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"Sciences - *Curriculum* Applied Biology"

"Rearing of bioconverting insects for the valorization of by-products into valuable substances"

Scientific Disciplinary Sector

"General and Applied Entomology - AGRI-05/A"

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ABSTRACT

Black Soldier Fly (BSF; *Hermetia illucens*, Diptera: Stratiomyidae) has emerged as a key species for the bioconversion of organic substrates within circular economy frameworks, offering sustainable solutions for food, feed, agriculture, and biomaterial production. Beyond insect biomass, BSF rearing generates valuable co-products, particularly larval frass, which can be exploited as an organic fertilizer and biostimulant, and protein-rich fractions that can be valorized for bio-based materials. Despite increasing interest, a comprehensive understanding of how rearing substrates, processing strategies, and application conditions influence the functional performance of BSF-derived products remains incomplete.

This doctoral thesis integrates a critical review of the current state of insect bioconversion with experimental investigations aimed at evaluating the agronomic and biotechnological potential of BSF-derived products. The first part of the thesis provides an overview of insect bioconversion within food and feed systems, with particular emphasis on *H. illucens*, addressing regulatory aspects, life cycle assessment, and the role of automation in scaling sustainable insect farming. The influence of larval rearing substrates on BSF performance, nutritional composition, and microbiological aspects is then examined, highlighting substrate-driven variability as a central factor shaping downstream product quality.

The experimental section focuses primarily on BSF frass and its multifunctional applications in agriculture. Frass extracts were evaluated as seed treatments in durum wheat (*Triticum durum* Desf.), assessing their effects on agronomic traits, antioxidant responses, and suppression of damping-off caused by *Fusarium sporotrichioides*. The presence of bioactive microorganisms was further investigated, leading to the isolation and characterization of plant growth-promoting bacteria (PGPB) from BSF frass. A large panel of isolates was screened *in vitro* for key plant growth-promoting traits, including phosphate solubilization, ammonia production, and phytohormone synthesis, and selected strains were tested *in vivo* on *Arabidopsis thaliana*, revealing differential effects on germination and early root development linked to substrate-dependent microbial abundance. In parallel, the agronomic suitability and potential phytotoxicity of BSF frass were assessed in lettuce (*Lactuca sativa*), identifying dose-dependent responses on germination, growth, and functional quality traits such as phenolic content and

antioxidant capacity. These results underline the importance of optimizing application rates and processing conditions to maximize benefits while minimizing inhibitory effects.

Finally, the thesis explores an alternative valorization pathway for BSF biomass, by investigating the production and characterization of protein-based bioplastic films derived from BSF larvae. Lipid removal, protein extraction, film formulation, and physicochemical characterization were conducted, followed by a preliminary evaluation of the films as biodegradable mulching materials, focusing on soil water evaporation control and macroscopic degradation behavior.

Overall, this work positions BSF bioconversion as a versatile and tunable platform, demonstrating how substrate selection, processing strategies, and application context critically determine the agronomic and biotechnological performance of BSF-derived products, thereby contributing practical insights for their sustainable integration into circular bioeconomy systems.

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AIM OF WORK

This PhD research was conducted at the Insect Physiology and Molecular Biology Laboratory, Department of Basic and Applied Sciences (DiSBA), University of Basilicata (UNIBAS), under the supervision of Prof. Patrizia Falabella, Dr. Rosanna Salvia and Dr. Carmen Scieuzo. The assessment of the microbiological component of *Hermetia illucens* (Black Soldier Fly, BSF) frass was carried out in collaboration with KU Leuven University (Belgium). While the optimization of protein extraction protocols, the development of protein-based films and their physicochemical characterization were performed in collaboration with the Sustainable Packaging Institute- SPI (Albstadt-Sigmaringen University, Germany) and the Department of Chemistry-Uniba (University of Bari, Italy).

The overall aim of this doctoral project was to contribute to the valorization of products and by-products derived from the rearing of the bioconverter insect BSF, within a circular economy framework. Specifically, the research focused on two main streams: (i) the agronomic, microbiological and functional exploitation of larval frass, a major by-product of BSF rearing, and (ii) the extraction and utilization of BSF larval proteins for the development of innovative, biodegradable biomaterials with potential agricultural applications.

In particular, this thesis aimed to: (I) investigate the biostimulant, antioxidant and antifungal properties of BSF frass extracts in crop systems, evaluating their effects on seed germination, plant growth, physiological performance and resistance to phytopathogenic fungi, using durum wheat (*Triticum durum* Desf.) as a model crop; (II) isolate, identify and functionally characterize plant growth-promoting bacteria (PGPB) associated with BSF frass, assessing their *in vitro* plant-beneficial traits and their effects on early plant development in *Arabidopsis thaliana*, while elucidating the influence of rearing substrate on microbial diversity and functional potential; (III) assess the phytotoxicity thresholds, agronomic performance and functional responses of horticultural crops to BSF frass and frass-derived extracts, with particular emphasis on lettuce (*Lactuca sativa*), integrating chemical, microbiological and physiological analyses to define safe and effective application conditions; and (IV) optimize protein extraction from BSF larvae and develop protein-based bioplastic films, characterizing their chemical, thermal and

mechanical properties and conducting preliminary evaluations of their performance as biodegradable mulching materials in agricultural soils.

On the whole, this work aims to provide an integrated and multidisciplinary evaluation of BSF-derived products, supporting their sustainable reuse in agriculture and contributing to the development of innovative strategies for waste reduction, resource efficiency and environmental sustainability.

CHAPTER 1

Transformative potential of insect bioconversion and its role in circular economy

What is reported in has been accepted and published in “Journal of Environmental Management”, in the form of a review titled “Transformative potential of insect bioconversion and its role in circular economy”.

1. Introduction

As the world population grows at an unprecedented rate, the demand for essential resources, particularly food and animal feed, continues to rise sharply. The global protein demand is expected to surge further, representing an increasing challenge for global food systems (UN, 2017; Aiking and de Boer, 2020). In response, the agri-food sector faces a difficult task: balancing the drive to increase production levels with the need to adhere to sustainable practices that align with the United Nations Sustainable Development Goals (SDGs) (UN, 2015) and major European policy frameworks. Specifically, the European Green Deal represents the EU’s roadmap for making the economy sustainable by turning climate and environmental challenges into opportunities. Within this framework, the 'Farm to Fork' Strategy is central to the Green Deal's goals, aiming to accelerate the transition to a fair, healthy, and environmentally-friendly food system (European Commission, 2020). This challenge requires innovative approaches to improve productivity without compromising environmental health and social equity. Among the 17 SDGs, one of the most important is the Goal 12: "Ensure sustainable consumption and production patterns". This underlines the need to focus on promoting the efficient use of resources, enhancing energy efficiency, supporting sustainable infrastructures, and expanding access to essential services. It also encourages a circular economy approach to reduce, reuse and recycle resources rather than immediately classifying them as waste. Modern lifestyle in developed countries, combined with inadequate processing and storage technologies in low-income regions, are major contributors to the annual loss of nearly one-third of global food production every year (Naikare, 2019). The term food waste refers to parts of plants and animals that are produced but are not consumed or used for their intended purpose. Leftover food is generated from different points of the agri-food production chain including agricultural production, postharvest handling and storage, further processing of food, wholesale and retail trade distribution, catering facilities and domestic kitchens (Edjabou *et*

al., 2016). The United Nations Environment Programme (UNEP) estimates that food waste and losses could reach 1.05 billion tonnes annually. Improper disposal further contributes to environmental pollution and global greenhouse gas (GHG) emissions (UNEP, 2024). This agri-food waste, along with other organic byproducts, represents a valuable raw material that can be converted into products with economic value even higher than the original products (Ajila *et al.*, 2012). In most of the recycling processes such type of waste resources typically demands a highly efficient process to recover as many nutrients as possible, like phosphorus, nitrogen and organic carbon, but at the same time there is a need for an environmentally friendly and sustainable process (Van Der Wiel *et al.*, 2019). Traditional food waste management methods, such as composting, have been widely explored for nutrient recycling and GHG mitigation (Kamyab *et al.*, 2015). Offering the additional benefits of producing other high-value products, many researchers have proposed insect-based bioconversion as an effective and marketable solution for the above-mentioned problems (Pastor *et al.*, 2015; Van Huis and Oonincx, 2017; Ojha *et al.*, 2020). The great diversity of insect species includes many detritivorous and herbivorous groups highly specialized in exploiting different organic substrates. Some of these substrates resemble food byproducts from the agri-food chain, so this natural behaviour can be exploited to develop sustainable methods for the food waste management (Fowles and Nansen, 2020). This concept aligns with recent works, where insect-mediated waste valorisation has been evaluated as a practical circular economy tool. For instance, Pang *et al.* (2020) and Mostafaie *et al.* (2025) demonstrated that Black Soldier Fly (BSF) (*Hermetia illucens*) larvae can significantly reduce GHG emissions while transforming organic wastes into valuable biomass and fertilizer products. By combining the traditional knowledge about insect applications and consumption with modern scientific research, insects could offer a significant opportunity in both developed and developing countries (Devi *et al.*, 2024). Insects offer numerous advantages over plants and livestock animals as a food and feed source, making them a promising alternative for several reasons. They generally have a short life cycle, which means they can reproduce and grow faster. This allows for quick and efficient production, making them a potential source of protein with faster turnover. Moreover, land and water requirements are lower than traditional livestock (Van Huis and Oonincx, 2017). The main by-product of insect rearing is the insect frass (excrement). European Regulation No 1925/2021 defines insect frass as the mixture

of insect excrements with parts of dead insects and unconsumed feeding substrate. Depending on insect species, rearing diet and conditions, frass production could vary from 2 to 4 kg per kilogram of insect biomass produced at the end of the bioconversion process (Hénault-Ethier *et al.*, 2024). This rearing product should not be considered a waste, as it can be employed for agricultural purposes with many benefits, aligning with the principles of the circular economy. Due to its animal origin, its chemical composition and physical properties, insect frass is biologically equivalent to animal manure and could be a promising alternative to commercial fertilizers. The idea of applying insect frass to agricultural lands allows to return nutrients in the food chain (Ojha *et al.*, 2020). In addition to its interesting composition (N-P-K ratio) comparable or even better than other conventional fertilizers, depending on the insect species and rearing substrate, this product also contains beneficial bacteria that act as plant growth promoters and biostimulant, thereby improving plants' health and facilitating their absorption of nutrients (Poveda *et al.*, 2019). As highlighted by Lomonaco *et al.* (2024), the valorisation of insect frass as a biofertilizer exemplifies how insect rearing can simultaneously address waste management challenges and promote sustainable soil productivity. This review integrates current findings from environmental, technological, and policy perspectives, drawing upon peer-reviewed studies, reports, and regulatory documents related to insect farming and waste bioconversion. By providing a comprehensive understanding of the current landscape and future directions in insect rearing, it positions insect-based systems as pivotal drivers of the circular bioeconomy, in direct alignment with the journal mission to advance sustainable development and integrated environmental solutions.

2. Bioconversion for food and feed

In the context of the circular economy, an encouraging and innovative approach involves using agri-food waste and by-products as a substrate for large-scale rearing of insects. Insects represent a potentially valuable solution to two growing challenges: the increasing amount of organic waste and by-products, which generates disposal costs and environmental pollution, and the rising global demand for proteins, both for food and for feed purposes (Salomone *et al.*, 2017). The trend in the number of scientific publications demonstrates the growing interest in the insect-based bioconversion. The search of the keywords "insect bioconversion" or "insect waste valorisation" on Science Direct, one of the major peer-reviewed

literature platforms (https://www.sciencedirect.com/), gave the results shown in Fig. 1. The number of scientific publications on this topic has increased substantially over the last 10 years, with a drastically increase of 530% between 2019 and 2022.

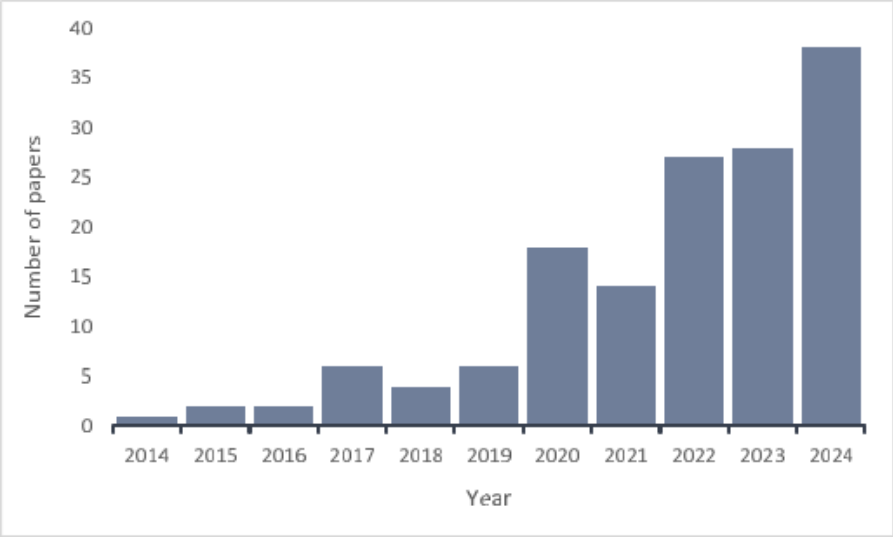


Figure 1. Distribution of research papers published in Science Direct containing the keywords "insect bioconversion" or "insect waste valorisation" within Title, Abstract and Keyword fields, by year (https://www.sciencedirect.com/, accessed: October 2024).

The bioconversion of food waste through insects replicates the natural biological decomposition of organic materials in ecosystems. In nature, various organisms such as insects, earthworms, diverse invertebrates, fungi, and bacteria naturally inhabit and decompose organic waste, transforming nutrients to sustain their metabolic and reproductive needs. Under controlled environmental conditions, some insect species can intensify this natural behaviour, making it exploitable for the development of urgently needed bio-circular economy systems (Ortiz *et al.*, 2016; Fowles and Nansen, 2020). The establishments of insect-based industries could represent a promising solution for valorizing food waste and producing sustainable animal feed in a growing circular economy perspective (Van Huis, 2013; Madau *et al.*, 2020). In developing countries, the local insect rearing for food and animal feed also represents an important economic opportunity. The potential for insect farming to provide a cost-effective, nutrient-rich protein source is particularly relevant in regions that are resource-constrained, unsuited for

conventional agriculture, and characterized by limited access to sustainable feed (Barragan-Fonseca ´ *et al.*, 2020; Verner *et al.*, 2021). Insects' ability to thrive on low-value organic waste further supports their suitability for small-scale, decentralized production systems. One promising insect species used for bioconversion is the BSF, whose larvae can efficiently convert a variety of organic waste, including agricultural byproducts, food scraps, and even manure, into high-quality protein and fat-rich biomass (Franco *et al.*, 2022; Franco *et al.*, 2025; Scieuzo *et al.*, 2023; Vozzo *et al.*, 2025). Their high feed conversion ratio makes them a highly productive and sustainable option for transforming organic waste into valuable products, like animal feed or raw material for other applications like biodiesel and organic fertilizer production (Gold *et al.*, 2018). BSF larvae exploitation as animal feed is constrained by regulatory frameworks. For example, the European Regulation n. 893/2017 allows this type of animal feed only if BSF larvae have been reared on vegetable by-products and/or specific permitted animal by-products. Other types of insect-feeding substrates (such as mixed food scraps or catering services wastes, animal manure or sewage sludge) are not permitted under current regulations. This strict regulation aims to guarantee animal and human health safety. The insect farming sector is increasingly attracting interest from both the food and feed industries as a means of reducing waste management costs and creating value-added products. This aligns with the goals of a circular economy, in which waste is minimized, and resources are continually reincorporated into the production cycle to close nutrient loops (Paris *et al.*, 2024). Insect-based bioconversion systems can also mitigate some of the environmental impacts associated with traditional waste disposal methods, like landfilling and incineration, which contribute significantly to GHG. Studies suggest that insect farming can reduce emissions, lower energy consumption, and decrease the reliance on synthetic fertilizers when the resulting insect biomass is used as feed supplement or its frass as soil improver (Joly and Nikiema, 2019; Katchali *et al.*, 2024).

3. Edible insects

I Insects have been part of human nutrition since ancient times, even in Australopithecus's diet millions of years ago (Sponheimer *et al.*, 2005). Entomophagy as traditional practice started about 7000 years ago (Naseem *et al.*, 2021). Today, insects are considered traditional and commonly consumed foods in more than 100 countries of Asia, Africa, and Central and South America. Since the

early 21st century, they have been also consumed in European and other American countries. Mexico, China, Thailand, and India are among the leading countries in terms of quantity and diversity of edible insect species consumed (Ianniciello *et al.*, 2024). More than 2100 species have been reported as edible worldwide, with the most commonly consumed genera belonging to Coleoptera (31% of total reported consumption), Lepidoptera (18%), Hymenoptera (14%) and Orthoptera (13%) (Fig. 2) (FAO, 2021; Jongema, 2017).

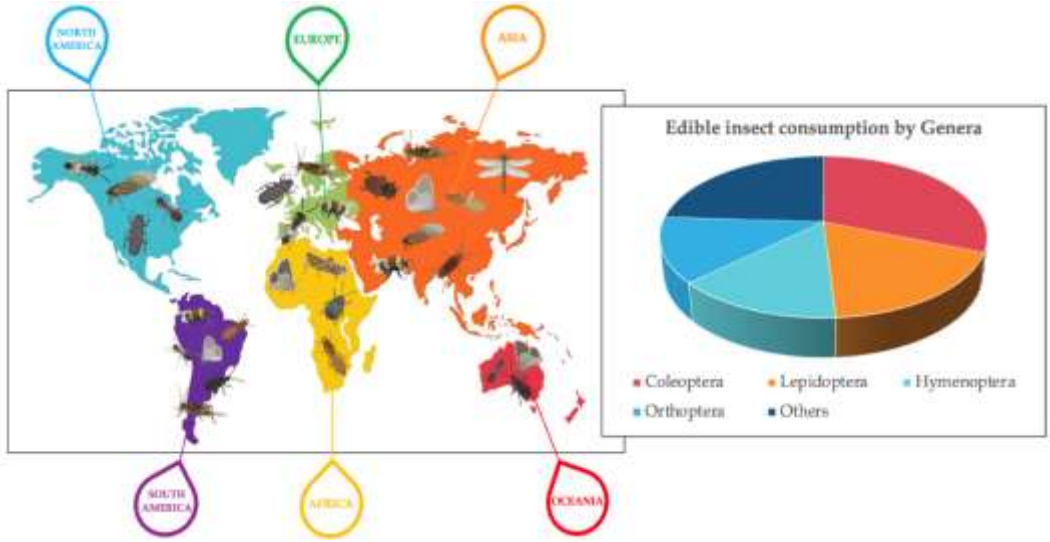


Figure 2. Global map showing the distribution of the main edible insects by continent. The insect icons on the map serve as representative illustrations of the main groups found in each region, while the pie chart details the specific global consumption proportions by insect Genera.

It is challenging to identify a person as "insect-eater" or entomophagist, as insect consumption could be occasional throughout the year or a lifetime, or even unintentional through food contamination. Combined with the lack of global statistics, it is not possible to determine the actual number of people who consume insects worldwide (Van Huis *et al.*, 2022). Generally, insects represent a very interesting source of high-quality animal proteins, containing all the essential amino acids, often with higher protein amounts than other conventional sources such as meat, fish or plants (Table 1). Insects also contain considerable amounts of lipids, with a predominance of unsaturated and polyunsaturated fatty acids, as well as vitamins, minerals and other bioactive compounds (Lange and Nakamura, 2021). Insect nutritional value is highly variable, and it depends on several factors, including species, development stage, rearing substrate, environmental conditions, and processing methods (Table 1) (Van Huis, 2013).

Common name	Species	Protein [g]	Fat [g]	Fiber [g]	Cholesterol [mg]
House cricket (Adults)	*Acheta domesticus*	62.6 - 70	12.2 – 22.8	8 - 22	98.5
Field cricket (Adults)	*Gryllus bimaculatus*	58.3	11.9	9.5	195
Migratory Locust	*Locusta migratoria*	48.7- 61.3	13.4 - 38.1	8.8 – 9.6	-
Mealworm (Larvae)	*Tenebrio molitor*	47.2 – 57.1	32.4 – 43.1	5 – 14.9	51.3
Superworm (Larvae)	*Zophobas morio*	43.1 – 46.8	35 – 42	6 - 13	45
Mopane worm	*Gonimbrasia belina*	35.2	15.2	-	-
Black soldier fly (Larvae)	*Hermetia illucens*	34.9 - 39	27.93 - 32.6	7.5 – 12.4	-
Silkworm (Larvae)	*Bombyx mori*	53.7 – 69.8	8 – 9.5	5.9 – 6.3	-
Wax moth (Larvae)	*Galleria mellonella*	34 – 41.2	51.4 - 58	8.9 – 12.1	75.3
Pork shoulder	*Sus scrofa domesticus*	16.89	7.05	-	50.02
Beef sirloin	*Bos taurus*	20.1	3.5	-	59
Chicken breast	*Gallus domesticus*	21.5	1.3	-	58

Table 1. Average nutritional values of some of the most diffused, consumed or commercially available worldwide edible insects and of meat expressed per 100 g of edible portion (Rumpold and Schlüter, 2013; Meyer-Rochow *et al.*, 2021; Orkusz, 2021; Zhou *et al.*, 2022).

One of the major concerns regarding insect consumption relates to safety and potential allergy risks, especially in countries where entomophagy is not part of food traditional dietary practices (Belluco *et al.*, 2013). According to literature, two main risk categories exist for developing a food allergy to insects. The first consists of people who are frequently exposed to edible insects due to professional reasons, such as insect farmers or producers and researchers, who may develop primary sensitization, typically manifesting as respiratory symptoms (e.g. bronchial hyperreactivity, asthma or allergic rhinitis) (Ganseman *et al.*, 2023). As safety and prevention measurements, infrastructural improvements to insect rearing facilities, such as ventilation and dust collectors, as well as proper personal protective equipment, can significantly reduce these allergenic symptoms (Ganseman *et al.*,

2023). The second risk category involves cross-reactivity in individuals allergic to other arthropods, like crustaceans or dust mites (De Marchi *et al.*, 2021). This cross-reactivity is due to high-sequence identity and taxonomic relation between insect allergenic proteins and those detected in related taxa. Due to protein biological structure, food processing could directly influence the allergenic potency: thermal treatments may alter the protein three-dimensional structure, potentially increasing antigen exposure, while fermentation and enzymatic processing are known to reduce allergenicity and could be applied to insect-based ingredients. (De Marchi *et al.*, 2021; Aguilar-Toala ´ *et al.*, 2022). The recent introduction of edible insect-based products into Western nations food supply, it is expected to increase the number of food allergy cases recorded (Ribeiro *et al.*, 2021). Despite insects' great nutritional values and the low environmental footprint of their rearing, the other main obstacle to the acceptance of insect as novel food in Western countries is the negative consumer perception, often burdened by cultural and psychological barriers, such as prejudice and disgust (Looy *et al.*, 2014). A useful strategy to overcome this aversion is processing insects into an unrecognisable or more conventional and familiar forms (for example chips or snacks), supported by educational initiatives to promote acceptance and awareness of their nutritional and environmental benefits (Franco *et al.*, 2024). Insects, dried and reduced in powder (insect meal), could be used to enrich existing foods such as crisps, bread, pasta, and similar products (Van Huis *et al.*, 2013; Baiano, 2020). Sensory improvements, such as enhancing flavours (e.g. mixing with other familiar and appreciated spices or ingredients) and textures, have shown promising results, with consumer acceptance strongly influenced by taste expectations and tasting experiences (Alhujaili *et al.*, 2023). The food service industry, including restaurants and chefs, can play a vital role in normalizing insect consumption. Innovative culinary presentations and gourmet insect dishes are already gaining popularity in high-end restaurants and food festivals, contributing to a gradual shift in consumer perception (House, 2016). When insects are introduced as exotic or luxury items, consumers may view them as trendy rather than unconventional, easing the cultural transition. The best strategy to change this aversion will be an important commitment from policy-makers and the education of the new generations. The described challenges are directing insect production to be integrated as animal feed (Baiano, 2020). This help in familiarizing the general public with the concept of insects as part of the food chain. If insect-based feeds become mainstream in livestock, aquaculture, and

domestic animals, it may pave the way for a broader acceptance of insects in human diets (Van Huis, 2020).

