followed by Tukey's post hoc test or, where assumptions of normality were not met, by Kruskal–Wallis's test followed by Dunn's multiple comparison test.

The application of thermally treated TMF (TT-TMF) did not affect plant survival at any of the tested concentrations. Differences were observed among the various TT-TMF application rates (shown in blue in Figure 6) with respect to the measured growth parameters. A comparison was also made with a commercially available TT-TMF product. Since the results were fully comparable with those obtained using the laboratory-produced TMF, only the latter are shown to avoid redundancy.

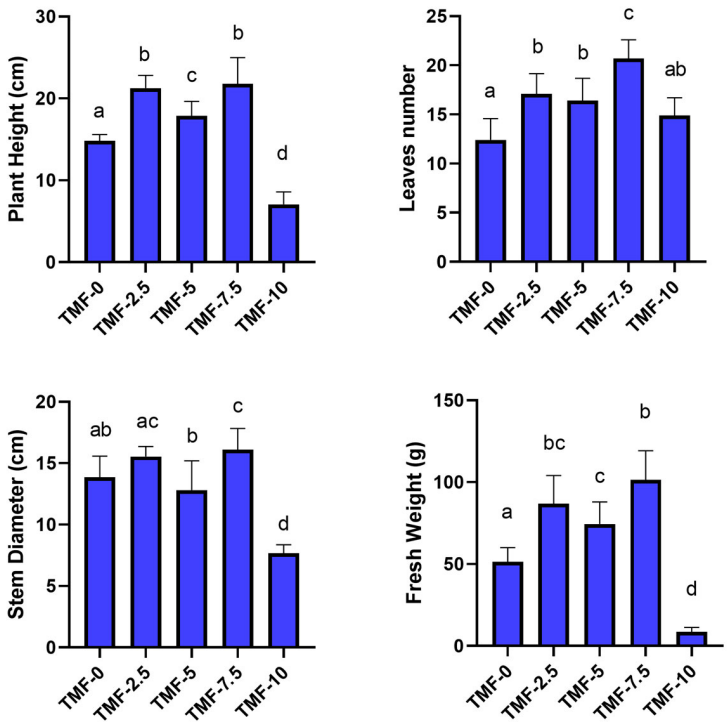

Figure 6. Growth parameters of *Lactuca sativa* plants cultivated with different percentages of TT-TMF. Bars represent mean values ± standard deviation. Different letters above the bars indicate statistically significant differences between treatments ($p < 0.05$), as determined by one-way ANOVA followed by Tukey's post hoc test or, where assumptions of normality were not met, by Kruskal–Wallis's test followed by Dunn's multiple comparison test.

Figure 7 highlights the differences between UT-TMF and TT-TMF in terms of plant development. For each of the tested frass concentrations, TT-TMF (blue bars) consistently outperformed UT-TMF (orange bars) across all parameters except for the LN for TMF-2.5 and TMF-5. UT-TMF tended to show lower and more variable performance, with some concentrations resulting in suboptimal growth. These results suggest that thermal treatment enhances the agronomic performance of TMF, likely by reducing the potential phytotoxic effects and improving nutrient availability.

In light of the improved results obtained with TT-TMF, an additional trial was conducted to investigate the application rates ranging from 1% to 5%. The outcomes of this dose-response experiment are presented in Figure 8.

The concentrations tested are within a very narrow range, leading to similar results for almost all parameters. However, some significant differences can be observed. The plant height (PH) is very similar for concentrations of 2% and above, while both the control and TMF-1, comparable to each other, show lower growth. For leaf number (LN), only TMF-2 is significantly lower. When it comes to stem diameter (SD), only TMF-1 and TMF-2 exhibit lower values. The fresh weight in TMF-1 is significantly lower than in the control group, while TMF-4 shows higher values compared to both the control and the other concentrations tested.