4. Insect-based feed production

Insect-based feed production refers to the process of producing animal feed using insect biomass as a primary ingredient. In recent years, there has been growing interest in using insects as a sustainable protein source for animal nutrition (Aragão *et al.*, 2020). Insects can effectively feed on low-value substrates such as bio-waste and organic side streams, converting them into a valuable source of nutrition for animals (Terova *et al.*, 2020). This approach not only promotes the principles of a circular economy but also reduces the environmental impact associated with conventional animal farming. One of the main advantages of insect production lies in its high feed conversion efficiency. Crickets, for example, require only about 2 kg of feed to produce 1 kg of body mass, whereas beef animals need at least 20 kg of corn and soybean to produce the same amount of meat (Elhassan *et al.*, 2019). Insects also require less land for cultivation compared to traditional livestock. As a natural component of the diets of many vertebrates, insects could be utilized as a sustainable feed option for farmed animals, including fish, poultry, and pigs (Grassi *et al.*, 2025; Sangiacomo *et al.*, 2025; Sogari *et al.*, 2019). Research also shows that pets, particularly dogs, can effectively utilize nutrients from insect-based meals, particularly amino acids, which are highly digestible, healthy, and do not negatively impact the gut flora (Valdés *et al.*, 2022). Insects are widely used as feed for exotic pets, including birds, reptiles, amphibians, aquarium fish, and small mammals (Ahmed *et al.*, 2022). The ongoing research is exploring different feeding substrates and rearing techniques to optimize insect biomass production and its nutritional composition. The future goal is to formulate balanced diets that maximize rearing performance and to assess the potential use of the different insect meals as a feed ingredient (Straub *et al.*, 2019; Leyo *et al.*, 2023). An ideal insect production and processing system fits perfectly into the food chain by recycling waste from food production, processing and/ or consumption as feed for insect rearing (Fig. 3). These insects are then re-introduced into the food chain as whole organisms or as nutrient-rich ingredients, while the byproducts from insect rearing and processing can be utilized in other fields, as frass that can be used as natural fertilizers to support sustainable agricultural crop production. Among other side stream products of the insect rearing, lipids extracted from insects, particularly from species like

BSF, have shown strong potential for biodiesel production (Caivano *et al.*, 2025; Ojha *et al.*, 2020).

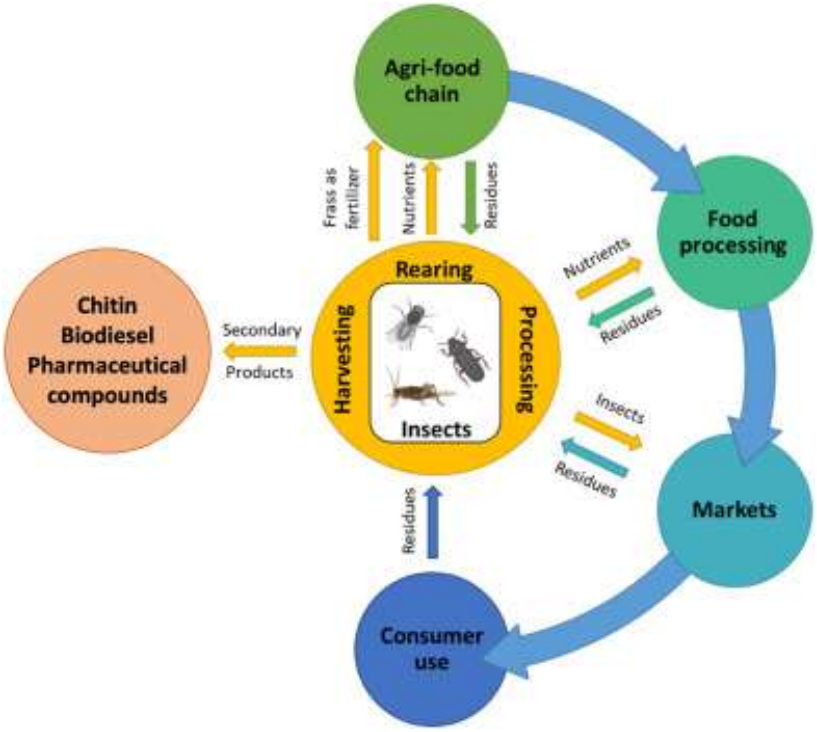


Figure 3. Concept of an ideal insect production and processing system integrated in the agri-food chain.

In Europe, the most relevant species used for animal feed are the yellow mealworm (*Tenebrio molitor*), the lesser mealworm (*Alphitobius diaperinus*) and BSF. This last represents almost 80% of the total European insect-feed market (Derrien and Boccuni, 2018). A notable example of large-scale BSF rearing for the feed sector is the model proposed by Innovafeed (https://innovafeed.com/). This company is a leading player in the European insect protein industry by pioneering sustainable and large-scale production of BSF-derived products. Its portfolio includes insect oil for poultry and swine nutrition, insect protein for aquaculture feed, and frass as an organic fertilizer. The core of the company is its facility in Nesle, France, which is the largest insect feed plant in the world with an annual capacity of 15,000 tons of insect protein (INNOVAFEED, 2024a).

5. Legislative status of insects for food and feed

The legislative status of insects for food and feed varies widely and continues to evolve globally, reflecting different levels of acceptance and regulatory approaches as governments address food security, safety, and sustainability concerns (Table 2). In the European Union, the regulation of insects as food falls under the European

Novel Food Regulation 2015/2283, updated in 2018 to explicitly include insects within the novel food category. This regulation requires that any insect species intended for human consumption must undergo a rigorous safety assessment by the European Food Safety Authority (EFSA). EFSA evaluates factors such as allergenic potential, microbial contamination, potential chemical hazards, and nutrient composition to ensure compliance with EU standards for food safety and quality before market authorization. Since the regulation update, a few insect species have received approval for sale within the EU market as protein sources for human food (EFSA Panel on Nutrition, Novel Foods and Food Allergens, 2021). In particular, four species have been authorized: *Tenebrio molitor* larvae (dried, frozen and in powder as a food ingredient), authorized by European Regulation 882/2021; *Locusta migratoria* (frozen, dried, and powdered), authorized by European Regulation 1975/2021; *Acheta domesticus* (frozen, dried, and powdered), authorized by European Regulation 188/2022; *Alphitobius diaperinus* larvae (frozen, paste, dried, and powdered), authorized by European Regulation May 2023. Regarding the animal feed sector, the EU amended its legislation in 2017 to allow the use of processed insect proteins specifically for aquaculture feed under European Regulation 2017/893. This amendment represents an important step forward toward reducing the environmental impact of traditional animal feed, as insects provide a more sustainable protein alternative. Further implementations, thanks to the Reg. CE n.1372/2021, extended the authorization of insect proteins also to other livestock categories, such as poultry and swine. However, the use of reared insects as animal feed remains strictly dependent on the type of substrate utilized for the rearing. The EU cautious approach reflects concerns about possible contamination risks and the need for more comprehensive studies on the safety of insect proteins in livestock production (Gasco *et al.*, 2018). North America adopts a more flexible but less centralized regulatory framework. In the United States, there are no specific federal regulations for insects as food; instead, insect production falls under the jurisdiction of the Food and Drug Administration (FDA). The FDA regulates edible insects as conventional food items, meaning they must adhere to existing safety, labelling, and sanitation requirements under the Federal Food, Drug, and Cosmetic Act (FD&C Act). According to this framework, insect-based foods must meet the same general standards as other food products for human consumption, including the absence of contaminants, proper labelling, and adherence to Good Manufacturing Practices. The lack of insect-specific guidelines

and definitions under federal law has resulted in regulatory ambiguity, leaving individual states responsible for defining their own standards. For example, California has been more proactive in setting detailed guidelines for insect rearing and processing, aimed at ensuring consistency and safety. The Association of American Feed Control Officials (AAFCO), working closely with the FDA, has been developed standards for insect-based animal feed to provide clarity for producers. However, federal regulations in the U.S. remain relatively undeveloped, posing challenges for the large-scale insect farming, due to differing state-level requirements (Larouche *et al.*, 2023). Canada has taken a more proactive stance, establishing clear guidelines through the Canadian Food Inspection Agency (CFIA). The CFIA permits the use of specific insect species, including crickets and mealworms, as ingredients in food and feed products. Canada framework for insect-based food and feed requires adherence to rigorous safety, labelling, and traceability requirements similar to those applied to conventional food items. These standards address essential aspects such as nutritional composition, microbiological safety, allergen labelling, and contaminant mitigation (Larouche *et al.*, 2023). The Canadian regulatory environment is distinguished by its clarity and coherence, which has helped the country position itself as a leading player in the global insect farming industry and also attracting international interest, from companies in Europe, Asia, and the U.S. In Asia, legislative approaches to insect-based foods vary widely. Countries such as Thailand and Vietnam have longstanding historical traditions of entomophagy and insect consumption face fewer cultural and regulatory barriers compared to Western countries. These countries have relatively permissive regulatory frameworks for insects as food, largely focusing on ensuring hygiene and quality in production rather than imposing strict barriers. Thailand, for example, one of the major worldwide producers and exporters of edible insects, developed one of the most advanced and productive cricket farming system (Halloran *et al.*, 2017). It became the first country in the world to issue official guidelines for cricket farming in 2017 and has also established basic regulatory standards covering insect rearing, processing, and packaging to ensure safety compliance with foreign markets, including the European Union (Deguerry *et al.*, 2023). In Vietnam, the approach is similar but more informal, with fewer insect-specific established regulations. Insects are treated as a common food and producers must comply only with general safety standards applied to other livestock species (Thao *et al.*, 2024). Most insect farming remains small-scale and oriented toward

local consumption, supporting local markets and some export activities, especially to neighbouring Asian countries where entomophagy is also common. China is also exploring insect farming as part of its sustainability initiatives, as it has a long historical tradition of entomophagy, with almost 324 species of insects currently consumed (Lin *et al.*, 2023). Edible insects in China are regulated under general food safety laws managed by the National Health Commission and China Food Safety Law, which outlines standards for hygiene, safety, and labelling. However, insect-based products are not specifically addressed in the existing regulatory framework, leading to legal ambiguity for producers and international standards (Feng *et al.* 2018). In the feed sector, the lack of insect-specific guidelines has led producers to adopt standards typically applied to conventional feed ingredients, which are not always suitable for insect proteins. The government is actively supporting research on insect-based feed to support aquaculture and livestock industries. Policymakers are exploring how specific safety standards could be implemented for insect-based feeds, potentially paving the way for comprehensive regulations tailored to the sector (Gahukar, 2016). As Asian countries, also many African nations have long-standing traditions of insect consumption, particularly in rural areas where edible insects contribute to food security and nutrition. However, formal regulatory frameworks to support the industry growth and safety standards remain underdeveloped. Majority of the Sub-Saharan African countries (91.7%) currently lacked food safety policies (Nakimbugwe *et al.*, 2021). The absence of specific regulations for insect-based products can be attributed to a shortage of comprehensive scientific data on the biological, chemical, and physical safety. This regulatory gap may increase health risks for consumers and limit the economic potential of insect-related food and feed industries (Nakimbugwe *et al.*, 2021). As one of the most developed African countries, in South Africa there is growing governmental interest in establishing policies to support commercial insect farming for both domestic consumption and export. The country aims to align its production with global food safety standards (Van Huis and Oonincx, 2017). The South African Bureau of Standards has started drafting guidelines, though comprehensive, enforceable regulations are still under development.

Region	Regulatory Status (Insect as Food)	Regulatory Status (Insect as Feed)	Key Notes / Examples	References
European Union	Novel Food Regulation (EU) 2015/2283; EFSA safety assessment mandatory	Regulation (EU) 2017/893 and 1372/2021 for aquaculture, poultry, swine	Only certain species approved; cautious approach; rearing substrate restrictions applied	(EFSA 2021; Gasco *et al.*, 2018)
United States	No insect-specific federal law; FDA regulates under FD&C Act	AAFCO developing insect feed standards; state-level variability	Regulatory ambiguity; producers navigate state-specific requirements	(Larouche *et al.*, 2023)
Canada	CFIA permits certain species (e.g., crickets, mealworms); strict safety, labelling, traceability	Guidelines parallel food regulations	Clear, coherent framework supports industry growth	(Larouche *et al.*, 2023)
Asia	Varies by country; Thailand & Vietnam have permissive, safety-focused guidelines; China general food safety laws	China: standards for conventional feed used; research ongoing	Thailand: official cricket farming guidelines; Vietnam: informal regulations; China: exploration phase	(Halloran *et al.*, 2017; Deguerry *et al.*, 2023; Lin *et al.*, 2023; Feng *et al.*, 2018)
Africa	Mostly informal; majority of Sub-Saharan countries lack insect-specific policies	Early-stage development; mostly informal or research-based	South Africa developing standards via SABS; general food safety policies limited	(Nakimbugwe *et al.*, 2021; Van Huis & Oonincx, 2017)

Table 2. Summary of the current legislative status of insects for food and feed across different regions worldwide. The table highlights regulatory frameworks and ongoing initiatives, providing a comparative overview.

6. Life cycle assessment (LCA)

G Although insect farming is emerging as a promising solution due to its potential to provide high-quality protein, one of the major challenges for industrial insect production is to scale-up rearing. Indeed, as every other type of industrial activity, industrial insect production has environmental impacts. Data availability is still

limited due to the novelty of the sector in the Western countries in comparison to other wellestablished feed and food industries (Smetana *et al.*, 2021). A practical tool to evaluate the environmental load associated with a production activity is the Life Cycle Assessment (LCA). LCA of insect farming helps to identify key environmental hotspots associated with the entire lifecycle of insect production, from the sourcing of feed substrates to the processing and application of insect-derived products. By standardizing these elements, LCA provides a comprehensive and comparable analysis of the environmental sustainability of different insect farming practices, ultimately guiding improvements and promoting more sustainable production systems. Most of the literature on insect farming LCA employs an attributional approach, which quantifies the environmental burdens directly associated with the product system by accounting for the flows of materials and energy within its defined boundaries (ISO 14040; ISO 14044). This approach helps identify specific hotspots along the life cycle, such as global warming potential (GWP), agricultural land occupation, water, soil and energy use, fossil fuel depletion, acidification, eutrophication, and eco-toxicity. Among these, GWP, expressed as CO_2-equivalents (CO_2-eq), is a key metric for evaluating the environmental impact of insect production. Insect farming generally generates lower CO_2-eq emissions compared to conventional livestock (broiler, beef, pork), due to the higher feed conversion efficiency of insects and their ability to thrive on organic waste streams (Table 3) (Smetana *et al.*, 2019). While attributional LCA dominates current studies, interest is increasing in consequential LCA, which evaluates the broader environmental consequences of changes in production or demand, including market-mediated and indirect effects. This approach is particularly relevant when scaling up insect production, as it can capture shifts in conventional agricultural systems, such as reduced demand for livestock feed crops and associated reductions in land use or deforestation. Applying consequential LCA in the context of insect farming provides policymakers and industry stakeholders with a more comprehensive understanding of potential systemic and long-term environmental impacts, complementing the insights from attributional assessments. For example, increased insect protein production may indirectly influence agricultural practices by reducing demand for traditional livestock feed crops, leading to positive cascading effects on land use, reduced deforestation rates and GHG emissions (Alexander *et al.*, 2017; Van Huis and Gasco, 2023).

	Species	GWP [*kg* CO_2-eq]	GWP % vs Beef	AC [*g* SO_2-eq]	ALOP [m^2]	LU [m^2]	FFE [*MJ*-eq]	Reference
1kg of Mealworm edible mass	*Tenebrio molitor larvae*	2.8	92-97% ↓	21.88	3.07	-	29.36	(Dreyer *et al.*, 2021)
1kg of Mealworm protein	*Tenebrio molitor larvae*	5.77	82-94% ↓	39.38	-	6.35	217.37	(Thévenot *et al.*, 2018)
1kg of BFSL meal	*Hermetia illucens larvae*	1.16	96-99% ↓	5.3	-	0.48	17.9	(Smetana *et al.*, 2019)
1kg of Cricket edible protein	*Gryllus bimaculatus* and *Acheta domesticus*	1.55-2.57	97-98% ↓	0.05 – 0.08 (mol H^+ eq)	-	-	-	(Halloran *et al.*, 2017)
1 kg of beef meat		33.3 – 99.5		318.8 - 343.6	-	43.2 – 326.2	-	(Harwatt *et al.*, 2024)
1kg of pork meat		12.3		142.7	-	17.4	-	(Harwatt *et al.*, 2024)
1kg of broiler edible meat		4.5		98.37	12.5	-	43.96	(Dreyer *et al.*, 2021)
1kg of soybean protein meal		4.09		17.61	-	4.34	31.17	(Thévenot *et al.*, 2018)

Table 3. Comparison of some life cycle assessment (LCA) impact categories between main reared insects and other conventional protein sources (GWP: global warming potential; AC: acidification; ALOP: agricultural land occupation; LU: land use; FFE: fossil fuel depletion).

Despite these advantages, several phases of the insect production lifecycle remain environmentally burdensome. High energy inputs required by insect rearing in climate-controlled environments, but also for feed production, remains certainly a major contributor to GHG emissions (Van Huis *et al.*, 2015). For example, energy use in maintaining constant and optimal temperature and humidity can vary greatly depending on the species and scale of production; some setups in regions with cold winters and/or hot summers can require substantial electricity inputs. Feed sourcing also plays a pivotal role in determining the overall environmental performance of insect farming. While many insects can be reared on low-value by-products or organic waste, large-scale commercial farms may rely on high-protein feed sources for insects, which can increase the overall carbon footprint, particularly when these inputs are derived from resource-intensive crops like soy or maize (Ryba, 2024).

Future LCA studies should focus on variations in energy consumption for climate-controlled environments and investigate alternative energy-efficient technologies. Using renewable energy sources could significantly reduce CO_2-eq emissions. In this perspective, the model of industrial symbiosis realized by the Innovafeed company represents an important step toward more sustainable production standards (INNOVAFEED, 2024b). Production plants are located directly nearby agricultural processing plants; this ensures a direct supply of by-products to feed insects, while reducing transportation costs and carbon emissions. Through automated sensors and technologies, the plants are also able to recover the waste energy (energy which is usually dissipated in the atmosphere) from nearby industries, such as hot water from a refrigerating facility. This energy is recovered to warm up BSF rearing units or to power transformation plants, perfectly aligning with global sustainability goals. This energy and feed supply model enables the company to emit 80% less CO_2 than traditional livestock feed production. Although LCA studies of insect farming have provided valuable insights into environmental hotspots, methodological fragmentation and data scarcity still hinder robust benchmarking. Most analyses rely on attributional frameworks, while consequential approaches remain limited (Cámara-Ruiz *et al.*, 2023). Recent research highlights the need for integrated assessments of insect rearing within circular bioeconomy systems, encompassing waste valorisation and market dynamics (Hamam *et al.*, 2024). A harmonised and transparent LCA framework is essential not only for ensuring scientific comparability but also for guiding policymakers in developing evidence-based regulations, sustainability standards, and incentives for low-impact insect production. Due to the novelty of the topic, further research is needed. Future studies should therefore prioritise standardized methodologies, regional data integration, and the inclusion of techno-economic and policy dimensions to support sustainable industry growth.

7. Automation role in scaling BSF rearing for a sustainable future

In response to the limitations of traditional small-scale facilities, such as labor-intensive and high associated costs, modern automated systems have been developed. Automation is becoming increasingly essential for advancing and scaling up BSF rearing, as it reduces manual work and increases high efficiency in industries focused on a more cost-effective and sustainable waste management (Amoshie *et al.*, 2024). These systems employ advanced modern technologies that

enable precise monitoring and control of every phase of the BSF lifecycle, from incubating eggs to harvesting larvae. A key feature is the integration of sensors and control technologies that regulate every environmental parameter such as temperature, environmental/substrate humidity and artificial light cycles. As reported by Amoshie *et al.* (2024), an integrated system made up of Internet of Things (IoT) devices and computer vision could easily allow for a non-invasive monitoring and real-time optimization of multiple parameters. Another example is proposed by Erbland *et al.* (2021), consisting of an innovative prototype, designed to minimize labor and energy consumption by automating processes like moisture management via sensor-activated valves. The proposed system also allows to exploit metabolic heat naturally generated by larval bioconversion activity to maintain optimal conditions, a feature particularly useful to reduce energy input in colder climates. Collectively, these features give the potential to scale up the rearing system, improving BSF growth and productivity over time. Modern systems also integrate mechanized feeders to support large-scale production, dispensing precisely measured quantities of substrate tailored to the larvae's growth stage, ensuring uniform feeding and reducing waste. Such systems often include multiple rearing trays that facilitate consistent monitoring of larval growth and reduce the variability often encountered in manual setups (Pahmeyer *et al.*, 2022). In another study, Avila *et al.* (2022), proposed an innovative approach to update BSF larvae farming redeveloping already existing rearing bins, with a designed "smart lid" equipped with sensors, thermal imaging, and computer vision techniques. This ensures automated aeration of the larval feeding substrate and real-time monitoring of environmental conditions. The proposed system maintains optimal conditions and tracks larval growth, significantly reducing manual labor requirements, producing high-quality larvae at the same time. Machine learning algorithms are also often combined in these systems to optimize operations, enhancing productivity, while minimizing resource consumption (Zaalberg *et al.*, 2024). The most modern systems now incorporate technologies to measure and mitigate GHG emissions (including CO_2, CH4, and NH3) produced during the decomposition and bioconversion of organic waste, contributing to more sustainable rearing practices. Studies have shown that environmental factors, like pH levels in larval feed, significantly impact GHG emissions, and IoT-based systems enable real-time adjustments to these variables. For instance, experiments have highlighted that controlling feed pH can reduce emissions, as demonstrated by lower CO_2 and CH4

levels at specific pH values (Soontronprasatporn *et al.*, 2024). Recently, Coudron *et al.* (2024), proposed a new method for quantifying both ammonia and CO_2 using an accumulation chamber equipped with dedicated sensors. Monitoring ammonia emissions is a key aspect, as they could affect both human health and local ecosystems (Leip *et al.*, 2015; Wyer *et al.*, 2022). The proposed approach allows real-time monitoring of emissions across different stages of larval growth, facilitating optimal ventilation rates and substrate composition. When combining with climate-controlled environments, these technologies not only enhance overall efficiency ensuring optimal growth conditions for the larvae, but also aligns with green industrial goals by minimizing environmental impact (Fig. 4).

Figure 4. Modern Black Soldier Fly (BSF) breeding system with a high-tech, automated setup. The facility has vertically arranged rearing trays and breeding cages equipped with sensors and cameras, for real-time monitoring of environmental and rearing parameters, and robotic arm, to handle trays. The system also provides controlled artificial light cycles to optimize larval growth conditions.

8. Future challenges

Insect rearing for feed and food offers a promising pathway to more sustainable protein sources, but several significant challenges need to be addressed for the effectively scale up. One primary challenge is the economics of production. Indeed, the cost of insect-based meal remains too high for a wide use in the farming system. Current insect farming methods often rely on small-scale or semi-automated systems, which contribute to higher production costs compared to traditional animal feed sources. Recent studies indicate that the production cost of mealworms

represents a significant barrier to their widespread adoption as an alternative protein source. Compared to soybean meal and fishmeal, which are commonly used as protein sources in animal diets, mealworm larvae remain less competitive in terms of availability and cost (Hong *et al.*, 2020). As reported by Siddiqui *et al.* (2024), producing insect protein has costs comparable to meat for the European market and mealworm feed could reach prices 40 times higher than conventional soybean meal. High expenses are primarily attributed to manual labour, energy-intensive climate control for rearing facilities for large-scale production, and the need for optimized substrates to ensure efficient protein conversion rates. Developing advanced technologies for automation, efficient breeding, and optimized feed conversion is essential to achieve economies of scale and lower costs. Innovations in housing design, such as vertical farming systems, can maximize space efficiency and lower facility costs, further reducing the economic burden of insect-meal production (Van Huis *et al.*, 2013; Dossey *et al.*, 2016). Improving the efficiency of feed substrates also plays a key role in lowering costs. Research indicates that utilizing low-cost, locally available organic waste as a feed source can reduce feed expenses and increase the sustainability of insect farming (Parodi *et al.*, 2018). Nevertheless, the quality and safety of insect feed are also of primary importance. Just like conventional livestock, the diet and rearing conditions of insects significantly affect the final product quality. Ensuring pathogen-free insect biomass requires robust safety protocols and monitoring systems. The risk of disease outbreaks within insect colonies, such as viral or bacterial infections, presents another challenge, necessitating effective biosecurity measures (van der Fels-Klerx *et al.*, 2018). Another important concern is the potential accumulation of heavy metals, pesticides, and veterinary antibiotics in insect tissues (Malematja *et al.*, 2023). In modern automated systems for insect farming and processing, technical fluids such as lubricants may pose a risk of chemical contamination, potentially affecting product safety and quality (Gałęcki *et al.*, 2023). Ensuring that substrates are sourced from controlled, contaminant-free waste streams or plant-based inputs is essential to prevent unintended chemical exposure and safeguard product quality and consumer health. As the insect farming industry is relatively new, another challenge lies in the regulatory framework, that could vary widely across countries and regions. This fragmentation complicates the establishment of standardized safety and quality guidelines, which are crucial for gaining consumer trust and ensuring the safety of insect-based products (Van der Spiegel *et al.*, 2013).

Producers must navigate a complex web of regulations concerning food safety, labelling, and environmental impact (Mancini *et al.*, 2022). Policy-makers should lead the change by supporting research, development, and marketing strategies for insect-based products. In Europe, the EFSA approval of specific insect species for human consumption has marked an important step toward broader acceptance (EFSA Panel on Nutrition, Novel Foods and Food Allergens, 2021). Government incentives, such as subsidies or tax reductions for companies involved in sustainable insect production, could also promote the growth of the sector and make insect-based products more competitively priced. Another challenge that needs regulation are the environmental concerns related to scaling up insect farming. Although insects generally have a lower ecological footprint than traditional livestock, issues like resource use (water, energy) and waste management still need optimization. As the sector grows, more data will be available to better quantify its environmental impacts and to identify best practices that can minimize trade-offs, ensuring that insect farming fulfils its potential as a sustainable protein source (Spykman *et al.*, 2021). In this context, in-depth LCA studies, that are relating to the specific market context, will play a pivotal role in guiding policy decisions and industry standards, helping to optimize insect farming practices and improve resource efficiency (Sokame *et al.*, 2024). Expanding the scope of LCA to consider consequential approaches, which take into account market dynamics and broader system-level changes, could provide even deeper insights into the long-term environmental implications of scaling up insect production (Nakimbugwe *et al.*, 2021). Lastly, consumer perception also remains a considerable barrier, particularly in Western countries, where entomophagy is not culturally ingrained. To overcome these cultural and psychological barriers, a multiple approach is essential. Education plays a critical role in changing perceptions, particularly among younger generations. Studies have shown that early exposure to novel foods, including insects, can positively influence attitudes and reduce neophobia over time (Labyak *et al.*, 2021). Educational campaigns targeting younger demographics could lay the groundwork for long-term cultural acceptance of insect-based foods. Clear and transparent educational campaigns about the environmental benefits and the sustainability of insect farming, compared to traditional livestock and agricultural practices, could be effective in shifting consumer perceptions (Verbeke, 2015). Social and psychological factors still play a key obstacle towards edible insects for Western consumers, especially for adults (Dagevos, 2021). As highlighted by

Mancini *et al.* (2022), most of the insect meal demand will regard the animal feed sector, with the pivotal role of pet food and aquaculture (IPIFF, 2024). Anyway, market studies show that introducing insect-based products in processed forms, such as powders or protein bars, can significantly improve consumer acceptance in Western countries (Sogari *et al.*, 2018).

9. Conclusions

In conclusion, the use of insects as a bioconversion tool offers a promising and innovative solution for sustainable food and feed production. Edible insects and insect-based feed production demonstrate substantial benefits in revalorizing agricultural waste and by-products and providing an alternative protein source as well as other products such as fertilizer, biodiesel, and chitin. Although the sector is advancing, global legislation remains uneven, particularly regarding food safety and quality standards, which are crucial for consumer acceptance and industry growth. While regions such as the European Union have made notable regulatory progress, frameworks in other parts of the world—including many African countries—are still emerging. As outlined, LCA studies have highlighted the efficiency and the environmental advantages of insect farming in reducing resource inputs and waste outputs compared to traditional livestock. On average, insectbased production shows over 80–90% lower global warming potential than beef meat (Smetana *et al.*, 2019; Harwatt *et al.*, 2024). By leveraging LCA to guide improvements emphasizing regional variations in energy and feed availability, the industry can scale responsibly and contribute to global food security goals. By integrating environmental management principles, emerging automation technologies, and evolving policy frameworks, this review provides a holistic synthesis of the current state of the field and identifies knowledge gaps essential for advancing research and industrial scalability. The novel contribution of this work lies in connecting scientific, technological, and legislative dimensions to illustrate how insect rearing can serve as a strategic lever in transitioning toward circular and resource-efficient agri-food systems. Looking forward, continued research and collaboration across regulatory bodies, researchers, and industry stakeholders will be essential to overcoming the future challenges as well as education to overcome perception and psychological barriers. With coordinated efforts, insects could play a transformative role in creating resilient food systems and meeting the growing global demand for sustainable protein sources.

CHAPTER 2

The role of the growing substrate

What is reported in has been accepted and published in "In F. Bovera & P. Falabella (Eds.), The Black Soldier Fly (*Hermetia illucens*): Sustainable Applications in Food, Feed, and Beyond", in the form of a chapter of a book titled "The role of the growing substrate".

1. The importance of growing substrate for *Hermetia illucens*

One of the primary advantages of utilizing larvae of *Hermetia illucens*, commonly known as black soldier flies' larvae (BSFL), is their remarkable capacity to degrade a wide variety of substrates, including organic by-products from the agri-food industry. The larvae are highly efficient in converting organic waste into valuable biomass, rich in proteins and fats, which can be repurposed as animal feed. This ability to convert waste into high-value nutrients is well-documented (Gold *et al.*, 2020). Studies suggest that BSFL can achieve significant waste reduction while producing nutrient-dense biomass, making them a promising solution for both waste management and enhancing food security (Joly *et al.*, 2021). However, the selection and composition of the growing substrate play a critical role in determining the growth performance, development, and productivity of *H. illucens*. The substrate provides not only essential nutrients but also the environmental conditions necessary for the larvae to thrive. It acts as both a food source and a growth medium, making it a pivotal factor in the successful rearing of BSF larvae.

1.1 Influence of environmental conditions

First of all, the moisture content of the rearing substrate plays a critical role. BSFL benefit from substrates with higher moisture levels, which soften solid components and facilitate ingestion (Kim *et al.*, 2010). Indeed, as scavenger insects, BSF larvae have a particular mandibular-maxillary complex that enables them to ingest only semiliquid food with fine parts from any possible decaying organic matter (Bruno *et al.*, 2020). Meneguz *et al.* (2018) found that a moisture content of approximately 70% was optimal for larval growth, while substrates with less than 40% humidity were not suitable for larval development, affecting subsequent fecundity and mating rates of flies (Cammack & Tomberlin, 2017). When the substrate's moisture content is very high (90–97.5%), thick clots may form, hindering larval feeding. As a result, larvae exhibit reduced final body weight and, more importantly, experience a significantly increased mortality rate (Diener *et al.*, 2011; Lalander *et al.*, 2020). In addition, environmental factors such as pH and temperature also play a significant role in the performance of BSFL. Although the larvae are resilient to a range of

environmental conditions, certain studies have suggested that an optimal pH range of 6.0 to 8.0 is preferable for larval development (Chia *et al.*, 2020). Excessive variations in pH can affect the efficiency of digestive enzymes, which in turn im pacts the larvae's ability to absorb nutrients. Substrates that deviate significantly from this pH range may lead to reduced growth rates and lower overall biomass output. Similarly, temperature is another critical factor in BSFL growth. Studies by Diener *et al.* (2009) have shown that the ideal temperature range for BSFL development is between 27 °C and 33 °C. Indeed, BSF is a subtropical species generally growing at high temperatures. Below this optimal range, larval development slows considerably, resulting in a decreased feeding efficiency and a longer developmental cycle. Extreme temperatures outside the optimal range can also negatively affect the larvae's metabolic activity and even survival rates for temperatures below 16 °C (Holmes *et al.*, 2016).

1.2 Macronutrient balance and larval health

The balance of macronutrients—proteins, lipids, and carbohydrates—within the sub strate is crucial not only for optimal growth rates but also for maintaining the health of the larvae. Insufficient protein content in the substrate has been shown to reduce growth rates and overall larval biomass. Barragán-Fonseca *et al.* (2019) emphasize that protein-rich diets are necessary to support robust larval development, while an excess of fats in the substrate may interfere with digestion and nutrient absorption, leading to suboptimal growth (Spranghers *et al.*, 2017). Moreover, the research by Meneguz *et al.* (2018) demonstrated that BSFL reared on a mixture of vegetable and fruit by-products reached the prepupal stage more quickly than those fed only fruit by-products. The mixed substrate also yielded superior results in terms of survival rate, substrate reduction, and larval growth (weight and length). These improvements are likely due to the high crude protein content and moisture level in the mixed substrate, which provide the necessary nutrients for enhanced development. Other studies confirm that diets richer in protein improve larval performances (Cammack & Tomberlin, 2017; Oonincx *et al.*, 2015a). The black soldier fly, as an insect, could be considered as a single-stomached animal. Due to this, not only the total amount of protein in its diet is important, but also the quality of the protein intended as amino acid composition. For this reason, with the increase in interest in insect breeding, attention is growing toward the nutritional aspects of substrates, and, in the last few years, the first

scientific research has been published aimed at evaluating the amino acid requirements of BSF larvae. In this regard, there are a couple of considerations to make. First of all, we start from the assumption that insects share with other animals the inability to synthesize some amino acids which, therefore, also become "essential amino acids" for them. Starting from this assumption, the standard diets used in industrial or semi-industrial production (e.g., Gainesville diet or broiler feed) generally have high contents of essential amino acids and for this reason they ensure good growth of the larvae. The problem of an amino acid deficiency could be linked to the use of substrates, such as production waste or similar, in which the amino acid profile could be unbalanced. Spanghers *et al.* (2024) observed that the addition of lysine, methionine, threonine, phenylalanine, and tryptophan improves the growth performance of BSF larvae during the first week of the nursery period. In partial confirmation of these results, Lemme and Kluber (2024) observed that only a severe reduction of the amino acids level in a substrate is able to reduce the growth rate of BSF larvae; however, the supplementation of the substrate with methionine improved growth performance, suggesting a possible role of "first limitant" for this amino acid. However, studies in this field are still in their infancy, and future research will help us better understand many aspects related to optimizing the growth potential of insects. Furthermore, Meneguz *et al.* (2018) observed that the lipid content of BSFL is not correlated with the lipid content of the substrate. In fact, larvae reared on a fruit based diet showed an increase in lipid content compared to those reared on a more complex diet (mixture of fruit and vegetables). This could be explained by the higher levels of carbohydrates (starch and sugar) in the fruit-based diet. Low protein and high carbohydrate content enhance the fatty acid biosynthesis in the larvae (Franco *et al.*, 2021a; Pimentel *et al.*, 2017). This thesis is also supported by Scala *et al.* (2020), who observed higher lipid content in larvae reared only on fruit than a mix of fruit and grain. In fact, mixing spent grain led to an increase in protein availability for larvae. This indicates that certain beneficial nutrients can be synthesized or concentrated by the larvae, potentially enhancing the nutritional value of BSF larvae as animal feed. The lipid profile of BSFL is composed mainly of saturated fatty acids (up to 70% of total fatty acids). The most abundant fatty acid is the lauric acid, followed by palmitic, myristic, and stearic acids (Franco *et al.*, 2021a). As for other Diptera species, this could reflect the less adaptation of BSF to low temperature (Bennett & Lee Jr, 1997). Regarding the BSF prepupae, Spranghers *et al.* (2017) observed that the

substrate only partially affects the fatty acid profile, which is characterized by high levels of saturated fatty acids. Notably, lauric acid (C12:0) was found in significant quantities in the prepupae, even when the substrate contained only trace amounts of this fatty acid. The fiber content of the substrate is another crucial factor in larval development. Studies by Tschirner and Simon (2015) demonstrated that high-fiber substrates significantly slow larval growth, and in some cases, excessive fiber can inhibit development entirely. Overly fibrous substrates may reduce the larvae's ability to efficiently process nutrients, leading to suboptimal growth and productivity. Indeed, fiber absorbs water from the substrate by reducing the availability of nutrients for the larvae, which need semiliquid food as said before. Another drawback of high fiber content is the excessive fungal growth on the surface of the substrate, which decreases moisture and nutrient availability (Tschirner & Simon, 2015). This suggests that balanced substrate composi tions, specifically with controlled fiber levels, are essential for ensuring optimal larval performance. Moreover, micronutrients, particularly calcium, play an essential role in larval development. Calcium is necessary for proper exoskeleton formation, and substrates deficient in calcium have been associated with deformities in BSFL (Van Huis *et al.*, 2013). This highlights the importance of both macronutrient and micronutrient balance in creating an ideal substrate for larval development.

1.3 Substrate contamination and bioaccumulation

While the quality of the substrate plays a critical role in ensuring safe and pro ductive BSFL rearing, potential contamination is an ongoing concern. Contaminated substrates, such as those containing mycotoxins, heavy metals, or pesticides, can compromise the safety of the larvae as feed or food sources. Heavy metals like cadmium, lead, and arsenic, if present in the substrate, pose particular risks due to their potential to bioaccumulate in larvae and enter the food chain. Nevertheless, some research has shown that *H. illucens* larvae can excrete or avoid the accumulation of certain heavy metals (Mao *et al.*, 2019), suggesting that bioaccumulation risks can be mitigated through proper substrate management. Moreover, larvae have been shown to m3etabolize certain organic contaminants, including pesticides, which limit their accumulation in the larvae's biomass (Schmitt *et al.*, 2019). This capacity to process and neutralize contaminants offers an added layer of safety in the rearing of BSFL, particularly in systems that utilize

waste substrates. The composition and quality of the growing substrate for *H. illucens* larvae are pivotal factors that influence their development, growth rates, and overall productivity. A balanced substrate, rich in protein, with appropriate moisture levels and limited f iber content, can maximize larval performance, while ensuring that contaminants, such as heavy metals and pesticides, are minimized or managed effectively. Addition ally, environmental factors such as temperature and pH must be carefully controlled to optimize larval growth. Given the larvae's ability to process organic waste and produce high-quality protein, BSFL offers significant potential for sustainable waste management and animal feed production. Future research should focus on identifying alternative substrates that enhance larval performance while minimizing environmental impact, thus supporting the development of more sustainable and efficient BSF farming systems.

1.4 Microbiological aspects of black soldier fly larvae rearing substrate

The role of the microbiota in the substrate is particularly significant in the BSFL rearing process. Microorganisms naturally present in the substrate can influence the bioavailability of nutrients and contribute to the degradation of organic matter. This ef fectively complements the larval enzymatic activities, positively influencing BSF larval growth performances. Beneficial microbes can produce metabolites that enhance larval digestion, suppress pathogenic bacteria, and even boost larval immunity. Substrate pretreatment methods, such as fermentation or microbial inoculation with beneficial strains, are therefore emerging as promising strategies to optimize substrate quality while mitigating risks of contamination (Lin and Shelomi, 2024; Ruschioni *et al.*, 2024). For example, recent studies have shown that substrates pretreated with beneficial microbes or enriched with probiotics such as Lactobacillus species can enhance larval growth rates and improve overall biomass quality (Lin and Shelomi, 2024). Moreover, the microbial composition of the substrate can influence the BSFL gut microbiome, which plays a crucial role in nutrient assimilation and pathogen resistance. Indeed, when reared on waste or decaying organic matter, BSFL and their gut microbiome are exposed to intense bacterial pressure. As adaptive mechanisms, both insects and their microbiome have developed defensive strategies to manage and mitigate the effects of these high bacterial loads. In particular, some genera of the BSFL microbiome, such as Morganella and Providencia, showed a proactive role against potential substrate pathogens, such as Pseudomonas and Escherichia coli (Tegtmeier *et al.*, 2021).

BSFL relies on their dynamic gut microbiome to adapt to diverse substrates, but a poorly balanced microbial ecosystem in the substrate can impair this adaptability. For instance, optimal microbial activity in the substrate can lead to the decomposition of carbohy drates, proteins, and lipids, creating a nutrient-rich environment. This supports robust larval growth and significantly enhances overall rearing efficiency through synergistic interactions between the insect gut microflora and organic waste bacteria (Mazza *et al.*, 2020; Rehman *et al.*, 2019; Shao *et al.*, 2024). Among the microorganisms that play a pivotal role in accelerating the bioconversion process, multiple bacterial strains, such as Kocuria, Proteus, *Bacillus subtilis*, and Lactobacillus, have been identified as key contributors (Kariuki *et al.*, 2023). Conversely, substrates contaminated with harmful microorganisms pose risks of microbial infections and reduced larval performance. Pathogens in poorly managed substrates can proliferate, leading to contamination of the larvae and their by-products. These pathogens can pose risks to downstream applications, especially when BSFL derived products are used in animal feed or other food-related processes. For instance, pathogenic bacteria such as *Salmonella* spp., Listeria monocytogenes, or E. coli may persist in substrates and are only partially reduced during larval digestion. As reported by Wynants *et al.* (2019), some pathogens not typically associated with BSFL, like *Salmonella* and Bacillus cereus, have been found in bioconversion residues. Thus, this type of biological contaminant has been targeted as a potential risk in BSF farming systems, highlighting the need for controlled substrate handling and stringent microbiological monitoring (Lin & Shelomi, 2024). Substrate pretreatments such as pasteurization/thermal treatment, prefermentation, the use of controlled microbial in oculants or even a mix of different treatments are therefore critical for compliance with safety standards, lowering contamination risks and enhancing rearing performances at the same time (Heussler *et al.*, 2023; Peguero *et al.*, 2022). Even downstream decontamination processes are often required for the final BSFL biomass, as well as for the other feed ingredients of animal origin. The microbiological quality of the growing substrate is as important as its nutritional composition. Ensuring a beneficial microbial ecosystem while minimizing pathogenic risks is essential for the sustainable and efficient rearing of *H. illucens*. This involves careful substrate selection, pretreatment strategies, and continuous monitoring to achieve the dual goals of waste valorization and high-quality biomass production.

2. Nutritional composition

H. illucens larvae are an excellent source of nutrients, which include fat, protein, high-quality amino acids, and micronutrients, while they are deficient in carbohydrate.

Macronutrient	Mean (%)	Standard deviation (±)
Ashes	10.36	5.47
Crude protein	42.72	7.37
Crude fat	31.80	6.77

Table 1. Macronutrient composition of BSFL, presented as mean values with standard deviations. The table includes ashes, protein, and fat contents expressed as percentages of dry matter. From Fitriana *et al.* (2022).

These characteristics make them particularly interesting both for the feed industry and for the development of innovative foods for human consumption. Their ability to convert organic waste into nutrients positions them as a sustainable resource to address global challenges related to food security (Siddiqui *et al.*, 2024). Larvae can transform a wide range of organic materials, including food waste, into a nutrient-rich biomass (Gao *et al.*, 2019). This ability to biodegrade materials such as agricultural residues, by-products of the food industry (Salomone *et al.*, 2017), and even manure (Franco *et al.*, 2022; Zhou *et al.*, 2013) makes them an environment-friendly solution to reduce waste. This peculiarity contributes to the closure of production cycles and the reduction of environmental impact, serving as a concrete example of a circular economy (Bortolini *et al.*, 2020). Scientific interest has focused on their nutritional composition, demonstrating their potential both as an ingredient in animal feed and as a human food resource, especially in contexts with poor availability of animal proteins. The use of *H. illucens* larvae in animal nutrition has already shown positive results in sectors such as aquaculture and poultry farming, improving animal growth and meat quality (Mohan *et al.*, 2022; Murawska *et al.*, 2021). Larvae are characterized by a high protein and lipid value, enriched by the presence of essential fatty acids, amino acids, minerals, and vitamins (Lu *et al.*, 2022; Scieuzo *et al.*, 2022). Their well balanced amino acid profile, containing lysine, methionine, and threonine (Huang *et al.*, 2019), makes them a valid alternative to traditional proteins such as soy and fish meal. The

chapter will delve into the main chemical components of *H. illucens* larvae, with particular attention to proteins, lipids, carbohydrates, vitamins, and minerals. It should be emphasized that the content of the different types of nutrients is very flexible and varies depending on various parameters. The nutritional composition of larvae is primarily influenced by several key factors: the type of substrate used for rearing, the developmental stage of the larvae, and the rearing conditions, including temperature and relative humidity (Fitriana *et al.*, 2022; Ribeiro *et al.*, 2022). The length of the rearing cycle and postharvest treatments also significantly influence nutritional quality. For example, the use of high-lipid substrates can increase the fat fraction of larvae, while protein-rich substrates promote protein synthesis (Ewald *et al.*, 2020; Nekrasov *et al.*, 2016). It is therefore useful to consider the composition of the main macronutrients of *H. illucens* larvae, which provides valuable information on their nutritional value. Table 1 present the general chemical composition of BSFL, as reported by Fitriana *et al.* (2022). As previously noted, the developmental stage significantly impacts the nutritional composition of BSF. In particular, the larval and prepupal stages are typically the richest in bioavailable nutrients, although these levels vary based on the growth substrates used (Matsakidou *et al.*, 2024; Smets *et al.*, 2020). The compositional differences emerge throughout the BSF life cycle due to feeding activities concentrated in the larval and prepupal phases. During these stages, the insect accumulates energy reserves essential for the pupal and adult phases, during which feeding ceases (Liu *et al.*, 2017). Additionally, morphological changes, including increased cuticle rigidity from larval to pupal stages, further influence the chemical composition and digestibility of BSF (Liao *et al.*, 2018). Given the importance of these nutrients, the following sections provide a detailed examination of BSF's nutritional composition.