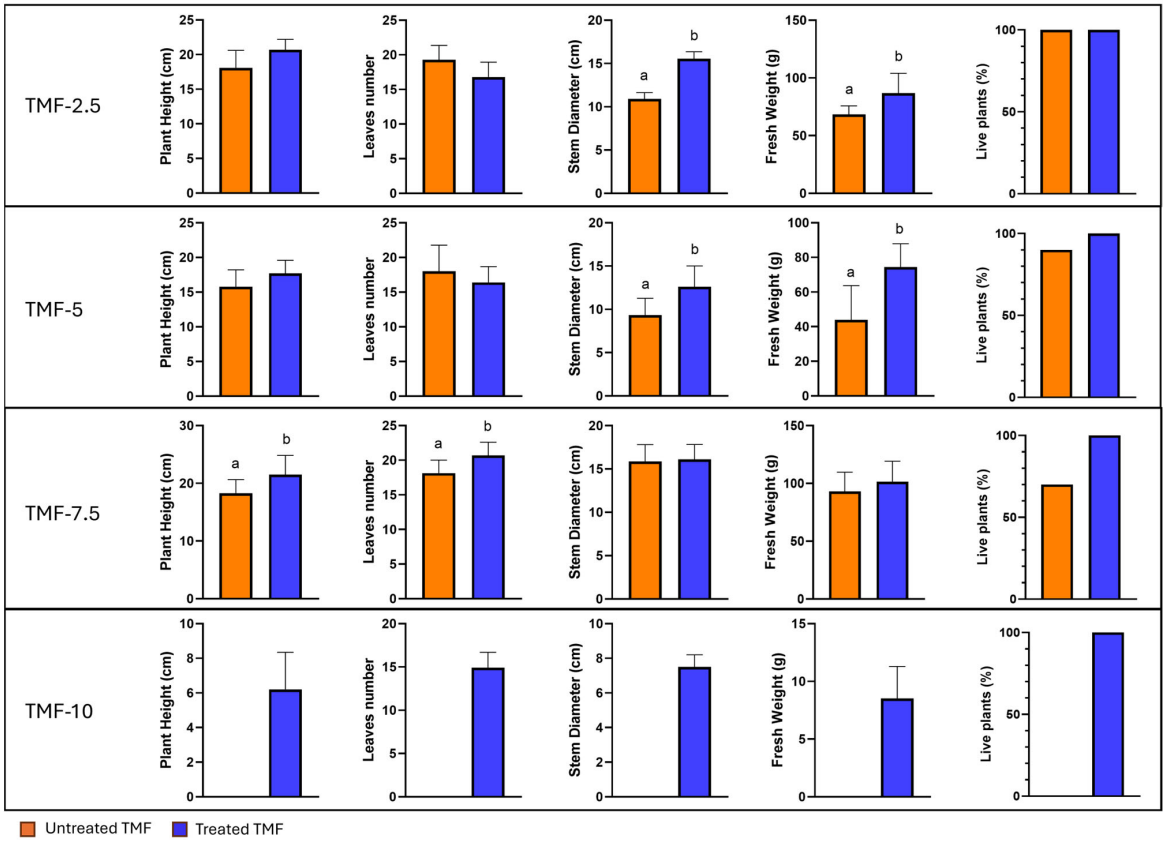

Figure 7. Comparison of UT-TMF (in orange) and TT-TMF (in blue) in terms of growth and survival parameters. Statistical differences between treatments were evaluated using an unpaired *t*-test ($p < 0.05$). Bars represent mean values ± standard deviation. Different letters indicate statistically significant differences.