2.1 Proteins

To examine the impact of the developmental stage, Table 2 summarizes protein content across BSF stages, generally measured using the Kjeldahl method (AOAC 978.02) with a nitrogen-to-protein conversion factor of 4.67 (or a specified alternative factor, where noted), as reported in various studies. Protein content on a dry matter (DM) basis, ranges from 31% to 41% in the larval stage, 31% to 43% in the prepupal stage, and 26% to 31% in the pupal stage. These variations are attributed to factors such as differences in substrate types across trials and the age

of BSF. For instance, studies by Intayung *et al.* (2021) and Liu *et al.* (2017) indicate that BSF protein content is influenced by the insect's age.

Protein content Dry basis (%w/w)	**Development stage**	**Growing substrate**	**References**
31.73 ± 0.65	Late-instar larvae from Greece	Wheat bran and cornmeal (1:1)	Matsakidou *et al.* (2024)
32.59 ± 0.11	Late-instar larvae from Portugal	Wheat bran and cornmeal (1:1)	Matsakidou *et al.* (2024)
38.86 ± 1.70	Larvae	Supermarket waste	Smets *et al.* (2020)
41.1 ± 0.3	Larvae	Chicken manure	Shumo *et al.* (2019)[a]
33.0 ± 1.0	Larvae	Kitchen waste	Shumo *et al.* (2019)[a]
41.3 ± 0.5	Larvae	Spent grain	Shumo *et al.* (2019)[a]
39.38 ± 0.16	Larvae	Organic agroindustry by-products	Zulkifli *et al.* (2022)
31.81 ± 1.26	Prepupae	Supermarket waste	Smets *et al.* (2020)
41.2 ± 0.6	Prepupae	Chicken feed	Spranghers *et al.* (2017)[b]
42.2 ± 1.4	Prepupae	Digestate	Spranghers *et al.* (2017)[b]
39.9 ± 0.2	Prepupae	Vegetable waste	Spranghers *et al.* (2017)[b]
43.1 ± 0.6	Prepupae	Restaurant waste	Spranghers *et al.* (2017)[b]
31.27 ± 0.92	Pupae	Supermarket waste	Smets *et al.* (2020)
26.62 ± 1.03	Pupae	Wheat bran and cornmeal (1:1)	Matsakidou *et al.* (2024)

a The nitrogen-to-protein ratio used is 4.76.

b The nitrogen-to-protein ratio used is 6.25.

Table 2 Protein content of BSF as affected by the development stage, presented as mean values with standard deviations.

To explore this further, Table 3 highlights findings from Intayung *et al.* (2021), who analyzed larval composition, and Liu *et al.* (2017), who assessed all developmental stages.

Life cycle phases	Crude protein Liu *et al.* (2017)	Crude protein Intayung *et al.* (2021)
1-d-larva	56.2 ± 0.06	–
4-d-larva	54.8 ± 0.28	–
6-d-larva	54.2 ± 0.15	49.5
7-d-larva	46.0 ± 0.21	–
9-d-larva	42.0 ± 0.10	–
12-d-larva	38.0 ± 0.35	42.4
14-d-larva	39.2 ± 0.06	–
18-d-larva	–	42.9
Early-prepupa	40.2 ± 0.15	–
Late-prepupa	40.4 ± 0.21	–
Early-pupa	46.2 ± 0.12	–
Late-pupa	43.8 ± 0.21	–

Table 3. Crude protein of BSF (% dry matter basis) in diverse life cycle steps.

These studies showed that crude protein content decreases gradually from hatching to the 12th day of larval development, with a notable increase in mature larvae. Both Intayung *et al.* (2021) and Liu *et al.* (2017) observed a slight rise, followed by a gradual increase and stabilization in later stages of the life cycle, including early prepupa (40.2%), late prepupa (40.4%), early pupa (46.2%), and late pupa (43.8%). Other variation in crude protein content can be attributed to the rearing substrates, as previously underlined, rearing conditions and also processing; in this regard, defatting causes a concentration of nutrients different from lipids in BSFL that can reach the outstanding amount of 65% of protein (on dry weight basis) in defatted larvae (Schiavone *et al.*, 2017). The human body needs nitrogen (N) and amino

acids from food-based proteins to create and maintain the roughly 25,000 proteins encoded in the human genome as well as other metabolically active nitrogenous substances; this is essential for the functioning of the immune system and the production of enzymes and hormones essential for regulating metabolism (EFSA Panel on Dietetic Products, Nutrition and Allergies [NDA], 2012). Twenty of the naturally occurring amino acids are referred to as proteinogenic amino acids because they are used by living organisms as the building blocks to biosynthesize proteins. Nine of these twenty (i.e., histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine) are cate gorized as essential in humans since the body cannot synthesize them from naturally oc curring precursors (EFSA Panel on Dietetic Products, Nutrition and Allergies [NDA], 2012). The intake of these amino acids through food is therefore crucial to maintain homeostasis and ensure growth and vital functions (Wu, 2009). BSFL contains almost all the amino acids. The essential amino acid content of BSFL is comparable with soybean meal and fishmeal (Heuel *et al.*, 2021). Among these, the most relevant essential amino acids are leucine, lysine, and valine. Indeed, these have an average content of 44.6, 38.8, and 40.1 g/kg, respectively, which is even higher than soybean meal (Lu *et al.*, 2022). Lysine is essential for protein synthesis (Dwight, 2020), while methionine acts as a precursor to cysteine, which is necessary for the formation of structural proteins such as keratin (Rehman *et al.*, 2020). Liu *et al.* (2017) found that a range of essential amino acids were synthesized across all developmental stages of BSF, from egg to adult. Notably, lysine, a commonly limiting amino acid in plant-based proteins, was present in relatively high amounts throughout BSF's life cycle (from 19.0 ± 0.09 to 29.8 ± 0.28 g/kg on DM basis). Additionally, the study showed that amino acid levels were generally highest in the early larval phase (4th–6th day) and then steadily declined as larvae matured. This is consistent with the data reviewed by Lu *et al.* (2022) in which all the reported amino acids were higher in BSF prepupae than in larvae. Irrespective of the life stage, the least prevalent essential amino acids are tryptophan, methionine, and cysteine. Hence, the high-quality protein content of BSFL (English *et al.*, 2021), with the advantage of a more sustainable and economically advantageous production (Van Huis *et al.*, 2013), makes them particularly suitable for replacing or integrating the protein sources in animal feed. In addition, the resource efficiency of larval protein production is higher than that of traditional livestock, requiring less

water, land, and resources and contributing to the reduction of greenhouse gas emissions (Van Huis *et al.*, 2013).

2.2 Fats

H. illucens larvae are also an interesting source of lipids, which provide concentrated energy and essential fatty acids (Nekrasov *et al.*, 2022). These lipids not only represent a fundamental energy reserve, but also play structural and functional roles, being key components of cell membranes and precursors of bioactive molecules (Wro´ nska *et al.*, 2023). The lipid content of BSF larvae, which makes up to 15% to 49% of total dry weight, is exceptionally high. The absolute crude fat content increases rapidly, peaking in mature larvae and early prepupal stages. Liu *et al.* (2017) observed that crude fat content reached its maximum in mature larvae (14 days old; 28.4 ± 0.06 g/100 g DM basis) and early prepupae (28.0 ± 0.25 g/100 g on DM basis), consistent with findings from Intayung *et al.* (2021) and Smets *et al.* (2020). Specifically, fat content ranged from 17.2% to 37.8% in larvae aged 6 to 18 days (Intayung *et al.*, 2021) and from 40.97% to 39.85% on DM basis in BSF larvae and pupae. The increase in fat accumulation aligns with heightened lipase activity observed during larval development. This variability depends on factors such as the feeding substrate and the devel opmental stage of the larvae, offering the possibility of optimizing the lipid content for specific applications (Addeo *et al.*, 2024a; Li *et al.*, 2021). In addition to fat quantity, the quality of the fat is crucial, as it significantly influences the overall fatty acid (FA) composition. Lipids present in *H. illucens* larvae contain a mixture of saturated (SFAs), monounsaturated (MUFAs), and polyunsaturated (PUFAs) fatty acids (Almeida *et al.*, 2022). These FAs are essential components for numerous metabolic processes in living beings, including energy production and cellular function (Dooley & Ryan, 2019). The predominance of one type of FA over another can significantly influence the nutritional and functional properties of the final product. According to literature and differently than other insects, there are more SFAs than PUFAs (Franco *et al.*, 2021b). Approximately, the contents of SFAs, MUFAs, ω-6 PUFAs, and ω-3 PUFAs vary from 362.0 to 782.9 g/kg, 85.5 to 287.0 g/kg, 80.0 to 314.0 g/kg, and 9.8 to 36.0 g/kg, respectively (Lu *et al.*, 2022). The most relevant saturated fatty acids are lauric (C12:0) and palmitic (C16:0) (Surendra *et al.*, 2016). Lauric acid is known for its antimicrobial properties as it is able to damage the surface of bacteria, compromising both the cell wall and the

membrane (Kaczor *et al.*, 2022). This makes larval lipids not only a valuable source of energy but also a potential ingredient in nutraceutical applications. Oleic, palmitoleic, linoleic, and linolenic acids represent the most common MUFAs and PUFAs (Lu *et al.*, 2022). Omega-3 (ω-3) and omega-6 (ω-6) are crucial for the health of all mammals (Biagi *et al.*, 2004). ω-3, such as alpha-linolenic acid, are precursors of bioactive compounds that regulate inflammatory processes (Calder, 2010). Considering the life cycle, the FAs composition varied considerably from one stage to another, even if this is particularly true for specific fatty acids (Liu *et al.*, 2017; Smets *et al.*, 2020). Focusing on the difference between mature larvae, prepupae, and pupae, it is possible to affirm that both SFAs and MUFAs, especially palmitic and oleic acids, sharply diminished between larval and prepupal stages, while they tended to remain almost unchanged between prepupal and pupal ones. Regarding the most representative fatty acid, C12:0, markedly increased during the life cycle peaking up to 65% of the total fatty acids. However, the difference in FA composition can be much larger in studies regarding rearing substrates (Barragán-Fonseca *et al.*, 2020; Ewald *et al.*, 2020; Oonincx *et al.*, 2015b) than the difference observed in the three life stages. For instance, when BSF larvae were fed on a mixture of organic waste, the fat content of the BSF prepupae showed a higher proportion of short-chain SFAs, particularly lauric acid and palmitic acid, accounting for 67% of the total fatty acids. In comparison, feeding them coconut oil and palm kernel oil resulted in a slightly lower proportion, with 55% to 57% of total FAs (Surendra *et al.*, 2016). Additionally, when ω-3 FAs are present in their diet, BSF prepupae may present some type of this, such as linolenic acid or eicosapentaenoic acid (St-Hilaire *et al.*, 2007). This demonstrates how diet significantly affects the fatty acid profile and other nutritional attributes of BSFL. Among fats, sterols make up only a small portion of the total. The average con centration of cholesterol in larvae is approximately 0.005% to 0.025% of the total lipid fraction (Caligiani *et al.*, 2019; Matthäus *et al.*, 2019; Ramos-Bueno *et al.*, 2016). Regarding phospholipids, the only ones isolated from BSF larvae were phosphatidyl choline and N-acyl phosphatidylethanolamine, both with very low concentrations (Leyva-Gutiérrez *et al.*, 2022). These, although present in small quantities, are crucial for the formation and functionality of cell membranes (Sun *et al.*, 2022; Terova *et al.*, 2005), adding further nutritional and biological value to the lipids of the larvae.

2.3 Carbohydrates

Although BSF larvae and edible insects in general are rich in protein and fat, they are poor in carbohydrates. However, they represent a special animal group regarding dietary fiber (Nowak *et al.*, 2016). Indeed, according to Lu *et al.* (2022) the average level of crude fiber in BSF larvae was 95.4 g/kg, with most of these represented by chitin (61.7 g/kg). Chitin is a linear biopolymer of N-Acetyl-D-glucosamine (Glc NAc), structurally similar to cellulose, and is one of the main constituents of the insect exoskeleton (Triunfo *et al.*, 2022). However, the total amount of fiber and especially chitin is highly variable naturally according to the stage of development. In general, this amount increases as the adult stage is approached (Wang *et al.*, 2020). Smets *et al.* (2020) proposed values equal to 3.85 ± 0.24, 4.72 ± 0.76, and 6.31 ± 0.14 g/100 g on DM basis for larvae, prepupae, and pupae, respectively, whereas Spranghers *et al.* (2017) found values from 5.6 ± 0.15 to 6.7 ± 0.13 g/100 g on DM basis for prepupae reared on chicken feed or restaurant waste, respectively. Chitin is a viscous dietary fiber that binds cholesterol, thereby reducing its absorption and leading to the excretion of excess of cholesterol (Shahidi *et al.*, 1999). Chitin, when deacetylated into its derivative chitosan, can be digested by humans and is recognized as a functional food component. Chitosan offers various health ben efits, including antimicrobial properties, immune modulation, cholesterol reduction, wound healing, and support in the treatment of chronic diseases (Singh *et al.*, 2018). Chitin, therefore, has great potential to improve intestinal health in humans and animals by regulating the gut microbiota as a dietary fiber (Kipkoech, 2023). Indigestible fiber is the main source of energy for the gut microbiota, which generates signals influencing metabolism, immune function, and overall health (Berding *et al.*, 2021; Rio-Aige *et al.*, 2021; Teng *et al.*, 2021). Studies have shown that a diet consisting of BSF larvae can positively influence the gut microbiota, improving gut health in quails (Atallah *et al.*, 2023). In addition to nutritional use, chitin has multiple applications in other sectors. Chitin is particularly interesting for the production of bioplastics due to its abundant reserves, biodegradability, film-forming properties, nontoxicity, and biocompatibility (Jing *et al.*, 2021). In summary, although *H. illucens* larvae are mainly known for their protein and fat content, their contribution in terms of carbohydrates, especially in the form of chitin, offers multiple opportunities for use. From a nutritional, technological, and environmental point of view, chitin is a

highly valuable component with applica tions ranging from nutrition to biotechnology, contributing to the promotion of insects as a multifunctional resource.

2.4 Minerals and vitamins

H. illucens larvae are also a significant source of essential minerals (Table 4) and vitamins (Zulkifli *et al.*, 2022), crucial as they play essential roles in a variety of basic metabolic pathways that support fundamental cellular functions (Tardy *et al.*, 2020). The mineral profile of BSFL showed high concentrations of essential macro and microelements, such as calcium (Ca), magnesium (Mg), potassium (K), iron (Fe), zinc (Zn), and selenium (Se), demonstrating their nutritional richness and potential for animal feed applications (Addeo *et al.*, 2024b; Barragán-Fonseca *et al.*, 2017; Zulkifli *et al.*, 2022). According to Dierenfeld and King (2008), BSFL also shows richer mineral content than other insects used to feed animals. However, the concentration of Na in BSF larvae is low compared to other insects. Table 4.4 shows the average composition of some of the main minerals present in BSF larvae, expressed as a percentage of DM. This data illustrates the richness of essential minerals such as calcium (Ca), phosphorus (P), magnesium (Mg), and iron (Fe), as well as the presence of trace elements such as copper (Cu) and zinc (Zn). The variation in the reported values reflects differences due to breeding factors and analysis methods. Considering the life stages, the mature larva (14 days old) resulted poorer in Ca and P than the prepupal stage, probably due to the cuticle formation in the prepupal period, while Na, Fe, and Zn were more abundant in larval stage (Liu *et al.*, 2017). These data were confirmed by Lu *et al.* (2022). However, as reported before for other nutrients, the type of processing methods and variations in the diets being utilized for the rearing also have a significant impact on the micronutrient content of BSF larvae (Fasakin *et al.*, 2003; Newton *et al.*, 2005; Zulkifli *et al.*, 2022), that results in more relevant differences than those observed during the development of BSF. For example, while Ca content was 2900.0 ± 13.57 and 3000.0 ± 18.45 mg/100 g in mature larvae and prepupae, respectively, Spranghers *et al.* (2017) reported values that ranged from 123 mg/100 g to 6615 mg/100 g in prepupae fed vegetable or restaurant wastes, respectively. Vitamins are essential in small amounts in many animal feeds, as they con tribute to the maintenance of normal metabolic and physiological functions (Pillay & Kutty, 2005). Larvae contain several vitamins, both water-soluble and fat-soluble,

including B vitamins (such as B1 and B2) and vitamin C (Zulkifli *et al.*, 2022). Vitamin B1 is particularly important, as it acts as a coenzyme in carbohydrate metabolism, which is essential for energy production (Zulkifli *et al.*, 2022). Furthermore, vitamin C in BSFL larvae plays a crucial role in supporting optimal growth and optimizing feed utilization in farm animals (Adewolu & Aro, 2009). According to the analysis conducted on BSF larvae by Zulkifli *et al.* (2022), vitamin A (β-carotene) was absent in all the samples; on the opposite, ascorbic acid (vitamin C) was present in all the samples with a mean concentration of 0.26 mg/100 g. Vitamins B1 and B2 were also detected (Zulkifli *et al.*, 2022). Vitamin E was the only determined by Liu *et al.* (2017); its content during larval and prepupal stages was 6.7 ± 0.64 and 3.3 ± 0.42 mg/100 DM, respectively.

Minerals	Mean (%)	Standard deviation (±)
Calcium (Ca)	1.45	1.12
Phosphorus (P)	0.62	0.39
Magnesium (Mg)	1.18	0.91
Potassium (K)	1.86	0.74
Copper (Cu)	0.045	0.03
Manganese (Mn)	0.33	0.19
Sodium (Na)	0.8	0.83
Iron (Fe)	1.15	1.14
Zinc (Zn)	0.29	0.39

Table 4. Micronutrient (mineral) composition of BSFL, presented as mean values with standard deviations. The table includes key minerals such as calcium, phosphorus, magnesium, and trace minerals like iron, copper, and zinc, expressed as percentages of dry matter. From Fitriana *et al.* (2022).

3. Conclusion

H. illucens represents a remarkable resource in the global effort to promote sustainable practices in waste management, food security, and animal feed production. The larval ability to efficiently convert diverse organic waste streams

into high-value biomass rich in proteins, fats, and essential nutrients underscores their potential as a cornerstone of a circular economy. The critical role of substrate composition, environmental conditions, and nutrient balance has been extensively highlighted, showing that these factors directly influence larval growth, productivity, and nutritional composition. From macronutrients like pro teins and fats to micronutrients and functional compounds such as chitin, the nutritional richness of BSFL makes them a versatile and valuable resource. Furthermore, the capacity of larvae to bioaccumulate or metabolize contaminants reinforces the need for careful substrate management and monitoring to ensure their safety and quality. Additionally, the microbiological aspects of BSFL rearing underline the importance of beneficial microbes in enhancing nutrient bioavailability (Newton *et al.*, 2005) and suppressing pathogens, opening new pathways for substrate optimization through microbial pretreatments. In conclusion, BSFL offers a sustainable, adaptable, and economically viable solu tion to address modern challenges in waste valorization and nutrition. Future research should focus on refining rearing techniques, exploring novel substrates, and expanding applications to fully harness the potential of this innovative resource. By integrating *H. illucens* into agricultural and industrial systems, we can advance toward more resilient and eco-friendly food production frameworks.

CHAPTER 3

Exploring the agronomic traits, antioxidant and antifungal properties of *Hermetia illucens* frass extract in durum wheat (*Triticum durum* Desf.)

What is reported in has been accepted and published in "BMC Plant Biology", in the form of scientific article titled "Exploring the agronomic traits, antioxidant and antifungal properties of *Hermetia illucens* frass extract in durum wheat (*Triticum durum* Desf.)".

1. Introduction

Building on the bioconversion potential of *Hermetia illucens* (L.) (BSF) extensively discussed in Chapters 1 and 2, it is well-established that BSF larvae can efficiently transform various organic by-products into high-value biomass (Gold *et al.*, 2018; Scieuzo *et al.*, 2023). However, beyond the production of proteins and fats for animal feed, a secondary but equally significant output of this sustainable process is the frass. This material, consisting of a mixture of larval excrement, undigested organic waste, and shed exoskeletons (Bohm *et al.*, 2023; Tan *et al.*, 2021). Frass could be useful in various industrial processes like biogas production (Wedwitschka *et al.*, 2023), but due to the balanced organic composition represents also a viable alternative to commercial fertilizers with beneficial effects on different crops (Lomonaco *et al.*, 2024). Wheat (*Triticum spp.*) represents the main nourishment for a large part of the world's population (Royo *et al.*, 2017). Overall, it is the most widely cultivated species worldwide, even surpassing rice in terms of harvested area in 2022 (FAO, 2023). Wheat is a strategic crop for food safety as it is a key source of dietary calories (worldwide, approximately 20% of calories and 55% of carbohydrates are provided by wheat). Durum wheat (*Triticum durum* Desf.) is widely cultivated in the Mediterranean area, and studies carried out with both ancient and modern varieties have provided valuable information on the characteristics of the development of the root system when a biostimulant is used (Bochicchio *et al.*, 2022), even under use of biotic stress conditions (Vitti *et al.*, 2022). Climate change, associated with rising global temperatures and extremes in rainfalls, is significantly impacting wheat production in many parts of the world (Ishaque *et al.*, 2023). Biotic stresses, like *Fusarium* head blight (FHB) disease, caused by more than twenty fungal species belonging to *Fusarium* genus, are responsible for significant yield losses, and are also able to produce mycotoxins (Ishaque *et al.*, 2023), in particular *F. sporotrichioides* (Chtioui *et al.*, 2022; Nazari

et al., 2019). Mycotoxins content in grain induced by this pathogen, and *F. sporotrichioides* itself, can be reduced by the application of fungicides, but also using some biocontrol agents (BCAs). Among BCAs, *Trichoderma spp.*, opportunistic plant symbionts, are fungi commonly isolated from soil and rhizosphere, and possess the ability to control soil-borne pathogens, playing a key role in resistance induction in planta (Harman, 2006), and also to enhance plant growth (Vitti *et al.*, 2022; Ishaque *et al.*, 2023; Mironenka *et al.*, 2021; Sempere Ferre & Santamarina, 2010; Sood *et al.*, 2020). The systemic response induced by *Trichoderma spp.* has been observed on a wide range of species and may be temporally and spatially distant from the time and site of inoculation (Harman, 2006; Kthiri *et al.*, 2020; Vitti *et al.*, 2016). Wiśniewska *et al.* (2011) (Ishaque *et al.*, 2023) found that *T. harzianum* AN4 has been useful in the control of *F. sporotrichioides*, by reducing its growth and inhibiting mycotoxins accumulation in grain. In a former study (Coviello *et al.*, 2024), pasteurized frass extract deriving from BSF larvae demonstrated the potential to be used as a sustainable tool to induce biostimulation and antifungal activity in *Triticum durum* Desf. var Simeto against the soil-borne pathogen *F. sporotrichioides*, also in combination with the known BCA *Trichoderma* afroharzianum T22, by the priming treatment of seeds. The disease reduction has been attributed to both enzymatic and non-enzymatic responses, because differences in total phenolic content and superoxide dismutase activity (SOD) in seedlings derived from treated seeds have been observed. Effectively, clear evidence of the biotic stresses perception by the plant is the overproduction of reactive oxygen species (ROS), which cause oxidative stress and whose detoxification by antioxidant enzymes like catalase (CAT) and SOD, is essential to ensure plant production (Sofo *et al.*, 2015). As a consequence, the regulation of the activity of the pool of antioxidant enzymes is crucial to fine-tune the adaptation mechanisms in conditions of oxidative stress induced by phytopathogens. In another study, the ability of frass deriving from the insect Tenebrio molitor to promote the tolerance of chard plants against various stresses has been demonstrated and attributed to the presence of microorganisms in the frass with growth promoting activity (Poveda, 2021). Furthermore, the application of frass to potted soil has been able to influence the plantassociated soil microbial communities stronger than a conventional compost used as fertilizer (Fuhrmann *et al.*, 2022). In continuation to the above-mentioned work (Coviello *et al.*, 2024), the aim of the present study was to further evaluate the ability of frass as a green and

sustainable strategy, by moving from a plate system studied during our first research, to a system that involved germination in soil. For this purpose, the effect of frass extract, alone or combined with *T. afroharzianum* T22, on potted wheat seedlings derived from primed seeds, was evaluated in terms of agronomic traits, reduction of damping-off due to *F. sporotrichioides*, and activity of the pool of antioxidant enzymes involved. In addition, the presence of microorganisms in the frass extract with possible plant growth promoting and/or protection activity, was searched in order to obtain, for the first time, a possible biological relevance of the frass extract to be employed for seed priming in sustainable agricultural systems.

2. Materials and methods

2.1 Plant material, frass extract from *Hermetia illucens*, and fungal isolates

Seeds of durum wheat (*Triticum durum* Desf. var Simeto) were considered. This variety consists in an Italian tetraploid modern durum wheat released in 1988 (Capeiti-8/ Valnova), and was obtained by mutagenesis and crosses involving old wheat materials. Seeds were surface sterilized for 2 min in 1% sodium hypochlorite solution, and rinsed three times with sterile distilled water (dH2O). Frass were provided by Xflies s.r.l. (Potenza, Italy) starting from *Hermetia illucens* hatched eggs, and neonates reared on the standard Gainesville diet (Mangimi Losasso s.r.l.—Balvano, Potenza, Italy), according to Coviello *et al.* (2024). Frass aqueous extract was prepared according to Coviello *et al.* 2024. Briefly, solid frass was pasteurized at 70 °C for 1 h, according to EC Regulation No 2021/1925 (European Commission Regulation, 2021), and frass extract (pFE) was prepared adding 10 g of pasteurized frass in 100 mL (1:10 w/V) of sterile 0.5% NaCl physiological saline solution. After incubation on an orbital shaker at 150 rpm and 27 °C, the suspension was ultracentrifugated at 6,000 × g (15 min). Finally, the supernatant was recovered by filtering through a double layer of sterile gauze. *Fusarium sporotrichioides* PZ2 strain (FS) was provided by Prof. Antonio Ippolito of Department of Soil, Plant and Food Sciences, University "Aldo Moro", Bari, Italy. Trianum P (Koppert Italia S.r.l., Viale delle Nazioni 7, Bussolengo, Verona, Italy) was used as source of *Trichoderma harzianum* strain T22 (T22), renamed *T. afroharzianum*, according to Chaverri *et al.* (2015). Botrytis cinerea isolate (BC) was supplied from the fungal collection of DAFE (Department of Agricultural, Forestry, Food and Environmental Sciences, University of Basilicata, Potenza, Italy). *Fusarium oxysporum* f.sp.

lycopersici (FOLYC) was provided by Dr. Catello Pane, Consiglio per la Ricerca in Agricoltura e L'analisi dell'Economia Agraria (CREA), Centro di Ricerca Orticoltura e Florovivaismo, Pontecagnano Faiano, Salerno, Italy. Fungal mycelia were grown on potato dextrose agar (PDA) medium for 7 days in the dark at 23–26 °C.

2.2 Experimental design

2.2.1 Seed priming with frass extract and *T. afroharzianum* and infection with *F. sporotrichioides*

Sterile seeds were primed with different solutions following the procedure described in Coviello *et al.* 2024. Priming solutions were constituted from pasteurized frass extract (pFE) diluted at 10% in dH2O (v/v) and/ or spore suspension of *T. afroharzianum* T22 (T22) at 1×106 conidia mL-1. The infection suspension was prepared using spores of *F. sporotrichioides* at 1×106 conidia mL-1. The resulting four or five theses for biostimulation (1–4) and protective (1, 5–8) trials, respectively, were: (1) untreated and not infected (control CTRL); (2) frass extract (pFE); (3) *T. afroharzianum* (T22); (4) frass extract and *T. afroharzianum* (pFE+T22); (5) *F. sporotrichioides* (FS); (6) frass extract and *F. sporotrichioides* (pFE vs. FS); (7) *T. afroharzianum* and *F. sporotrichioides* (T22 vs. FS); (8) frass extract, *T. afroharzianum* and *F. sporotrichioides* (pFE+T22 vs. FS). The presence of FS and/or T22 on the coated seed of each thesis was verified by fungus re-isolation on PDA medium, and mycelium and conidia characterization under optical microscope observation.

2.2.2 Growth conditions and agronomic and physiological analyses

Twenty primed seeds were sown in aluminum trays (210×280×60 mm, L×W×D) filled with autoclaved (at 121 °C for 30 min on two consecutive days) soil substrates (COMPO SANA® Universal Potting Soil, COMPO Italia s.r.l., Italy) and arranged in complete randomized design with four replicates for each experiment. Throughout the experiment, seedlings were kept in a growth chamber with a 16/8 h photoperiod, light/dark (average T of 22 °C; average relative humidity of 60%), and tap watered every 3 days until the field water capacity was reached. The emergence was monitored daily. At 21 days after sowing (DAS), seed germination was evaluated by counting the number of germinated seeds for each tray, and the height, measured from ground level to the tip of the apical shoot for each seedling, was

recorded. The SPAD index (SPAD502 Chlorophyll meter, Konica Minolta Sensing Europe B.V., Cinisello Balsamo, MI, Italy) was measured on the fully expanded leaves with three measurements per plant. Seedlings were carefully removed from the tray, soaked in water to remove root attached substrate particles, dried with paper towels to remove excess water and separated into shoot and root portions. The length of the longest root of each seedling was measured; the fresh and dry weights (oven-dried at 60 °C until constant weight) of the shoots and roots were recorded. Seed Germination Index (SGI) was calculated according to Vitti *et al.* (2024):

SGI% = [(n. seeds germinated in sample/n.seeds germinated in control)] ×100

2.2.3 Effect of seed treatment on damping-off caused by *F. sporotrichioides*

The presence of FS and/or T22 on the wheat root was verified at the end of the survey (21 DAS) by re-isolating the fungi on PDA and observing their mycelia and conidia under optical microscope. The effect of pFE and T22, both alone and combined, was evaluated as damping-off (DO) caused by FS on the emerged seedlings, according to the formula of Veeken *et al.* (2005):

% DO = [(HPo − HPi) / HPo] × 100

where: HPo is the number of healthy seedlings under the untreated experimental condition and HPi is the number of healthy seedlings derived by treated seeds and inoculated with FS.

2.2.4 Total antioxidant activity

Total antioxidant capacity was assessed using the Antioxidant Assay Kit (item No. 709001, Cayman Chemical, Ann Arbour, MI, USA) according to the manufacturer's instructions. The kit allows to assess the antioxidant activity of both aqueous- and lipid-soluble antioxidants and relies on the ability of those molecules to inhibit the oxidation of ABTS to $ABTS^{\cdot+}$ by metmyoglobin. Four tissue samples replicates were made for each thesis (n=4) deriving by 500 mg of frozen tissues (leaf+root) from five seedlings, randomly chosen from each tray. The samples were homogenized in 5 mL of cold 5 mM potassium phosphate, pH 7.4 containing 0.9% sodium chloride and 0.1% glucose, and centrifuged at 10000 × g for 15 min at 4 °C. The resulting supernatant was recovered and absorbance was read at 750 nm with a microplate reader model Multiskan FC (Thermo Scientific, Fisher Scientific,

Segrate, Italy). Antioxidant molecules in the sample caused a suppression of absorbance proportional to their concentration and were expressed as mM Trolox equivalents g-1 FW.

2.3 Isolation, identification and antifungal activity of microorganisms derived from frass extract

2.3.1 Microorganisms isolation from pFE

Fifty µL of pFE were spread on Petri plate containing Luria-Bertani Agar (LBA) and PDA media for bacterial or fungal isolation, respectively. The plates were incubated in the dark at 30 °C for 24 h (for bacteria) or between 23 and 26 °C for 72 h (for fungi). No fungal colonies were observed after incubation. The species resembling bacteria was isolated and grown on nutrient broth agar with glycerin (NGA) medium. Subcultures were performed by transferring the bacteria on the same medium to obtain pure cultures.

2.3.2 Molecular identification of the bacterial isolate

The pure bacterial cultures were cultivated overnight in LB liquid medium at 30 °C and under agitation (150 rev/ min). Subsequently, they were aliquoted and conserved at -80 °C in 15% w/v glycerol (Merck KgaA, Darmstadt, Germany). The total genomic DNA (gDNA) was extracted from nine single pure cultures using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following manufacturer's instructions with minor modifications as described by Camele and Mang (2019) and Mentana *et al.* (2019). The quantity and quality of the genetic material were checked by readings at ND-1000 spectrophotometer (Thermo Fisher Sci. Inc., MA, USA) and the gDNA was kept at -20 °C until further use. The extracted gDNA was amplified with three pairs of primers: the fD1 and rD1 amplifying a fragment of the 16 S rRNA gene (Weisburg *et al.*, 1991); the gyrA-42 F and gyrA-1066r amplifying a fragment of the gyrA gene (Rooney *et al.*, 2009) and the rpoB-2292f and rpoB-3354r amplifying a portion of the rpoB gene (Rooney *et al.*, 2009) using the Phire Plant Direct PCR Master Mix (Thermo Scientific Inc., USA) and the protocols fully described in previous studies by Frisullo *et al.* (2015) and Mang *et al.* (2022). PCR outcomes were observed by electrophoresis in a 1.2% agarose gel run at 50 V in Tris-acetate-EDTA (TAE) 1X running buffer. The amplicons were directly sequenced, in both directions, using the same primers as for the PCR assay. The obtained nucleotide sequences were aligned against the National Center for

Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (BLAST) program and the option Blastn for precise species identification.

2.3.3 Phylogenetic analysis of the bacterial isolate

The 16 S rRNA, gyrA and rpoB partial genes sequences were first aligned by the ClustalW program in the MEGA11 phylogeny package, and the alignments were manually corrected (Tamura *et al.*, 2021). Only the single barcode phylogenetic analysis using Maximum Likelihood (ML) method and Kimura-2 model (Kimura, 1980) was performed, by the same phylogeny package, due to different isolates/strains (for each DNA barcode) data availability in the NCBI database. Stability of trees' branches was tested by bootstrap analysis with 1000 replicates (Felsenstein, 1985).

2.3.4 *In vitro* antagonistic effect of *Paenibacillus polymyxa* against phytopathogenic fungi

The inhibitory effect of bacterial isolate was evaluated according to Jiang *et al.* (2014) with slight modifications. Conidial suspension of phytopathogenic fungi (Botrytis cinerea, *F. sporotrichioides* and *F. oxysporum* f.sp. *lycopersici*) was obtained by flooding a 7 days old PDA culture plate with dH2O. The suspension was filtered through double layered sterile gauze to remove mycelium and the conidial concentration was adjusted to 106 conidia mL-1 using a hemocytometer (Thoma counting chamber, BLAUBRAND(R), Wertheim, Germany). Forty µl of conidial suspension of each phytopathogenic fungus were spread on a 90 mm PDA Petri plate. Thereafter, an 8 mm diameter well was made in the center of the plate, and 100 µl of *P. polymyxa* liquid culture or sterile LB medium (as control) were added to the well. The fungal growth inhibition was calculated after 72 h of incubation at 26 °C by subtracting the average of two diameters (90° from each other) of the zone around the well where no fungal growth was observed from the diameter of the fungal growth seen in the control Petri dish. The experiment was carried out in triplicate.

2.4 Statistical analysis

Normal distribution of data was tested by the Shapiro-Wilk test at $p < 0.05$ and homoscedasticity was tested performing the Breusch-Pagan test ($p < 0.05$). When the assumptions were not met, the variable was z transformed or subjected to log10 (count data). Data from agronomic and physiological analyses, indicated as

percentages relative to the control (%), damping-off, antioxidant activity, and *in vitro* data, were expressed as mean (n=4 or n=3 for the *in vitro* test) ±SD and analyzed according to one-way ANOVA followed by Tukey's HSD test ($p < 0.05$). Data from the *in vivo* experiment were subjected to principal component analysis (PCA) using the "PCA" function from "FactoMineR" package, considering an eigenvalue > 1 as cutoff point for which PC was retained (Kassambara, 2017). R (version 4.2.3, R Foundation for Statistical Computing, Vienna, Austria) with the software RStudio IDE (release 2023.06.0+421) and packages Tidyverse (Wickham *et al.*, 2019) and MultcompView (Graves *et al.*, 2024), to write and run R code were used.

3. Results

3.1 Effect of seed treatment on agronomic traits

In order to gain specific information regarding the effect on growth parameters by the treatments as such or during the interaction also with the pathogen, we have considered separately the analysis of the agronomic traits, in presence or not of the infection. The biostimulation effect induced by priming with pFE, T22 and pFE+T22 on wheat seeds treated, but not infected, is summarized in Fig. 1; Table 1. Dimension 1 in PCA plot (Dim1) accounted for 52.5% of the variance, dimension 2 (Dim2) accounted for 18%, dimension 3 (Dim3) accounted for 11.5%, and their sum explained 82% of total variance. Dim1 was principally driven by root length (RL), plant height (PH), shoot fresh weight (SFW), root fresh weight (RFW), shoot dry weight (SDW), root dry weight (RDW). Dim1 clearly separated the control 85(CTRL) from all the other treatments, because CTRL was placed in the negative quadrant and characterized by significantly lower values of all above mentioned parameters (Table 1). On the contrary, pFE was the only one positioned across the two quadrants because it was not positively associated with high values of RL (Table 1). Differently from Dim1, Dim2 showed strong connection with the percentage of germinated seeds (G), SPAD, seed germination index (SGI) and root dry matter (RDM), with a negative relation between SPAD and SGI with RDM (Fig. 1a). Dim2 clearly separated both treatments with pFE and pFE+T22 from control but also from T22. In fact, this latter was entirely placed in the positive quadrant, while CTRL was across the axis, and pFE and pFE+T22 were in the negative quadrant. As shown in Fig. 1b, Dim3 was principally driven by shoot dry matter (SDM) and SPAD, that were strongly related to each other since their vectors

were overlapped (Fig. 1b). Because of a positive association with its high values of SPAD and SDM, this latter being significantly different from the control, the pFE+T22 was the only treatment placed almost completely in the positive quadrant (Fig. 1b; Table 1). On the contrary, pFE was positioned on the negative quadrant of Dim3 due to the negative association with high values of both SPAD and SDM, although never significantly different from those of control (Fig. 1b; Table 1).

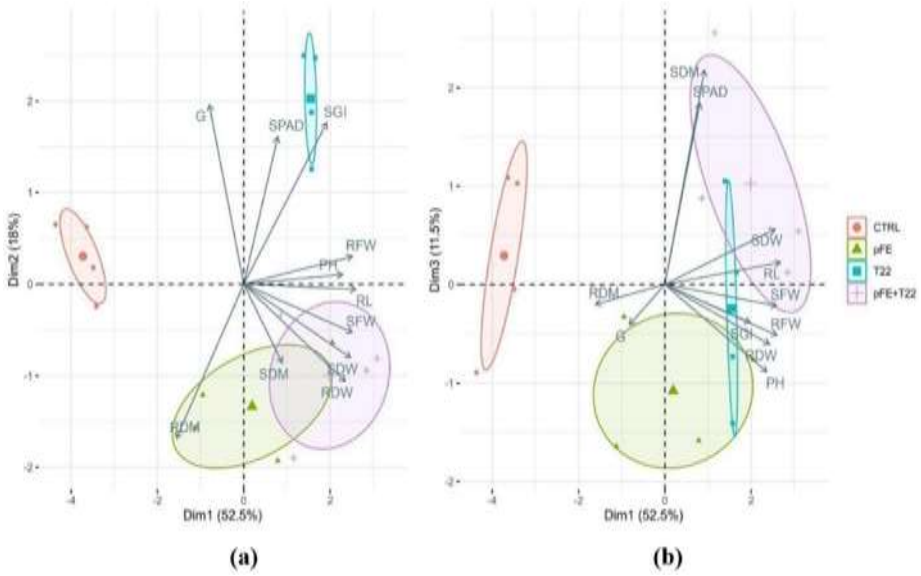


Fig. 1 Biplots for the principal component analysis (PCA) outputs for the effect on growth parameters in durum wheat (*Triticum durum* Desf. var *Simeto*) seedlings derived from: untreated seed (CTRL) or seeds primed with frass extract (pFE) and *T. afroharzianum* T22 (T22), alone or together (pFE + T22). **a**: biplots of dimension 1 (Dim1) and dimension 2 (Dim2); **b**: biplots of dimension 1 (Dim1) and dimension 3 (Dim3). G = percentage of germinated seed/ total sowed seeds (%), RL = radicle length (cm), SGI = seed germination index (%), PH = plant height (cm), SPAD = index of chlorophyll content in leaf, SFW = shoot fresh weight (g), RFW = root fresh weight (g), SDW = shoot dry weight (g), RDW = root dry weight (g), SDM = shoot dry matter (%), RDM = root dry matter (%).

Table 1 Effect on growth parameters in durum wheat (*Triticum durum* desf. Var *Simeto*) seedlings derived from: untreated seed (CTRL) or seeds primed with Frass extract (pFE) and *T. afroharzianum* T22 (T22), alone or together (pFE + T22)

Treatment	G (%)	SW *p*	RL (cm)	SW *p*	SGI (%)	SW *p*	PH (cm)	SW *p*	SPAD	SW *p*
CTRL	95.00 ± 10.00 a	0.082	9.27 ± 1.14 c	0.564	95.38 ± 7.29 c	0.929	25.23 ± 2.22 b	0.955	37.17 ± 1.96 a	0.373
pFE	75.00 ± 10.20 b	0.051	13.73 ± 1.43 b	0.557	112.56 ± 12.98 bc	0.299	29.72 ± 3.51 ab	0.507	33.25 ± 4.40 a	0.132
T22	100.00 ± 0.00 a	0.442	16.34 ± 1.81 ab	0.300	181.77 ± 16.38 a	0.602	30.41 ± 0.97 a	0.389	39.63 ± 4.12 a	0.256
pFE + T22	70.20 ± 11.58 b	0.355	18.08 ± 2.21 a	0.751	129.52 ± 15.91 b	0.828	30.31 ± 1.99 a	0.494	39.58 ± 1.53 a	0.073
Statistics	df = 3, 12; F = 10.40; $p < 0.01$*; BP $p = 0.183$		df = 3, 12; F = 20.43; $p < 0.01$*; BP $p = 0.183$		df = 3, 12; F = 30.02; $p < 0.01$*; BP $p = 0.183$		df = 3, 12; F = 4.44; $p = 0.03$*; BP $p = 0.183$		df = 3, 12; F = 3.38; $p = 0.05$; BP $p = 0.183$	

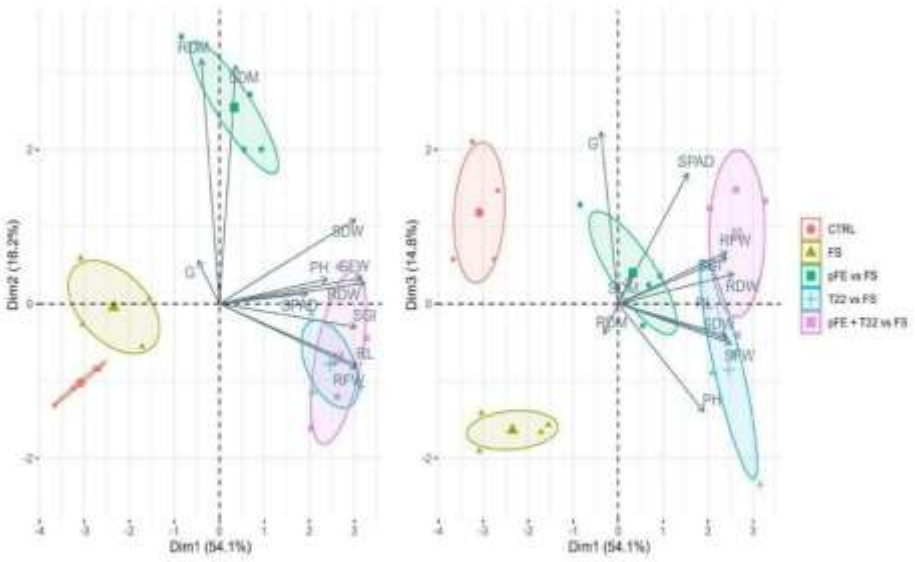
Treatment	SFW (g)	SW p	RFW (g)	SW p	SDW (g)	SW p	RDW (g)	SW p	SDM (%)	SW p	RDM (%)	SW p
CTRL	0.48 ± 0.05 b	0.785	0.11 ± 0.05 b	0.677	0.057 ± 0.007 b	0.246	0.017 ± 0.003 b	0.789	11.601 ± 0.445 b	0.287	18.00 ± 2.0 a	0.383
pFE	0.65 ± 0.08 a	0.304	0.34 ± 0.05 a	0.499	0.077 ± 0.01 a	0.436	0.051 ± 0.008 a	0.568	11.642 ± 0.382 b	0.397	17.00 ± 2.0 ab	0.107
T22	0.66 ± 0.06 a	0.571	0.41 ± 0.03 a	0.311	0.077 ± 0.005 a	0.885	0.041 ± 0.003 a	0.282	11.747 ± 1.112 ab	0.060	12.00 ± 2.0 b	0.943
pFE + T22	0.75 ± 0.12 a	0.118	0.34 ± 0.03 a	0.698	0.092 ± 0.011 a	0.385	0.048 ± 0.004 a	0.994	12.651 ± 1.132 a	0.086	16.00 ± 4.0 ab	0.578
Statistics	df = 3, 12; F = 7.10; $p < 0.01$*; BP $p = 0.183$		df = 3, 12; F = 39.68; $p < 0.01$*; BP $p = 0.183$		df = 3, 12; F = 11.15; $p < 0.01$*; BP $p = 0.183$		df = 3, 12; F = 35.61; $p < 0.01$*; BP $p = 0.183$		df = 3, 12; F = 4.49; $p = 0.02$*; BP $p = 0.183$		df = 3, 12; F = 4.86; $p = 0.02$*; BP $p = 0.183$	

G Percentage of germinated seeds/total sowed seeds, *RL* Radicle length, *SGI* Seed germination index, *PH* Plant height, *SPAD* Index of chlorophyll content in leaf, *SFW* Shoot fresh weight, *RFW* Root fresh weight, *SDW* Shoot dry weight, *RDW* Root dry weight, *SDM* Shoot dry matter, *RDM* Root dry matter.

Different letters indicate significant differences between values, according to one-way ANOVA followed by Tukey post-hoc test at $p < 0.05$.

Data are expressed as the mean of 4 replicates (each of 20 seeds) ± SD. df = degree of freedom (treatment, residuals), F = F statistic, *p* = *p value*, BP *p* = Breusch-Pagan *p value*, * = significance of the result, SW *p* = Shapiro-Wilk *p value*.