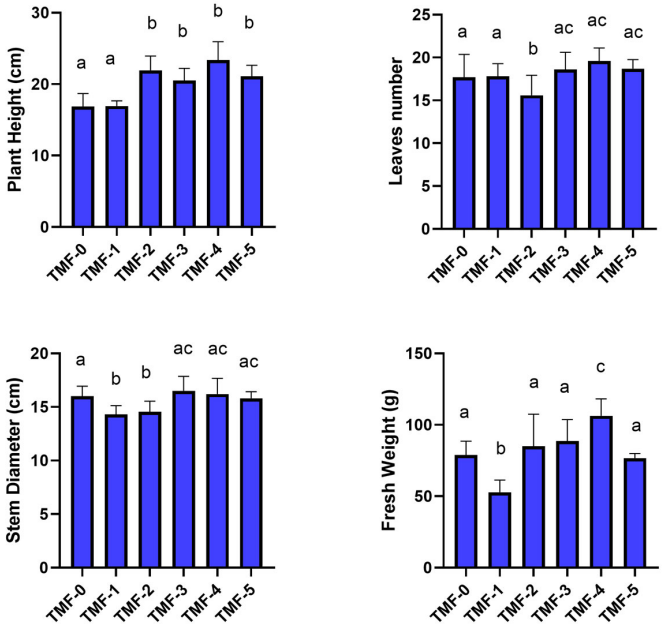

Figure 8. Growth parameters of *Lactuca sativa* plants cultivated with low percentages (1–5%) of TT-TMF. Bars represent mean values ± standard deviation. Different letters above the bars indicate statistically significant differences between treatments ($p < 0.05$), as determined by one-way ANOVA followed by Tukey's post hoc test or, where assumptions of normality were not met, by Kruskal–Wallis's test followed by Dunn's multiple comparison test.

The plants treated with various percentages of TT-TMF were photographed at the beginning and end of the tests to also carry out a visual examination. Figure 9 was created using these photos.

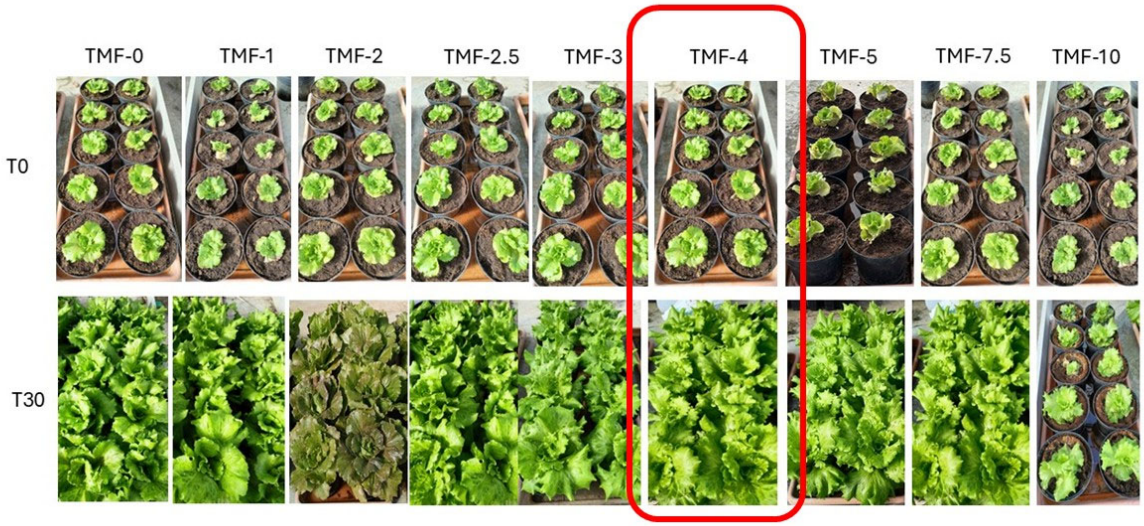

Figure 9. Growth of *Lactuca sativa* var. *capitata* at different TMF amendment levels (TMF-0, TMF-1, TMF-2, TMF2.5, TMF-3, TMF-4, TMF-5, TMF-7.5, TMF-10, dry weight basis) at day 0 (T0) and after 30 days (T30), by two tests. The 4% TMF treatment showed the best performance in terms of plant development.

The combination of quantitative growth data (Figures 6 and 8) and morphological observations (Figure 9) offers a comprehensive understanding of the effects of different TT-TMF concentrations on *Lactuca sativa* development. TMF-1 yielded results that were almost always identical to the control without TMF (TMF-0), except for SD and FW, where the results were lower than the control (Figures 8 and 9). Between TMF-2 and TMF-5, plants show increasing size, greener and more structured foliage, and higher uniformity. However, TMF-2 results in visibly smaller plants with reduced leaf development (Figures 8 and 9). TMF-2.5 shows intermediate results between TMF-2 and TMF-3 for all parameters analyzed (Figures 6 and 9). TMF-3, although acceptable in terms of height and diameter, shows lower fresh weight and slightly less homogeneity in canopy structure (Figures 8 and 9). TMF-4 stands out as the most balanced treatment: it shows consistent and robust growth, well-formed leaf architecture, and high fresh weight (Figures 8 and 9). TMF-5 was our internal control, as we repeated it in the two tests with TT-TMF. Although it gave good results, they are still inferior in many parameters to those obtained with lower percentages of TT-TMF (Figures 6, 8 and 9). TMF-7.5 also gave interesting results for all parameters, while TMF-10 is not worth considering as it showed the worst results (Figures 6 and 9). The strong agreement between quantitative growth data and morphological observations supports the selection of TMF-4 (4% frass on dry soil weight) as a promising application rate for Canasta lettuce cultivation under our tested conditions.