The effect on growth parameters induced by priming with pFE, T22 and pFE+T22 on wheat seeds when these were also infected by *F. sporotrichioides* (FS) is shown in Fig. 2; Table 2. Dim1 in PCA plot accounted for 54.1% of the variance, while Dim2 for 18.2%, and Dim3 for 14.8%, so that their sum explained 87.1% of total variance. Dim1 was principally driven by RL, SGI, and fresh and dry weights of both shoot and root. Dim1 clearly separated CTRL and also FS from all the other treatments, placing them in the negative quadrant because of their significantly lower values of all these parameters, except RL (in CTRL) and RFW, than values of treatments (Fig. 2a; Table 2). In particular, the treatment with pFE (pFE vs. FS) was across the axis of Dim1 due to the negative association with high values of RL and RFW, in fact not significantly different from those of CTRL and/or FS (Fig. 2a; Table 2). Meanwhile, when used in combination with T22, the frass extract (pFE+T22 vs. FS) showed significantly higher values of RL, RFW, SDW, and RDW with respect to the CTRL and FS (Table 2), so that it was completely placed, together with the treatment T22 vs. FS, in the positive quadrant (Fig. 2a; Table 2). Noteworthy, pFE vs. FS was the only treatment fully positioned in the upper side of Dim2 (Figs. 2a). This is because of its positive association with very high values of shoot and root dry matter (Fig. 2a; Table 2), that were the driver parameters for this dimension 2. Regarding the Dim3, driven by SPAD, G and PH, with a negative relation between these two latter, a significantly higher PH value for T22 vs. FS (Table 2) compared to CTRL, while lower G values for FS and T22 vs. FS, as well as lower SPAD values for FS in contrast to CTRL and/or to all the other treatments. These values determined the position, completely or almost completely, in the negative quadrant for FS and T22 vs. FS, respectively (Fig. 2b; Table 2).



(a) **(b)**

Fig. 2 Biplots for the principal component analysis (PCA) outputs for the effect on growth parameters in durum wheat (*Triticum durum* Desf. var *Simeto*) seedlings derived from: untreated seed (CTRL) or seeds primed with frass extract (pFE), *T. afroharzianum* T22 (T22), alone or together (pFE + T22) and infected with *F. sporotrichioides* (FS). **a**: biplots of dimension 1 (Dim1) and dimension 2 (Dim2); **b**: biplots of dimension 1 (Dim1) and dimension 3 (Dim3). G = percentage of germinated seeds/total sowed seeds (%), RL = radicle length (cm), SGI = seed germination index (%), PH = plant height (cm), SPAD = index of chlorophyll content in leaf, L = leaves number, SFW = shoot fresh weight (g), RFW = root fresh weight (g), SDW = shoot dry weight (g), RDW = root dry weight (g), SDM = shoot dry matter (%), RDM = root dry matter (%)

Table 2 Effect on growth parameters in durum wheat (*Triticum durum* desf. Var *Simeto*) seedlings derived from: untreated seed (CTRL), seeds infected with *F. sporotrichioides* (FS), and seeds infected and primed with Frass extract (pFE vs. FS) and *T. afroharzianum* T22 (T22 vs. FS), alone or together (pFE + T22 vs. FS)

Treatment	G (%)	SW *p*	RL (cm)	SW *p*	SGI (%)	SW *p*	PH (cm)	SW *p*	SPAD	SW *p*
CTRL	95.00 ± 10.00 a	0.153	12.10 ± 0.74 bc	0.564	102.30 ± 9.83 c	0.929	25.23 ± 2.22 b	0.955	37.17 ± 1.96 a	0.411
FS	75.00 ± 0.00 b	0.142	9.27 ± 1.14 c	0.949	95.38 ± 7.29 c	0.650	29.03 ± 1.40 ab	0.914	29.20 ± 2.46 b	0.354
pFE vs. FS	95.00 ± 10.00 a	0.109	12.99 ± 0.90 b	0.077	135.59 ± 10.74 b	0.101	29.28 ± 2.06 ab	0.716	38.60 ± 2.72 a	0.343
T22 vs. FS	75.00 ± 10.00 b	0.109	19.20 ± 2.88 a	0.582	155.60 ± 15.25 b	0.797	30.74 ± 2.07 a	0.381	39.00 ± 3.29 a	0.546
pFE + T22 vs. FS	95.00 ± 10.00 a	0.153	19.02 ± 1.36 a	0.859	199.87 ± 8.73 a	0.165	30.53 ± 1.41 a	0.926	40.03 ± 1.02 a	0.271
Statistics	df = 4, 15; F = 4.75; *p* = 0.01*; BP *p* = 0.468		df = 4, 15; F = 30.60; *p* < 0.01*; BP *p* = 0.691		df = 4, 15; F = 63.04; *p* < 0.01*; BP *p* = 0.668		df = 4, 15; F = 5.66; *p* = < 0.01*; BP *p* = 0.571		df = 4, 15; F = 13.10; *p* = < 0.01*; BP *p* = 0.913	

Treatment	SFW (g)	SW p	RFW (g)	SW p	SDW (g)	SW p	RDW (g)	SW p	SDM (%)	SW p	RDM (%)	SW p
CTRL	0.65 ± 0.06 c	0.785	0.11 ± 0.05 b	0.677	0.079 ± 0.007 b	0.246	0.017 ± 0.003 b	0.789	12.045 ± 0.366 b	0.326	18.00 ± 2.0 b	0.383
FS	0.48 ± 0.05 d	0.809	0.07 ± 0.03 b	0.513	0.057 ± 0.007 c	0.764	0.014 ± 0.005 b	0.578	11.601 ± 0.445 b	0.058	20.00 ± 4.0 b	0.941
pFE vs. FS	0.84 ± 0.11 b	0.837	0.14 ± 0.02 b	0.858	0.109 ± 0.013 a	0.839	0.034 ± 0.004 a	0.203	14.064 ± 1.037 a	0.808	31.00 ± 1.0 a	0.998
T22 vs. FS	1.01 ± 0.05 a	0.062	0.27 ± 0.03 a	0.515	0.112 ± 0.012 a	0.230	0.043 ± 0.004 a	0.522	12.210 ± 0.918 b	0.137	18.00 ± 2.0 b	0.862
pFE + T22 vs. FS	0.89 ± 0.05 ab	0.957	0.24 ± 0.03 a	0.689	0.118 ± 0.003 a	0.355	0.041 ± 0.007 a	0.797	11.762 ± 0.778 b	0.444	19.00 ± 2.0 b	0.765
Statistics	df = 4, 15; F = 38.22; *p* < 0.01*; BP *p* = 0.619		df = 4, 15; F = 24.10; *p* < 0.01*; BP *p* = 0.411		df = 4, 15; F = 33.63; *p* < 0.01*; BP *p* = 0.460		df = 4, 15; F = 31.37; *p* < 0.01*; BP *p* = 0.129		df = 4, 15; F = 6.93; *p* = < 0.01*; BP *p* = 0.510		df = 4, 15; F = 20.96; *p* = < 0.01*; BP *p* = 0.858	

G Percentage of germinated seeds/total sowed seeds, *RL* Radicle length, *SGI* Seed germination index, *PH* Plant height, *SPAD* Index of chlorophyll content in leaf, *SFW* Shoot fresh weight, *RFW* Root fresh weight, *SDW* Shoot dry weight, *RDW* Root dry weight, *SDM* Shoot dry matter, *RDM* Root dry matter.

Different letters indicate significant differences between values, according to one-way ANOVA followed by Tukey post-hoc test at $p < 0.05$.

Data are expressed as the mean of 4 replicates (each of 20 seeds) ± SD. df = degree of freedom (treatment, residuals), F = F statistic, *p* = *p value*, BP *p* = Breusch-Pagan *p value*, * = significance of the result, SW *p* = Shapiro-Wilk *p value*.

3.2 Control of damping-off (DO) induced by *F. sporotrichioides* in post-emergence wheat seedlings

The effect of priming on DO disease incidence due to *F. sporotrichioides* infection is summarized in Table 3. Seeds infection with the pathogen spores caused the highest DO in the positive control (FS), with 85% of diseased seedlings. The priming treatment with pFE reduced the DO by 31%, while T22 of 44%. The combination of pFE and T22 determined a reduction of the DO of 38%, not significantly different from that of pFE and T22 used alone.

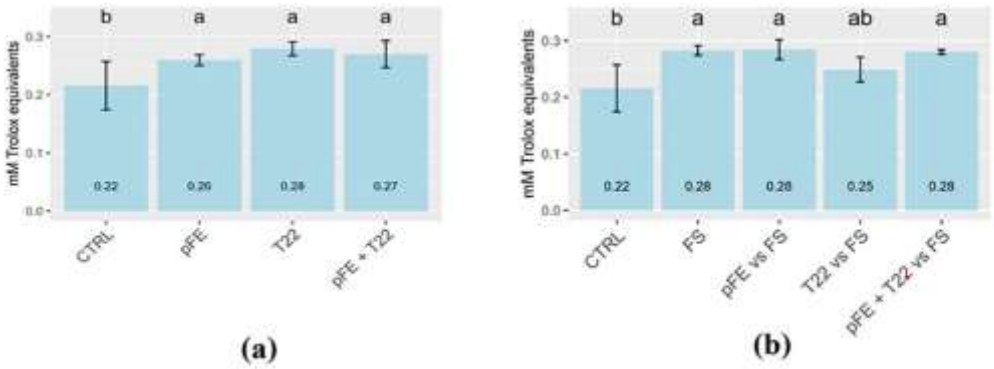
Treatment	DO (%)	SW *p*
FS	85.00 ± 4.08 a	0.458
pFE vs. FS	53.75 ± 4.79 b	0.383
T22 vs. FS	41.25 ± 2.50 c	0.126
pFE + T22 vs. FS	47.50 ± 2.89 bc	0.316
Statistics	df = 3, 12, F = 165.43, *p* = < 0.01*, BP *p* = 0.716	

Data are expressed as mean (n=4) ± SD. Different letters indicate significant differences between values, according to one-way ANOVA followed by Tukey post-hoc test at p.

Table 3 Damping-off (DO) incidence caused by *F. sporotrichioides* on wheat seedlings derived from: seed primed with Frass extract (pFE) and *T. afroharzianum* T22 (T22), alone or combined (pFE+T22) and infected with *F. sporotrichioides* (FSs summarized in Table 3. Seeds infection with the pathogen spores caused the highest DO in the positive control (FS).

3.3 Total antioxidant activity

The antioxidant activity is reported in Fig. 3. Priming treatment caused changes in the antioxidant system of the uninfected wheat seedlings, as shown in Fig. 3a. In particular, a significant increase of the antioxidant activity was found in all plantlets derived from treated seeds compared to control (CTRL). Meanwhile, also in the presence of *F. sporotrichioides* changes in the antioxidant system were induced (Fig. 3b). Indeed, seeds infected and primed resulted in a significant increase in the antioxidant activity with respect to CTRL, except for T22 vs. FS.



Treatment	SW *p*
CTRL	0.443
pFE vs FS	0.911
T22 vs FS	0.220
pFE + T22 vs FS	0.262
Statistics	df = 3, 12, F 0.= 6.02, $p < 0.01$*, Breusch Pagan $p = 0.087$

Treatment	SW *p*	SW *p* after *z* transformation
CTRL	0.653	0.292
FS	0.574	0.195
pFE vs FS	0.478	0.925
T22 vs FS	0.714	0.338
pFE + T22 vs FS	0.972	0.972
Statistics	df = 4, 15, F = 6.27, $p < 0.01$*, Breusch Pagan $p = 0.01$* (z transformed *p value* 0.835)	

Fig.3 Total antioxidant activity and statistics of wheat seedlings derived from seeds primed with pFE and T22, alone or combined **(a)**; seedlings derived from seeds primed with pFE and T22, alone or combined, and also infected by FS **(b)**. Bars indicate mean values (n = 4) ± SD significantly different according to one-way ANOVA followed by Tukey *post hoc* test ($p < 0.05$). df = degree of freedom (treatment, residuals), F = F statistic, p = *p value*, BP p = BreuschPagan *p value*, * = significance of the result, SW p = Shapiro-Wilk *p value.*

3.4 DNA extraction, PCR and sequencing

PCR reactions were successful yielding amplicons of expected sizes for each DNA barcode investigated. Nine nucleotide sequences (three/isolate and gene region) were obtained after direct sequencing. The BLASTn analysis showed a 99% similarity of the sequences obtained in this study to Paenibacillus polymyxa sequences already present in the NCBI database under accession numbers: ON329761.1, NR 114810.1 and NR112117.1 for the 16 S rRNA gene; CP109848.1, CP097770.3 and CP157284.1 for the gyrA gene and CP133768.1 and CP139198.1 for the rpoB gene. All nucleotide sequences obtained in this study were deposited in the NCBI GenBank database under the following accession numbers: PV162856 and PV162858 (16S RNA gene); PV893198, PV893199 and PV893200 (rpoB gene); PV893201, PV893202 and PV893203 (gyrA gene).

3.5 Phylogenetic analysis of 16 S rRNA, GyrA and RpoB genes

Phylogenetic trees of the three barcodes investigated (16 S rRNA, gyrA and rpoB) showed that the bacterial isolates obtained in this study grouped together with various strains of *P. polymyxa* from different sources and origins with 100% bootstrap support while closely related *B. subtilis* and *B. cereus* species, taken as outgroups, clustered separately, as expected (Fig. 4: a, b and c). Furthermore, the phylogenetic data allowed to identify all bacterial isolates as *P. polymyxa* based on 100% sequence identity (for all 16 S rRNA, gyrA and rpoB genes) with sequences of the same species already present in the GenBank. In particular, the *P. polymyxa* strains M2-1-N LWSANGYL (acc. no. ON329761.1), DSM36 (acc. no. NR114810.1) and IAM13419 (acc. no. NR112117.1) in case of the 16 S sRNA gene; the *P. polymyxa* strains K16 (acc. no. CP109848.1), R 4.5 (acc. no. CP09770.3) and F1 (acc. no. CP157284.1) for the gyrA gene and the *P. polymyxa* strains YT9 (acc. no. CP133768.1) and C2 (acc. no. CP139198.1) for the rpoB gene found in GenBank showed identical sequences to our isolates (Fig. 4a, b and c).

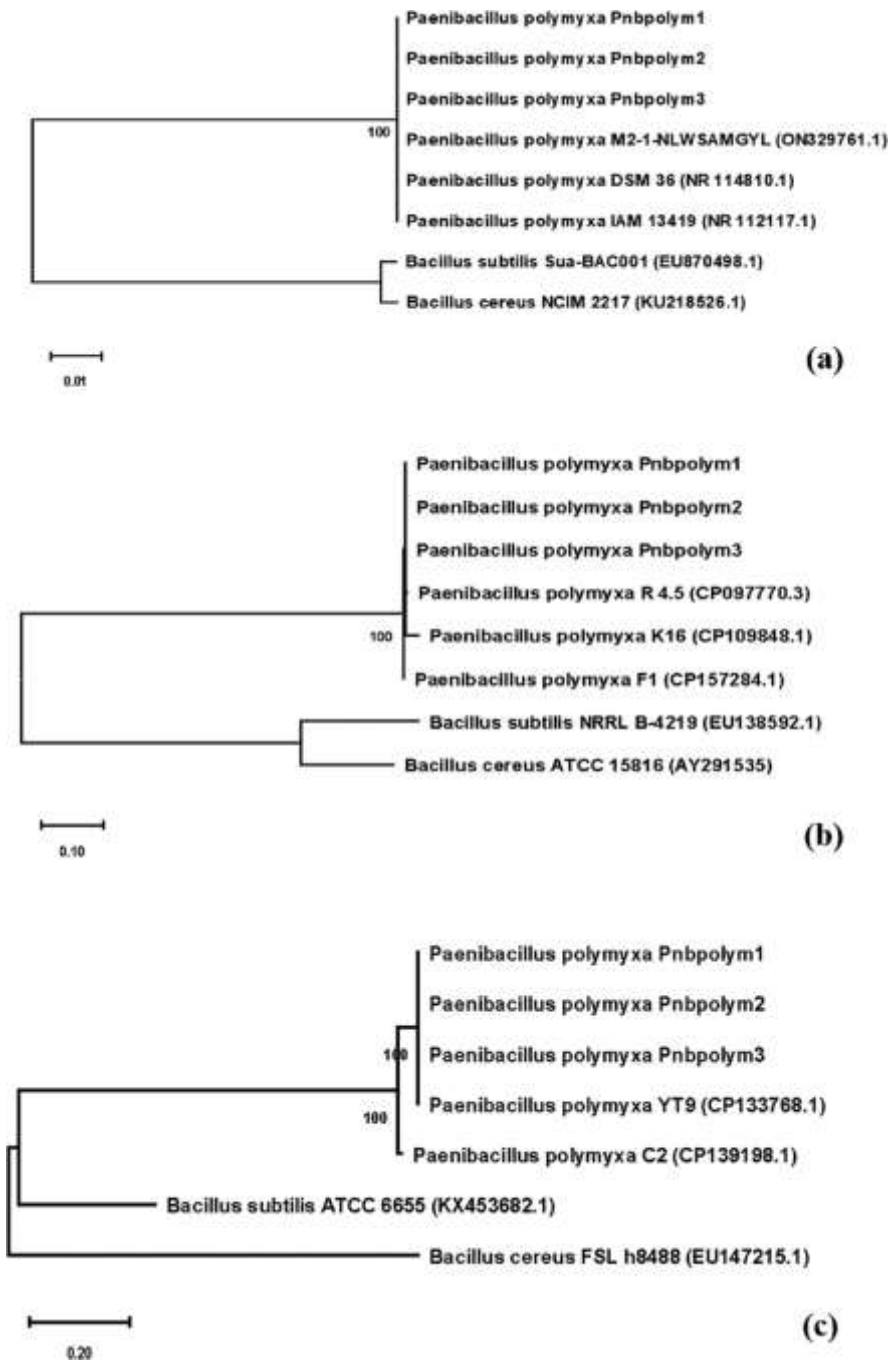


Fig. 4 Maximum Likelihood (ML) phylogenetic trees showing the relationship of partial 16 S rRNA (a), gyrA (b) and rpoB (c) barcodes of *P. polymyxa* isolates obtained in this study and other related species. Bootstrap values are indicated above the nodes. Bars 0.01, 0.10 and 0.20 represent substitutions per nucleotide site. *Bacillus subtilis* strain Sua-BAC001 (EU870498.1) and *B. cereus* strain NCIM2217 (KU218526.1) for the 16 S ribosomal RNA, *B. subtilis* strain NRRL B 4219 (EU138592.1) and *B. cereus* strain ATCC15816 (AY291535) for the gyrA along with *B. subtilis* strain ATCC6655 (KX453682.1) and *B. cereus* strain FLS h8488 (EU147215.1) for the rpoB gene were used as outgroups.

3.6 *In vitro* antagonistic activity induced by *P. polymyxa* isolated from frass extract against *B. cinerea*, *F. oxysporum* f. sp. *lycopersici* and *F. sporotrichioides*

The bacterial isolate of *P. polymyxa* (PP) found in pFE was assessed for its effects against the three phytopathogenic fungi, as summarized in Table 4. Mycelial growth was reduced by PP for all pathogenic fungi, showing the highest efficacy against BC and FOLYC (GI% of 32%) and a growth inhibition equal to 26% against FS.

Fungal species	DO (%)	SW *p*
BC	32.7 ± 3.5 a	0.477
FOLYC	32.4 ± 4.9 a	0.501
FS	26.4 ± 2.2 b	0.106
Statistics	df=2, 6, F=8.70, p=0.02, Bresch-Pagan p value=0.569	

Different letters indicate mean values (n=3) ±SD significantly different according to one-way ANOVA followed by Tukey post-hoc test ($p<0.05$).

Table 4 Percentage of mycelial growth Inhibition (GI%) of *B. cinerea* (BC), *F. oxysporum* f.sp. *Lycopersici* (FOLYC) and *F. sporotrichioides* (FS) induced by *P. polymyxa*.

4. Discussion

The farming of insects generates substantial amounts of by-products, like frass, that can be valorized and advantageously used to improve crop production thanks to its richness in macro- and micro-nutrients (European Commission, 2021; Baldacchino & Lamaj, 2025). Indeed, frass can be considered as an organic fertilizer after its pasteurization by a heat treatment at 70 °C for at least 1 h, according to EC Regulation No 2021/1925 to reduce safety risk and suppress foodborne pathogens, especially *Salmonella*, *Clostridium* and *Enterobacteriaceae* (Van Looveren *et al.*, 2021). Considering its content in microorganisms with potential plant growth-promoting activity (Romano *et al.*, 2022) and in chitin, that could act as defense elicitor, frass can be favorably used as biofertilizer, as well as to enhance plant resilience under biotic and abiotic stress conditions, also in wheat crop (Coviello *et al.*, 2024; Boudabbous *et al.*, 2023). In particular, some Mediterranean cultivars of durum wheat released after the Green Revolution, such as Simeto, are appreciated for several important characteristics (yield potential, grain quality, tolerance to heat and drought), but they are susceptible to *Fusarium* spp (Bochicchio *et al.*, 2022; Vitti *et al.*, 2022). In our previous research (Coviello *et al.*, 2024), a first evaluation

of frass extract as a possible sustainable tool for inducing biostimulant and/or antifungal activities against *Fusarium* spp. was carried out by preliminary *in vitro* test and in plate study on the same durum wheat cultivar used here (Simeto). The defined dilution of 10% pasteurized frass extract, obtained with a totally environmentally friendly method, resulted optimal for improving the growth performance and the seedling protection against *F. sporotrichioides* when used for seed priming, alone or combined with *T. afroharzianum* T22. For this reason, the same conditions were used in the current work, with the hope to gain further knowledge in the possible substitution of chemical fertilizers and/or pesticides in wheat production. In the present study, the effect of 10% pFE, alone or combined with T22, on potted wheat seedlings derived from primed seeds, was evaluated in terms of agronomic traits, reduction of damping-off due to *F. sporotrichioides*, and activity of the pool of antioxidant enzymes involved with the aim to further unravel the possible use of insect frass and valorize the economic feasibility and circularity of the insect-rearing industry (Safitri *et al.*, 2024). Regarding the agronomic traits, frass extract, both alone and combined with *T. afroharzianum*, also in presence of the *F. sporotrichioides* pathogen, positively influenced seedlings growth parameters. This is because all treatments determined significantly equal or higher values than untreated and not infected control (CTRL) and/ or infected positive control (FS) for all considered parameters, with the exception of percentage of germinated seed (G) for pFE, pFE+T22, and T22 vs. FS, and root dry matter (RDM) for T22 (see Tables 1 and 2). This was evident from the biplots for the principal component analysis (PCA) in the biostimulation trial (see Fig. 1), where CTRL was clearly separated from all treatments and placed in the negative quadrant of dimension 1 (Dim1), that was principally driven by root length (RL), plant height (PH), shoot and root fresh and dry weights (SFW, RFW, SDW and RDW), while pFE was placed across the two quadrants because it was not positively associated only with high values of RL (see Fig. 1; Table 1). This was probably due to the property of frass from *H. illucens* to be a slow-release nitrogen fertilizer, as already described by Beesigamukama *et al.* (2020) in a maize production. Moreover, Dim2 clearly separated both treatments with pFE (pFE and pFE+T22) from control, but also from T22, and was principally driven by G, SPAD, seed germination index (SGI) and RDM. Our results are in line with the study by Boudabbous *et al.* (2023) where potted plants of durum wheat (variety INRAT 100), germinated and grown in substrate fertilizer with BSFL frass, showed height, RDW and chlorophyll

indicators higher than those unfertilized. Meanwhile, the third dimension in the biplot (Dim3), principally driven by shoot dry matter (SDM) and SPAD, placed pFE completely on the negative quadrant due to the negative association with high values of both SPAD and SDM (lower values, but almost never significantly different from CTRL and/or other treatments, as shown in Table 1). This is probably because, as a slow-release nitrogen amendment, frass could promote in seedling a lower or slower accumulation of biomass in the epicotyl than hypocotyl (Beesigamukama *et al.*, 2020). On the other hand, SPAD and SDM resulted closely related because their relative loading vectors were almost overlapped in Dim 3 (see Fig. 2b). These parameters can be an expression of the nutritional status, being both influenced by nitrogen content in seedling (Kumar *et al.*, 2024). Interestingly, the combination with T22 resulted in a positioning of pFE+T22 almost completely in the positive quadrant of Dim3 (see Fig. 2b), suggesting that T22 added to pFE in the priming solution could be able to improve the nitrogen use efficiency by wheat seedlings (Visconti *et al.*, 2020). Safitri *et al.* (2024) advised that potential solutions to improve the efficacy of frass as fertilizer are to use it combined with earthworms, or nitrification inhibitors, or mineral nitrogen fertilizers to enhance the short-term recycling of nutrients, or prevent nitrite accumulation, or to compensate eventual deficiency of readily available nitrogen by frass itself. In such a way, an improved synchronization of nitrogen release could occur thanks to a good combination between the immobilization and rapid mineralization of nitrogen, resulting in an optimal plant growth (Dulaurent *et al.*, 2020; Beesigamukama *et al.*, 2021; Esteves *et al.*, 2022; Tanga *et al.*, 2022; Watson *et al.*, 2021). We can hypothesize that similar mechanisms could have been put in place when frass was used in combination with T22. *Trichoderma*-based biostimulants were already recognized as a tool to improve nitrogen use efficiency of leafy crops (Visconti *et al.*, 2020) and, therefore, T22 could be helpful for frass to improve the growth performance of seedling also in wheat. The seed infection with *F. sporotrichioides* resulted in seedlings with values for all growth parameters equal or lower with respect to the CTRL, as expected, so that FS was placed, together with CTRL, in the negative quadrant of Dim1, driven by RL, SGI, SFW, RFW, SDW, and RDW and of Dim 3, driven by SPAD, G and PH, in PCA biplots (see Table 2; Fig. 2). Noteworthy, the presence of pFE and T22, both alone and combined, seemed to restore the growth ability in infected seedlings because pFE vs. FS, T22 vs. FS, and pFE+T22 vs. FS showed significantly higher values of these parameters with respect to FS, except for G in