4. Discussion

The morphological changes observed through SEM analysis highlight the impact of thermal treatment on the structural organization of TMF.

In UT-TMF, SEM micrographs revealed multiple fibrous fragments, primarily attributable to residues of the cereal-based feeding substrate. These structures exhibited clear lamellar organization and a porous architecture, suggesting limited degradation during the digestive process. The identification of these components as plant-derived is supported by

the presence of elongated and stratified cell walls, typical of lignocellulosic tissues such as wheat bran. The observed surface roughness and longitudinal cavities, as revealed by SEM, are interpreted as consistent with partially degraded parenchymatous channels and fibrous bundles of cereal origin, likely derived from the wheat bran-based feeding substrate. This interpretation aligns with the research studies describing the presence of partially undigested lignocellulosic residues in mealworm frass [19,47].

Despite some localized compaction, the persistence of recognizable morphological features indicates that a significant portion of the substrate retained its structural identity throughout insect digestion and excretion. No morphological evidence of unhatched or dead eggs was detected in either sample. The absence of ellipsoidal or smooth-shelled elements typically associated with TM eggs may be due to their microscopic size and inherent fragility, which likely leads to degradation during rearing or digestion, or in their structural collapse during thermal treatment. Additionally, small spherical or sub-spherical particles observed in some untreated samples (e.g., A2–A3) are more likely to represent fecal aggregates or compacted fragments of plant-derived material. Their irregular surface, lack of distinct ellipsoidal morphology, and reduced size compared to TM eggs do not support their identification as viable or unhatched eggs. Nevertheless, this observation does not conflict with the regulatory definition of frass, which includes dead eggs among its acceptable constituents (Reg. EU 2021/1925).

Thermal processing at 70 °C for 60 min markedly altered the physical appearance of the frass (TT-TMF). The micrographs showed a compact, amorphous matrix devoid of clearly discernible fibrous residues. The surface morphology was dominated by dense agglomerates with fractures, depressions, and wrinkling features that may be indicative of dehydration and thermally induced shrinkage. In a few cases, residual lamellar structures remained visible, though partially embedded within the surrounding matrix, suggesting incomplete fusion between vegetal and organic components.

Overall, the SEM analysis demonstrates that thermal treatment transforms frass from a heterogeneous, fibrous material into a more homogeneous amorphous structure. Although no direct studies have yet established a link between frass microstructure and fertilization efficacy, evidence from pelletized or thermally treated organic fertilizers supports this hypothesis. For example, Alemi et al. reported that composts made from manure and urea, when pelletized to increase density and reduce porosity, led to slower nitrogen release, lower leaching losses, and enhanced nutrient uptake in crops such as wheat and basil [48]. Likewise, Das et al. and Rafique et al. showed that compact, microporous structures in biochar-based fertilizers enhanced nutrient retention and modulated release dynamics, resulting in better plant growth performance [49,50]. These findings support the idea that the microstructural transformation induced by thermal processing may contribute to the improved efficacy of TT-TMF. The smoother and denser surface may not only enable more controlled nutrient delivery over time but also stabilize the microbial microhabitat, facilitating colonization and reducing osmotic stress fluctuations [27,51]. This hypothesis finds indirect support in recent studies on BSF frass, where microbial-mediated biocontrol activity was preserved even after pasteurization. Specifically, *Bacillus* spp., known for their thermotolerance and antifungal potential, remained functionally active against pathogens such as *Fusarium oxysporum* and *Botrytis cinerea* [38–40]. Although TMF and BSF frass differ in composition and microbial profile, these results suggest that structural features post-treatment—such as compactness—might help retain beneficial microbial traits that are critical for plant health. Further research should explore how microstructure interacts with microbial viability and biocontrol efficacy under real soil conditions.

The results regarding pH and electrical conductivity are particularly noteworthy. For both TT-TMF and UT-TMF, the variation in pH with an increasing frass concentration fol-

lows a parabolic (or "hump-shaped") behavior: the pH initially increases at low application rates and then decreases as the percentage of TMF increases further (Figure 3). This pattern may be attributed to the interaction between the chemical composition of the frass and the buffering capacity of the soil. At low concentrations (e.g., 2.5%), frass appears to exert an alkalizing effect, raising the soil pH up to approximately 7.75. This occurs despite the intrinsic acidity of TMF (pH ≈ 5.8), likely due to the presence of alkalizing compounds, such as:

- ammonium ions (NH_4^+), which can release NH_3 (a basic compound);
- carbonates and bicarbonates formed during larval metabolism;
- basic components such as chitin, chitosan, and peptidoglycans.

At higher concentrations (≥5%), the acidifying effect becomes predominant. The larger amounts of frass introduce greater quantities of organic acids (e.g., lactic acid, acetic acid) produced in the larval gut, along with organic nitrogen that may hydrolyze and release protons (H^+). At this point, the buffering capacity of the soil is exceeded, and the pH progressively declines to values around 6.65–6.66, like the baseline pH of the unamended soil. A similar parabolic pH response was also observed by Antoniadis et al. in spinach cultivation trials using TMF [52].

Regarding frass conductivity, our results show that EC increases with the increase of TMF, both treated and untreated, in the substrates (Figure 4). This trend has also been observed by some authors [52]. However, Antoniadis et al. report EC values in samples with 1% TMF of 0.396 mS/cm, which are lower than ours, slightly below 0.90 mS/cm [52]. Additionally, another recent study shows lower frass conductivity, with a reported value of 7.85 ± 0.03 mS/cm for 100% TMF [28]. In contrast, our results range between 13 and 16 mS/cm. The difference in EC between treated and untreated frass is quite intriguing. The UT-TMF exhibits a significantly higher EC value than the TT-TMF. The observed phenomenon is likely a result of heat treatment, which leads to the formation of dense agglomerates (refer to Section 3.1.1) in the frass. These agglomerates can effectively retain the ions that contribute to electrical conductivity. However, the heat treatment can also cause the decomposition of ammonium ions to ammonia, thus reducing EC. Our results contrast with those of Praeg et al., who provide similar results for treated and untreated frass, close to 4.5 mS/cm and lower than our measurements [33]. Heat treatment can break down organic matter and release ions into the solution, thus increasing EC.

Growth parameter analysis on Canasta lettuce showed that TT-TMF is more effective than UT-TMF and, above all, does not cause mortality in the Canasta plants. As is known, biofertilizer effectiveness is influenced by various factors, including microbial and nutrient content, as well as structural characteristics [22,23]. The heat treatment required by law can affect these factors, thereby modifying the efficacy of frass as a biofertilizer. Unfortunately, there are few studies in the scientific literature investigating the effects of heat treatment on the frass effectiveness, particularly concerning TMF. To our knowledge, only Praeg et al. examined the changes brought about by heat treatment in commercial TMF [33]. These authors observed a substantial maintenance of the total microbial load in TMF, unlike what happens, for example, with BSF frass. Assuming that the findings of this study can be generalized to TMF produced under our growing conditions, it suggests that the microbial load is not the reason for the differences in mortality and growth observed in Canasta plants grown with UT-TMF compared to those grown with TT-TMF. There are other hypotheses regarding the causes of the differences observed in our tests. Heat treatment, while preserving the microbial activity of heat-resistant communities, may destroy any phytopathogens present in the frass, making it a more effective fertilizer than untreated frass. However, more in-depth and targeted studies are needed to support this hypothesis. Another explanation—supported by the results of Praeg et al.—is that heat treatment

leads to the decomposition and volatilization of compounds like ammonium ions, which are likely responsible for the death of Canasta plants when untreated frass is applied at high rates [33]. Furthermore, heat treatment can lead to the formation of agglomerates (Section 3.1.1) that can trap phytotoxic compounds.