T22 vs. FS, PH for all thesis and RFW in pFE vs. FS (see Table 2). Effectively, this latter parameter resulted significantly equal also to CTRL, differently from what was observed during the biostimulation trial (see Table 1). This probably was due to a competition for nutrients, and following less water and nutrient uptake by pFE vs. FS, which may have established between the pathogen and the bacterium *P. polymyxa* isolated from frass extract (Romano *et al.*, 2022). As a consequence, pFE vs. FS was the only thesis definitely placed in the positive quadrant of Dim 2 (see Fig. 2a) because it was the only one to show the highest, and significantly different from all the other thesis, values of SDM and RDM (see Table 2). On the other hand, the presence of this beneficial bacterium in pFE could have elicited the seedling defense mechanisms based on the reinforcement of cell walls with insoluble compounds, such as synthesis of lignin, callose deposition, phenolic compounds production, in order to impede, reduce and/or prevent the penetration and spread of fungal hyphae (Chrpová *et al.*, 2021; Dixon & Paiva, 1995), as previously assessed (Coviello *et al.*, 2024). Indeed, other than to restore the growth ability in infected seedlings, the priming with pFE ensured the significant reduction of damping-off (DO) by 31% when used alone, and almost by 40% in co-presence with T22, so strengthening the belief that some microorganisms belonging to *Bacillus spp.* and *Trichoderma spp.* have the capacity to foster plant growth and/or defense against pathogens during their life cycle, starting from seed germination (Tsolakidou *et al.*, 2024). Therefore, seed priming with both pFE containing *P. polymyxa* and T22 showed to be effective, at low cost and allowed the precision targeting of the pathogen *F. sporotrichioides* in durum wheat Simeto. The reduction of DO found here coincides with that observed in the plate system from our first study (Coviello *et al.*, 2024), and it is even a little better for pFE vs. FS and pFE+T22 vs. FS (from 28% to 37% of plate system to 31% and 38% in soil, respectively). Furthermore, this DO reduction is in line with a study showing that the amendment with BSF larvae frass reduced of 30% the DO caused by Sclerotiniaminor in garden cress (Setti *et al.*, 2019), and of 40% the dead plants due to *Fusarium* wilt disease in cowpea (Quilliam *et al.*, 2020). In addition to the growth and antifungal performances, seed priming with pFE and T22, both alone and together, also led to changes in the antioxidant system. This activation of the antioxidant system in plants is responsible for triggering the synthesis of antioxidant compounds before the exposure to stress (Kasote *et al.*, 2015). This is the reason why higher total antioxidant activity was found in seedlings derived from primed seeds even in

absence of the pathogen (see Fig. 4a). The enhanced antioxidant activity allowed seedlings to face biotic stress encountered in the case of *F. sporotrichioides* infection, probably by neutralizing ROS (Ashraf *et al.*, 2019). Indeed, all seedlings derived from primed seed had enhanced total antioxidants, compared to those of CTRL, except for T22 vs. FS (see Fig. 4b). This lower antioxidant activity, similar to those of healthy seedlings, could be explained by the mycoparasitism action performed by T22 against *F. sporotrichioides*, that have determined the reduction of disease pressure, as also demonstrated by the highest DO reduction in T22 vs. FS (see Table 3) (Mukherjee *et al.*, 2012). In summary, also in presence of *F. sporotrichioides*, the induction of antioxidant production due to priming with pFE and T22, both alone and combined, led to an improved tolerance by seedling against the pathogen (Khaledi *et al.*, 2016). In our previous experiment, using the same pathosystem and priming technique, we had established the defensive role of the phenolics and of superoxide dismutase (SOD), during fungal infection, as nonenzymatic and enzymatic key compounds in the possible decomposition of the fungal hyphae cell wall or in ROS detoxification (Coviello *et al.*, 2024). In light of the interesting increased total antioxidant activity revealed here, for the first time, as seedling "alert" response thanks to seed priming with pFE, also when combined with T22, it surely will be fascinating to unravel, in future studies, the behavior of post-priming mechanisms as antifungal responses related to other antioxidant compounds counteracting ROS, such as catalase or peroxidases, in wheat under field conditions. To better determine to which component of pFE address the antifungal activity found here, the bacterial species *P. polymyxa*, originally isolated from pFE and accurately identified based on phylogenetic data of three-locus DNA barcode, was tested for its activity against several phytopathogenic fungi. This species was able to inhibit mycelial growth of *B. cinerea* and *F. oxysporum* f. sp. *lycopersici* of 32%, while the growth reduction of *F. sporotrichioides* was about 26%. The antifungal activity of *P. polymyxa* is due to its ability to compete for nutrients, produce various antibiotics and enzymes, like fusaricidin, chitinases and glucanases, and its capacity to induce systemic resistance (Lal & Tabacchioni, 2009). In agreement to our results, Zhang *et al.* (2021) reported a similar mycelial growth inhibition of *B. cinerea*, while a slight lighter inhibition was found against *F. oxysporum*. Noteworthy, the *in vitro* activity of *P. polymyxa* against *F. sporotrichioides* was slightly lower to that found *in vivo* (DO reduced of 31%), suggesting that *P. polymyxa* was able to inhibit fungal growth similarly to the whole

extract, as observed by Arabzadeh *et al.* (2023). The authors attributed the inhibition of fungal pathogens mycelial growth by a frass extract to the presence of microorganisms able to produce antifungal and anti-oomycetes compounds and, in particular, to the Bacillus veleziensis found in their extract. Further studies should be performed to verify *in vivo* the antifungal activity against *F. sporotrichioides* by *P. polymyxa* alone, considering that this bacterium, commonly found in soil, rhizosphere and plant tissue, is able to produce antimicrobial peptides, to solubilize insoluble phosphate, to produce indole acetic acid (IAA), as well as to degrade lignin, cellulose and hemicellulose (Weselowski *et al.*, 2016) efficiently used the combination of the three bacterial strains *P. polymyxa*, Bacillus amyloliquefaciens, and *B. subtilis* against *F. graminearum* in wheat. In agreement, the results of our study obtained from the combined use of frass, naturally containing *P. polymyxa*, and the biocontrol agent *T. afroharzianum* T22, demonstrated that this strategy can be employed for an effective control of *F. sporotrichioides* in wheat plants using the priming technique and, at the same time, was also able to promote plant growth to a greater extent.

5. Conclusion

This study provided evidence on the role of frass extract as seed priming, emphasizing its capacity to positively affect agronomic traits, antioxidant and antifungal properties of wheat starting from the seed germination stage. Indeed, the interaction between pFE and the pathogen *F. sporotrichioides*, also in presence of *T. afroharzianum* strain T22, endowed wheat seedlings with an improved resilience, preparing them to effectively challenge and tolerate the biotic stress induced by the fungal pathogen. The insights gathered from this research, confirmed the possibility to use frass in priming technique, opening the door to promising solutions to harness the potential of sustainable agricultural practices and circular economybased green technologies. Our findings support the use of natural compounds, as frass, in a cost-efficiency contest, considering the ability of *H. illucens* larvae to feed on organic by-products and waste. In such a way, it will be possible to reduce feed input costs and to offer a solution to managing organic waste, mitigating their impact on disposal and, at the same time, giving the wheat crop greater growth and defense performances. Although the present study positively evaluated the effectiveness of this strategy by moving from a plate system, studied during our first research, to a soil system, further studies will be necessary to confirm the performance of these

seedlings in the field and during different plant phenological stages, as well as to better define molecular and biochemical mechanisms responsible of the frass extract activities. Surely, the identification of the *P. polymyxa* as a biocontrol agent further strengthens the biological relevance of the frass extract and adds value to the sustainable aspect of this strategy.

CHAPTER 4

Selection and *in vitro* assessment of plant-growth promoting bacteria from Black Soldier Fly frass

What is reported in Chapter 4, concerning the evaluation of the presence of plant growth-promoting bacteria in *Hermetia illucens* frass, is under revision on "ACS Agricultural Science & Technology" journal as a scientific article entitled "Selection and *in vitro* assessment of plant-growth-promoting bacteria from Black Soldier Fly frass.

1. Introduction

In line with the bioconversion framework established in Chapters 1 and 2 insect farming focus primarily on producing proteins and lipids for use in food and feed through the bioconversion of organic substrates. Beyond their primary role, insect farms also produce valuable by-products, such as frass, a nutrient-rich residue that can be repurposed as a soil amendment or fertilizer. As established in Chapter 3, leveraging such by-products not only reduces organic waste but also enhances the overall economic and environmental sustainability of the insect farming business model (Niyonsaba *et al.*, 2021; Lomonaco *et al.*, 2024; Lomonaco *et al.*, 2025). Frass is defined by Regulation (EU) 2021/1925 as "a mixture of excrements derived from farmed insects, the feeding substrate, parts of farmed insects, dead eggs and with a content of dead farmed insects not exceeding 5% in volume and 3% in weight". These leftovers are the primary byproduct of the bioconversion process (Klammsteiner *et al.*, 2020) and have been shown to have beneficial effects on plants (Ferruzca-Campos *et al.*, 2023; Houben *et al.*, 2020; Menino *et al.*, 2021). To exploit these benefits for plants, Regulation (EU) 2021/1925 reports on the requirements for placing frass on the market as a fertilizer. Farmers are obliged to heat-treat the excrement at 70 °C for at least 60 minutes (European Commission, 2021), to ensure microbiological safety. Indeed, Van Looveren *et al.* (2021) demonstrated that heat treatment of frass can successfully lower the amount of unwanted microorganisms, guaranteeing compliance with the safety regulation specified by European Union standards. Although a heat treatment guarantees the elimination of pathogens, it may also prevent microbial activity that could change its suitability as a soil fertilizer (Praeg & Klammsteiner, 2024). Several studies have highlighted the presence of Plant-Growth Promoting Micro-organisms (PGPM) in insect frass, framing it as a valuable resource for crop fertilization. However, these beneficial microbes may be compromised during the mandatory heat treatment

(Poveda *et al.*, 2019; Lopes *et al.*, 2021; Fuhrmann *et al.*, 2022; Green, 2023). PGPM are a diverse group of microbes that inhabit the rhizosphere, the micro-environment surrounding the plant roots, and typically consist of arbuscular mycorrhizal fungus, rhizobia, and various plant growth-promoting bacteria (Acharya *et al.*, 2024). They enhance plant development through multiple mechanisms, such as regulating phytohormones synthesis, improving soil nutrient availability, or increasing resistance to infections. Consequently, PGPM might reduce the need for artificial fertilizers, mitigate the impact of biotic and abiotic stressors, and boost plant yields (Abhilash *et al.*, 2016; Asghari *et al.*, 2020; Etesami, 2020). As a biofertilizer, PGPM can enhance nutrient availability by solubilizing soil minerals such as potassium and phosphorus, fixing atmospheric nitrogen, and producing phytohormones including auxins, cytokinins and gibberellins (Lopes *et al.*, 2021). Plants can directly benefit from these phytohormones. For example, auxins regulate various physiological and developmental processes, such as root and shoot growth, cell expansion, vascular tissue differentiation, pathogen defence, and root colonization of microbes (Duca *et al.*, 2014; Duca *et al.*, 2018; Poveda and González-Andrés, 2021). Cytokinins play a crucial role in cell division, photosynthesis, chloroplast differentiation, control of leaf senescence (Wang *et al.*, 2019), and nutrient metabolism (Cortleven *et al.*, 2019). They also help maintain meristem activity, particularly in roots and shoots (Kurakawa *et al.*, 2007; Sang *et al.*, 2018). Gibberellins primarily promote shoot growth but also influence several other developmental processes. They can accelerate leaves and fruits senescence, stop seed dormancy to stimulate germination, and enhance stem growth (Lee *et al.*, 2015; Orozco-Mosqueda *et al.*, 2023). At lower concentrations, gibberellins can also encourage root growth (Tanimoto, 2012). Collectively, PGPM contribute to increased plant biomass, improved root development, greater plant height, enhanced seed germination and seedling vigor, higher chlorophyll content, increased photosynthetic rates, and expanded leaf area (Lopes *et al.*, 2021). Although several studies report beneficial effects of frass on various aspects of plant physiology, the outcomes clearly depend on factors such as frass composition, application rate, and treated plant species (Lomonaco *et al.*, 2024). At the same time, only a limited number of studies have investigated the actual presence of PGPM in frass or how their abundance influences the effectiveness of frass applications. Poveda *et al.* (2019) identified in the microbial community within *Tenebrio molitor* frass several genera of

rhizobacteria, including *Pseudomonas*, *Acinetobacter*, *Pantoea* and *Brevibacillus*, microbes known to promote plant growth through mechanisms such as auxin and gibberellin production, siderophores synthesis and pathogen suppression. Building on the evidence that BSF larval development is strictly dependent on the growth substrate (as detailed in Chapters 2 and 3), further research is needed to understand how these dynamics affect the frass from the bioconverter *Hermetia illucens* (black soldier fly, BSF). Previous studies have shown that the microbial community composition of the BSF frass varies with the larval diet (Wynants *et al.*, 2019; Gold *et al.*, 2020), suggesting that the presence and abundance of PGPM may also differ, potentially leading to variable effects on plant growth. However, empirical data on PGPM occurrence and their plant growth-promoting activities in BSF larval frass remain scarce. To address this knowledge gap between the presumed presence of PGPM and actual data, this study aimed to isolate PGPM from the frass of Black Soldier Fly larvae (BSFL) (Raman *et al.*, 2022), using a rhizosphere mimicking agar. In addition, the influence of thefeeding substrate, previously identified as a major driver of frass microbial composition (Gold *et al.*, 2018; Wynants *et al.*, 2019; Osimani *et al.*, 2021), on the presence and abundance of PGPM, was also explored. Frass derived from BSFL reared on ten different diets was collected as the starting material for the isolation of microbes capable of colonizing the rhizosphere. Additionally, the effect of the heat treatment (1h at 70°C) on the presence and abundance of these microbes was assessed. In the second part of the study, the isolated bacteria were screened *in vitro* for several plant growth promoting traits: (I) growth in the presence of humic acids, (II) phosphorus solubilization, (III) ammonia production, and (IV) synthesis of phytohormones (auxins and gibberellins). The most promising isolates with PGP activities were identified using 16S rRNA gene sequencing, and the six most promising isolates bacteria were evaluated in an *in vivo* plant trial using *Arabidopsis thaliana* to assess their effect on germination and growth.

2. Materials and methods

A schematic representation of the methods described in the following subsections is provided in Supplementary Figure 1.

2.1 BSFL rearing

The frass used for the experiments was derived from the BSF colony reared by Xflies s.r.l. (Potenza, Italy). For six days, freshly hatched larvae were fed with a

standard Gainesville diet (50% wheat bran, 20% maize meal, and 30% alpha alpha) (Hogsette, 1992). After 6 days, the six day old larvae (6-DOL) were transferred to, and fed with, ten different substrates consisting of vegetable by-products: i) peppers (P), ii) broccoli (B), iii) artichoke (A), iv) fennel (F), v) turnip greens (T), vi) eggplant (E), vii) olive pomace (OP), viii) whey + seeds (WS), ix) spent barley (SB) and x) Gainesville diet (DS) according to the composition reported in Table 1. During the experiment, the BSFL were kept in complete darkness at a temperature of 27 ± 1°C with 65 ± 2% relative humidity. Larvae were kept on these diets till 30% of the larvae reached the prepupal stage. At the end of the trial, larval frass from each diet was separated from the larvae by manual sieving using a 3 mm mesh. The collected frass was divided into two portions: one was heat-treated for one hour at 70°C (HT) in accordance with EU Regulation 2021/1925, while the other remained untreated (NT). Heat-treated samples were subsequently stored at 16°C until bacterial isolation and identification.

Table 1 Abbreviation and composition of the used diets in this study

Abbreviation	Composition	Abbreviation	Composition
P	35% peppers + 65% old bread	**E**	35% eggplant + 65% old bread
B	35% broccoli + 65% old bread	**OP**	35% olive pomace + 65% old bread
A	35% artichoke + 65% old bread	**WS**	43% sheep whey + 57% wheat seeds
F	35% fennel + 65% old bread	**SB**	100 % spent barley
T	35% turnip greens + 65% old bread	**DS**	30% gainesville diet + 70 % water

2.2 Diet preparation

The vegetable by-products (peppers, broccoli, artichoke, fennel, turnip greens and eggplant) were sourced from ARPOR (Scanzano Jonico, Matera, Italy), while olive pomace was obtained from F.lli PACE (Pietragalla, Potenza, Italy). Seeds and whey were provided by a farm in Muro Lucano (Potenza, Italy), and spent barley was sourced from BYKES BEER (Rivello, Potenza, Italy). The experiment diets consisted of a mixture of 35% vegetable substrate (peppers, broccoli, artichokes, fennel, turnip greens, eggplant or olive pomace) and 65% of surplus bread collected

from catering facilities of University of Basilicata, with a dry matter (DM) content of 22.0%. Bread was included to absorb excess moisture from vegetables, which could otherwise affect larval survival. Spent barley was used as received (DM = 22%), while the whey + seeds substrate consisted of 43% sheep whey and 57% wheat seeds in order to obtain a DM content of 22%.

The standard Gainesville diet (DM = 87.5%) was hydrated with water to achieve 70% of moisture and served as the control.

2.3 Isolation of bacteria from frass

For the isolation of plant-growth promoting bacteria in frass, Rhizosphere Mimicking Agar (RMA), composed of synthetic root exudates, recalcitrant organic carbon sources, and salts, was prepared based on the method described by Brescia *et al.* (2020) with minor modifications. Specifically, all synthetic root exudates were sterilized using a 0.2 µm filter before being added to the medium RMA. Cycloheximide (100 mg/L) was added to the RMA medium (Vasseur-Coronado *et al.*, 2021). The pH of the RMA was adjusted to 6.5 prior to the addition of agar at a final concentration of 1.6 % (w/v). A total of 3 g of frass was weighed and added to 27 mL of sterile saline solution (0.85% w/v NaCl) in sterile 50 mL tubes. The tubes were then shaken at 200 rpm for 1hour at room temperature. Following agitation, serial dilutions were prepared from 10^{-1} to 10^{-7}. Aliquots of 100 µL from dilutions ranging from 10^{-3} to 10^{-7} were plated for microbial isolation. The prepared plates were incubated at 27°C for 3 days. After 3 days, various colonies were randomly selected from different dilutions for all types of frass and both treatments. The selected bacteria were grown in Nutrient Broth at 27°C for one day and subsequently stored in 50% glycerol at -20°C and -80°C.

2.4 Determining *in vitro* plant-growth promoting (PGP) activities

2.4.1 Compatibility with humic acids

To assess compatibility with humic acids, the protocol reported by Vasseur-Coronado *et al.* (2021) was used with some modifications. The bacteria were grown in 5 mL of Nutrient Broth (Lab Lemco 1g/L + Yeast extract 2g/L + Peptone 5g/L + NaCl 5g/L) at 27°C for 24 hours with orbital shaking at 200 rpm. After 24 hours, bacterial cultures were centrifuged at 4000 rpm for 2 minutes, and the pellets were resuspended in NaCl (0.85% w/v) to achieve a final optical density at 600 nm (OD600) of 0.1. Subsequently, the R2A (VWR chemicals, Belgium) growth

medium was prepared with the addition of 0.003% humic acids (Sigma Aldrich, Switzerland). Once the plates were prepared (R2A + 0.003% humic acids), a volume of 5 µL was spotted at 3 points, and the test was repeated in triplicate. The plates were then incubated at 28°C for 2 days (Vasseur-Coronado *et al.*, 2021).

2.4.2 Phosphate solubilization

To assess the phosphate-solubilizing ability of the bacteria, Pikovskaya agar (Himedia, Germany) was used as growth medium. For this analysis, the bacteria were grown in 5 mL of Nutrient Broth (Lab Lemco 1g/L, Yeast extract 2g/L, Peptone 5g/L, NaCl 5g/L) at 27°C for 24 hours with orbital shaking at 200 rpm. After 24 hours, the bacterial cultures were centrifuged at 4000 rpm for 2 minutes, and the resulting pellets were resuspended in 0.85% (w/v) NaCl to achieve a final optical density at OD600 of 0.1. Once the plates were prepared, 5 µL of the bacterial suspension was spotted in three separate locations and the test was performed in triplicate. The plates were then incubated at 28°C for 2 days (Vasseur-Coronado *et al.*, 2021). The development of a halo around the bacterial colony indicates the bacterium's ability to solubilize phosphate.

2.4.3 Ammonia production

For ammonia production, bacteria were cultured in sterile 15 mL tubes containing 5 mL of Nutrient Broth (Lab Lemco 1g/L, Yeast extract 2g/L, Peptone 5g/L, NaCl 5g/L), incubated at 27°C on an orbital shaker at 200 rpm. Overnight cultures (50 µL, OD600 = 0.2) were inoculated into 5 mL of 1% peptone broth (HiMedia) and incubated at 37°C in a shaking incubator at 150 rpm for 48 hours. The cultures were then centrifuged at 3000 rpm for 5 minutes. For quantitative ammonia estimation, 1 mL of Nessler’s reagent (Chemlab, Belgium) was added to 0.2 mL of cell-free supernatant, followed by 8.5 mL of distilled water. The spectrophotometric reading with Thermo Scientific Genesys 10S UV-Vis (Thermo Fisher Scientific, Waltham, MA, USA) was immediately taken at 450 nm. Uninoculated 1% peptone broth mixed with 8.5 mL of distilled water and 1 mL Nessler’s reagent was used as blank (Akoijam & Joshi 2023). The ammonia concentration was determined using a standard curve of ammonium sulfate with concentrations in the range of 0.6-8 µmol/mL.