Regarding the TT-TMF percentage leading to the most promising results (4% dry w/w), our findings contrast with several previous studies that suggested lower optimal application rates of TMF, typically ranging between 0.5% and 3% (v/v), depending on crop type, substrate, and agronomic goals [26,53]. The optimal dose identified in our experimental conditions appears to exceed this range, underlining the importance of adapting fertilization strategies to specific crop requirements and cultivation settings. The literature confirms that the efficacy of TMF as an organic amendment is highly context dependent [54]. For example, it was observed that a 3% (v/v) dose produced the best results in terms of germination and early growth in ginseng seedlings, while higher doses (>5%) provided no additional benefit and, in some cases, impaired plant development [54]. Similarly, another study reported that applying 2% TMF (w/w) increased soil fertility by enhancing phosphorus and potassium availability compared to lower dosages [25]. A study conducted by Hénault-Ethier et al. on vegetables, herbs, and flowers used a dose of 0.5% by volume of TMF, resulting in a significant increase in edible biomass (both the total height of the plant and the diameter of the stem), comparable to that of chicken manure. However, for certain crops such as carrots and beets, a reduction in seedling emergence was observed, suggesting potential phytotoxic effects or mechanical interference in early development [26]. The percentage we identified is slightly higher than those reported in the literature [26,53,54]. These results reinforce the notion that no universal optimal dose of TMF can be defined across crops and various types of soil. Instead, careful calibration is required, considering species-specific physiological responses, substrate buffering capacity, and the balance between nutrient release and possible phytotoxic effects. Our data highlight the value of integrating both quantitative growth indicators and structural (morphological) observations to evaluate the real agronomic potential of insect-derived soil amendments. This integrated approach enables more accurate optimisation strategies tailored to each crop–substrate system.

5. Conclusions

This study demonstrates that the thermal treatment of TMF is essential for its effective use in the cultivation of *Lactuca sativa*. SEM analysis revealed that the heat treatment induces clear morphological changes in the frass matrix, while greenhouse trials confirmed its impact on plant growth performance. These findings support the regulatory requirement and suggest that heat treatment may also influence its agronomic efficacy. Among the different application rates tested, a concentration of 4% TMF (on a dry soil weight basis) proved to be the most effective and consistent under our experimental conditions. This rate supported optimal plant growth and biomass accumulation, showing a balanced performance across all measured parameters.

Data on the microbial load of the TMF used in our study before and after heat treatment, as well as the related nutrient content, are currently unavailable. Various tests are underway in these areas to understand the changes in TMF caused by heat treatment and the effects of the TMF addition on soil characteristics. Nevertheless, the study should be regarded as an initial step. The trials were conducted under controlled greenhouse conditions in pots and may not fully reflect open-field dynamics, where climatic factors, soil heterogeneity, and biotic interactions can significantly influence outcomes. As such, further research is required to validate these results under real-world conditions.

Future studies should assess whether the percentage that proved most effective in our tests (4%) is effective across other crop species, including both leafy and non-leafy vegetables. They should evaluate the role of TMF in enhancing plant tolerance to abiotic stresses (e.g., drought, salinity, thermal fluctuations), verify the agronomic performance of TMF in open-field conditions and across different pedoclimatic contexts, and investigate the influence of insect diet on frass composition and, consequently, on its optimal application rate. In this context, preliminary efforts have been undertaken to collect and characterize frass obtained from larvae reared on alternative substrates, with the aim of evaluating whether the 4% application rate remains effective for *Lactuca sativa* cultivation when the insect diet is varied.

Overall, the results presented herein provide a valuable basis for the optimization of insect-based fertilizers within sustainable agricultural practices and contribute to the broader objective of integrating circular, bio-based inputs into horticultural production systems.

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Abbreviations

The following abbreviations are used in this manuscript:

TM	*Tenebrio molitor*
TMF	*Tenebrio molitor* Frass
TML	*Tenebrio molitor* Larvae
BSF	Black Soldier Fly
SEM	Scanning electron microscopy
PH	Plant height
LN	Number of edible leaves
SD	Stem diameter at the base of the plant
FW	Fresh weight
EC	Electrical conductivity

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