2.4.4 Indole-3-acetic acid production

For indole-3-acetic acid (IAA) production, bacteria were grown in sterile 15 mL tubes containing 5 mL of Nutrient Broth (Lab Lemco 1g/L, Yeast extract 2g/L,

Peptone 5g/L, NaCl 5g/L) and incubated at 27°C on a shaker set to 200 rpm. A 50 µL overnight culture with an OD600 of 0.2 was transferred into 5 mL of LB broth (prepared by mixing 10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl in 1 L of distilled water, adjusting the pH to 7.0 using 1 N NaOH, and sterilizing at 120°C for 25 minutes), supplemented with 0.1% tryptophan. The cultures were incubated for 3 days at 30°C (Kapadia *et al.*, 2022). Following incubation, the cultures were centrifuged at 3000 rpm for 5 minutes, and 2 mL of the supernatant was collected. This was combined with 4 mL of Salkowski's reagent (50 mL of 35% perchloric acid and 1 mL 0.5 M $FeCl_3$ solution) (Jayaprakashvel *et al.*, 2014) and incubated for 10 minutes (Kapadia *et al.*, 2022, Lahsini *et al.*, 2022). A blank was prepared by mixing 2 mL of uninoculated LB broth containing tryptophan with 4 mL of Salkowski's reagent. The colour intensity was measured using a spectrophotometer at 535 nm (Thermo Fisher Scientific, Waltham, MA, USA), and the IAA production for each sample was calculated using a standard IAA curve with concentrations ranging from 1 to 100 µg/mL.

2.4.5 Gibberellin production

For Gibberellin production, bacteria were grown in sterile 15 mL tubes containing 5 mL of Nutrient Broth (Lab Lemco 1g/L, Yeast extract 2g/L, Peptone 5g/L, NaCl 5g/L) and incubated at 27°C on a shaker set to 200 rpm. A 50 µL overnight culture with an OD600 of 0.2 was transferred into 20 mL of Nutrient Broth (NB, Oxoid) at 27 °C on an orbital shaker (200 rpm) for 5 days. After the incubation period, the tubes were centrifuged at 4000 rpm for 5 minutes, 15 ml of supernatant was taken and placed in a new tube. In addition to 15 ml of supernatant we added 2 ml of zinc acetate reagent (21.9 g zinc acetate + 1 ml of glacial acetic acid and volume was made up to 100 ml with distilled water) and after 2 minutes, we added 2 ml of potassium ferrocyanide (10.6% in distilled water). After that, it was centrifuged at low speed (2000 rpm) for 15 minutes. 5 ml of supernatant and 5 ml of 30 %HCI was added, and mixture was incubated at 20° C for 75 min (Sharma *et al.*, 2018). For blank 5 ml of 30 % HCl was used. Absorbance was read at 254 nm (Thermo Fisher Scientific, Waltham, MA, USA) concentration of gibberellins was calculated by preparing standard curve by using gibberellic acid (GA3, Hi-media) as standard (100-1000 µg/ml).

2.5 Isolate identification using 16S rRNA gene sequencing

Bacterial strains exhibiting beneficial properties were identified. Selected isolates were grown overnight on Plate Count Agar (PCA) from the stock culture collection. From each plate, one individual colony was suspended in 20 µl milli-Q water and the genomic DNA from each isolate was released through cell lysis by boiling. Next, the bacterial 16S ribosomal RNA region was amplified using the primers 27F (5′-AGA GTT TGA TCM TGG CTC AG-3′) and 1492R (5′-CTA CGG CTA CCT TGT TAC GA-3′), as previously described by Gorrens *et al.* (2021). PCR was carried out with a DreamTaq DNA polymerase (Thermo Scientific) according to the manufacturer's protocol. The PCR program was as follows: initial denaturation of 3 min at 95 °C, followed by 30 cycles of 30s at 95 °C, 30s at 50 °C, 2 min at 72 °C, and a final extension step of 10 min at 72 °C. A negative control for which the DNA was replaced by sterile milli-Q water was included in each PCR run. Specificity of the 16 rRNA gene amplification was checked using gel electrophoresis and if a single band was observed, the PCR product was purified using the GeneJet PCR purification kit. The obtained PCR products were sent for Sanger Sequencing at Eurofins Genomics, after which the obtained sequences were identified using blastn.

2.6 Inoculation of Arabidopsis seeds with promising bacteria

From the identified isolates, the six bacteria with the best *in vitro* PGP characteristics were selected to analyse their effects on the plant species *Arabidopsis thaliana.* For the *in vivo* assay on *Arabidopsis thaliana*, bacteria were grown in LB medium at 28°C for 24 hours with shaking at 200 rpm. The seeds were surface sterilized using 70% ethanol for 2 minutes, followed by 5% hypochlorite for 2 minutes, and then washed five times with sterile bi-distilled water. Subsequently, the seeds were immersed in the bacterial solution (OD 0.3) for 1.5 hours under orbital agitation (Giannelli *et al.*, 2022). The seeds were then collected and placed on agar medium composed of a standard solution (Tocquin *et al.*, 2003) with the addition of 1% agar. To assess germination and root elongation, the plates were placed vertically in a growth chamber The growth conditions were: 23°C/18°C light/dark temperature; light/dark photoperiod of 16/8 hours; photosynthetically active radiation of 130 µmol m^{-2} s^{-1}, and a relative humidity (RH) of 70%). Germination was monitored every 12 hours from the beginning of the assay, by counting the number of germinated seeds for each treatment. After 5 days, the

seedlings were collected to measure root length, stem length, and the number of root hairs per mm of root length with ImageJ 1.54 d software.

2.7 Identification of the bacterial community in the frass samples using 16S rRNA gene sequencing

From each of the ten BSF frass types, three replicate samples of approximately 250 mg were taken and subjected to DNA extraction using the E.Z.N.A. ® Soil DNA Kit (Omega Bio-Tek, Norcross, GA, USA) following the manufacturer's instructions. Concentration and purity of the DNA was verified using a Nanodrop device (ND-1000, Isogen Life Science, Utrecht, the Netherlands) before the samples were sent to Novogene Bioinformatics Technology Co., Ltd. for sequencing of the V4 region of the 16S rRNA gene using barcoded primers 515F (5'-GTG CCA GCM GCC GCG GTA A-3') and 806R (5'-GGA CTA CHV GGG TWT CTA AT-3'). The PCR procedure and bioinformatic analysis of the raw sequencing data is described in detail in Van Looveren *et al.* (2024). In short, paired-end 16S rRNA gene V4 region amplicon sequencing were merged and filtered before clustering. The UNOISE algorithm was used to cluster sequences into zero-radius operational taxonomic units (zOTUs), followed by taxonomic classification using the SINTAX algorithm, using a bootstrap confidence value of 0.80. The reads were further filtered by removing the reads with less than 0.1% abundance in each sample. Differences in bacterial communities among frass types were evaluated using the alpha diversity indices Chao1 and Shannon's diversity index. Beta diversity estimates based on Hellinger-transformed Bray-Curtis distances were analysed using a permutational analysis of variance (PERMANOVA) and visualized in a Non-metric Multidimensional Scaling (NMDS) plot, using the phyloseq and MicroViz packages in R version 4.3.0. The mean relative abundance of the bacterial community was further visualized in a dot plot, using the ggplot2 package in R.

2.8 Statistical analysis

For the six best bacteria the measurement was carried out three times, while for the plant growth parameters, measurements were carried out ten times. The normality of the data was assessed using the Shapiro-Wilks test. Data were analysed by one-way Anova and Tukey's *post-hoc* test at $P = 0.05$. Statistical analyses were performed using a GraphPad Prism version 6.0.0 for Windows (GraphPad Software, San Diego, California USA).

3. Results

3.1 Determining the load of microbes capable of growth in the rhizosphere

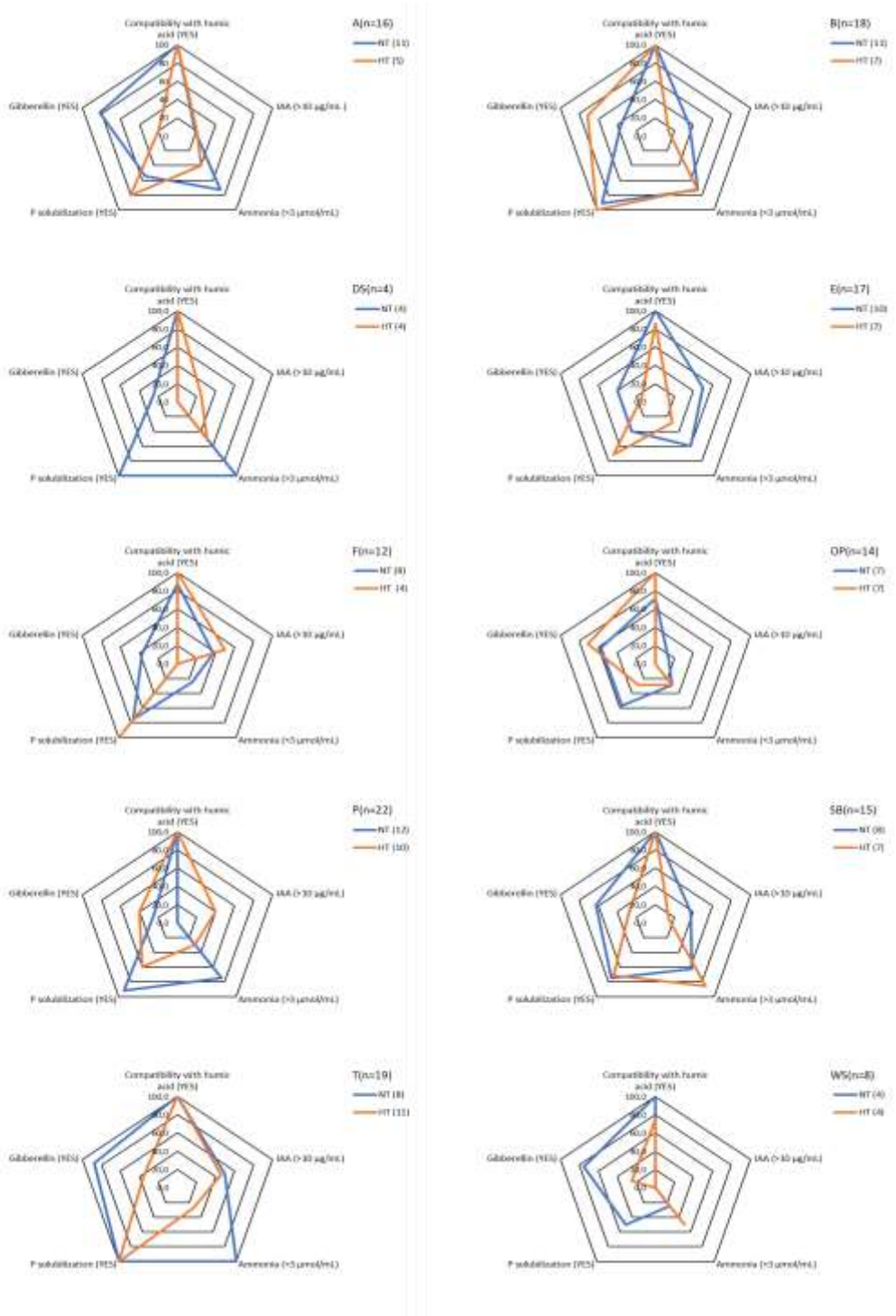
Plating the different types of frass on a rhizosphere mimicking agar (RMA) revealed a high number of potential bacteria (between 4,.57 log CFU/g frass and 8.08 log CFU/g frass) from frass in which this number clearly varied depending on the frass type, with untreated OP frass outperforming all other types (Supplementary Figure 2). Interestingly the effect of a heat treatment on this number was rather limited with 6.23 log CFU/g frass still persisting in the heat-treated samples. Specifically, the rhizobacterial population decreased only slightly from 6.52 log CFU/g in the untreated frass to 6.23 log CFU/g after treatment, corresponding to a retention of approximately 95.6% of the rhizobacteria. These values represent the average across all tested frass types. This suggests that the applied heat treatment had a minimal impact on the overall survival of the rhizobacteria.

3.2 Isolation and Assessment of potential plant-growth promoting Bacterial Strains

The total colony-forming units (CFU) number was quantified for each frass type. Using the same RMA agar, 149 bacterial isolates were randomly selected from the larger pool of colonies that developed (Supplementary Figure 3), originating from the 10 different types of frass (being either heat treated or not) for further testing. These 149 isolates were subjected to *in vitro* screening of PGP activities (Figure 1). The assessed parameters for the different bacterial isolates were: (I) compatibility with humic acids, (II) phosphate solubilization ability, (III) ammonia production, and (IV) the synthesis of IAA and gibberellins. For certain parameters, such as compatibility with humic acids, phosphate solubilization ability, and gibberellin production, the assessment was qualitative, determining whether the bacteria had this ability or not. In contrast, ammonia and IAA production were quantified. Ammonia production was then scored within a range of > or < 3 µmol/mL, while IAA production was evaluated within a range of > or < 10 µg/mL. An overview of the results obtained for all tested bacteria for the different types of frass can be found in Figure 1. For each type of frass, the same graph also compares the results for bacteria isolated from heat treated (HT, orange) and not treated (NT, blue) frass. Important differences were observed between HT and NT of the same frass typology. In all frass types, humic acid compatibility consistently reached 100% for

both treated and untreated isolates, this might indicate that this parameter was not affected by heat treatment but is more likely the result of already an initial selection for isolates with a tolerance to humic acids due to their presence in the RMA. Phosphorus solubilisation showed variability between different frass types and treatments. A, B, P, WS, DS, OP and SB frass showed consistently more isolates in NT frass than in HT frass, which suggests that heat treatment reduced the number of phosphorus-solubilising bacteria. In E and F frass, more isolates capable of phosphor solubilisation were present in the HT frass than in NT frass, while in T frass all selected bacteria were able to solubilise phosphate. The presence of isolates able to produce ammonia also differed between different frass types. For example, in samples A, B, DS, E, F, P and T, NT frass showed a higher prevalence of isolates with higher ammonia production than HT frass, whereas in samples WS and SB, the opposite was observed. Additionally, a general trend towards reduced IAA production in the isolates was observed in the treated frass samples. This was particularly evident in samples A, B, E, OP, and SB, where bacteria isolated from NT exhibited higher IAA production compared to those isolated from HT frass. However, in certain cases, such as DS, P, F, and T, the values were relatively similar, suggesting that some IAA-producing bacterial strains might be more resistant to heat treatment. Furthermore, all bacteria isolated from both T- and NT-WS frass produced IAA at levels below 10 µg/mL. A notable enrichment in IAA-producing strains was observed in frass E and SB, particularly in non-treated conditions. There was also a large variability in the ability to produce gibberellins. In some frass types, such as A, T, DS, E, F, WS and SB, a heat treatment appeared to reduce the number of bacteria capable of producing gibberellins. In other frass samples, such as B, OP and P, most of the bacteria selected after HT were able to produce gibberellins when compared with bacteria selected from NT frass. Ammonia production was relatively higher in bacteria isolated in frass B, OP, and T, though it decreased in heat-treated samples. One of the important observations is that heat treatment tended to reduce the occurrence of most PGP traits, including IAA and gibberellin production, phosphate solubilization, and ammonia release, suggesting a detrimental impact on beneficial microbial functions. However, no full reduction was observed either, indicating a significant fraction of PGPM’s are able to survive controlled heat treatments.

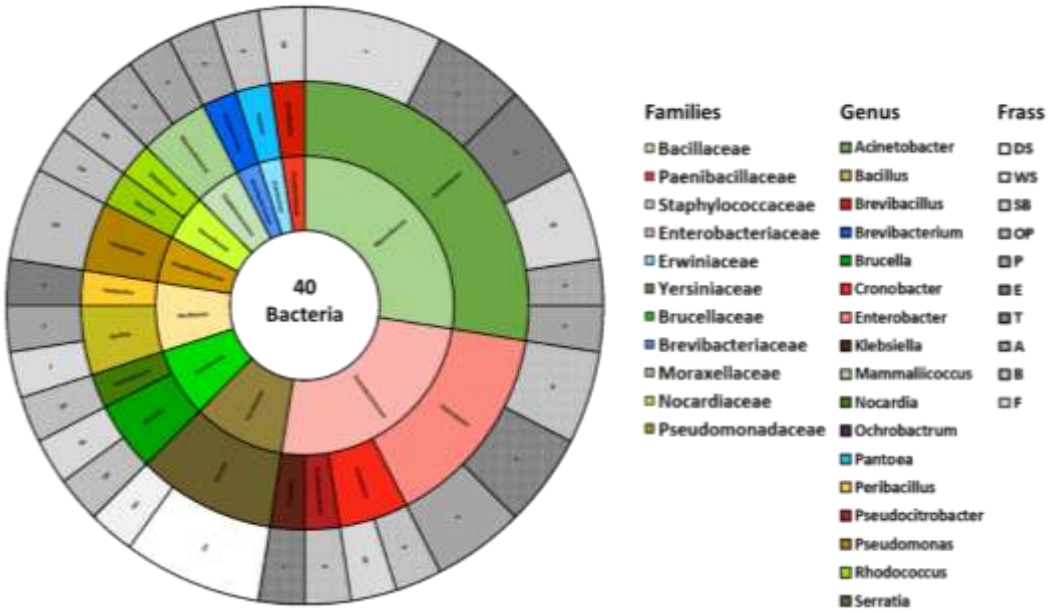
Figure 1 Characteristics of PGPR bacteria strains isolated from different frass types: peppers (P), broccoli (B), artichokes (A), fennel (F), turnip greens (T), eggplant (E), olive pomace (OP), seeds + whey (WS), spent barley (SB) and Gainesville diet (DS). For each frass type, isolates were obtained from both heat-treated (HT; treated at 70 °C) and non-treated (NT) samples, n indicates the total number of bacterial isolates screened per frass type, and percentages represent the proportion of isolates exhibiting a specific PGP trait within each group.



3.3 Sequencing results of the best performing bacteria isolated from treated and untreated frass

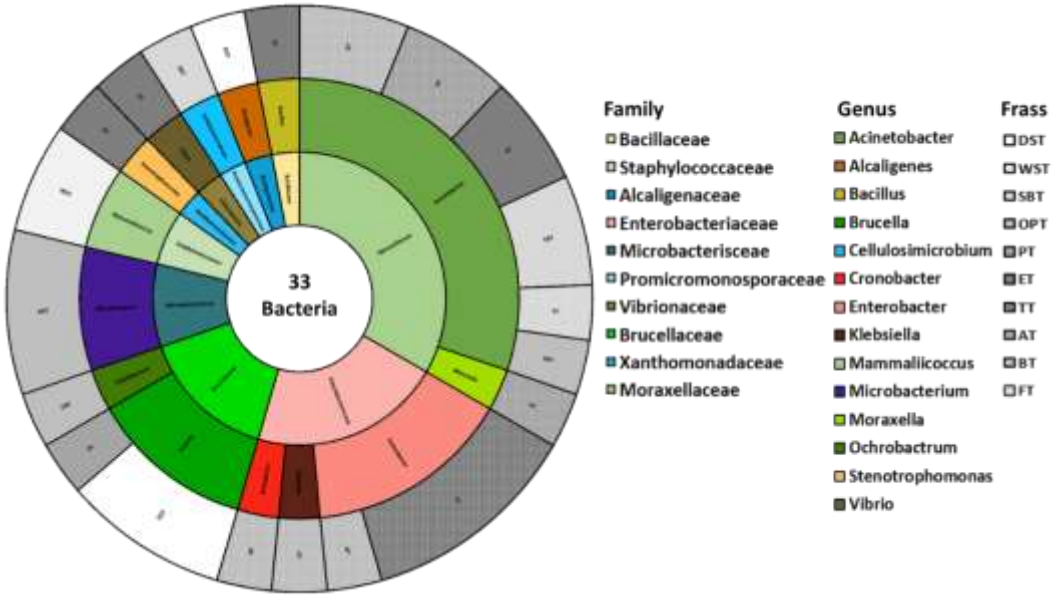
Out of the 149 bacterial isolates, 73 isolates were identified using full 16S rRNA gene sequencing. Because the colonies were randomly selected for this preliminary functional screening, these identified isolates represent only the subset showing relevant PGP activities and do not provide a comprehensive or quantitative characterization of the frass microbiota. Their selection was based on their overall performance across all evaluated PGP parameters, including indole-3-acetic acid (IAA) production, ammonia production, phosphate solubilization and gibberellin production. Strains showing the highest activity in one or more of these assays were retained for molecular identification and results are depicted for the isolates from the thermally untreated (Figure 2) and treated (Figure 3) frass separately. On both figures the bacterial family, genus, and the type of frass from which they were isolated is also depicted. For the non-treated frass (Figure 2), the most abundant bacterial family is Moraxellaceae, of which *Acinetobacter* is the sole genus identified. This genus was found across several types of frass, including F, T, E, SB, A, and P. The second most represented bacterial family is Enterobacteriaceae, which includes four distinct genera: *Enterobacter, Cronobacter, Pseudocitrobacter, and Klebsiella.* Interestingly, bacteria belonging to the genera *Enterobacter*, *Cronobacter* and *Pseudocytobacter* have been selected from the B frass. The third most representative family is Yersiniaceae, represented by the genus *Serratia*. Other families of bacteria have been isolated but their presence varies depending on the type of frass.

Figure 2 Overview of the identification for the 40 bacterial isolates retrieved from untreated frass at family-level (inner circle) and genus-level (middle circle), the outer circle depicts the type of frass they have been isolated from.



Looking at the heat-treated frass samples (Figure 3), the two most identified families remain the Moraxellaceae, Enterobacteriaceae. While the Yersiniaceae do not seem to survive the heat treatment, the Brucellaceae do and become the third most abundant family. For the family Moraxellaceae, in addition to the genus *Acinetobacter*, the genus *Moraxella* was also identified in bacteria isolated from AT frass. In the family *Enterobacteriaceae* there are no bacteria belonging to the genus *Pseudocitrobacter*, compared to the results of the NT frass. The genus *Brucella* and *Ochrobactrum* are the two genera belonging to the family Brucellaceae, and these were isolated from several frass types such as DST, PT and OPT.

Figure 3 Families (inner circle) and genera (middle circle) of the 33 bacterial isolates identified and isolated from different types of TREATED FRASS (outer circle).



As observed from the comparison between Figures 2 and 3, the genera most commonly found in NT frass were also present in HT frass., including *Bacillus*, *Acinetobacter*, *Mammalicoccus*, *Cronobacter*, *Enterobacter*, *Klebsiella*, and *Brucella*.

This indicates that the HT applied to the frass did not have important effects on these bacterial groups. Some families were present in the NT frass but not in the HT samples, such as Paenibacillaceae, Yersiniaceae, Brevibacteriaceae, Nocardiaceae, and Pseudobacteriaceae. Conversely, in the HT frass, certain bacterial families were identified also if they were absent in the NT samples, including Alcaligenaceae, Microbacterisceae, Promicromonosporaceae, and Xanthomonadaceae.

3.4 Characteristics of the six selected bacterial isolates with the most potent PGP activity

The six bacteria selected between those having the most potent rhizobacterial characteristics and their ability to grow in the presence of humic acids, solubilize phosphate, and produce gibberellins are depicted in Table 2.

As a reference for comparison, the rhizobacterium *P. polymyxa* was included, as it is documented in literature for its excellent rhizobacterial properties.[45] The selected

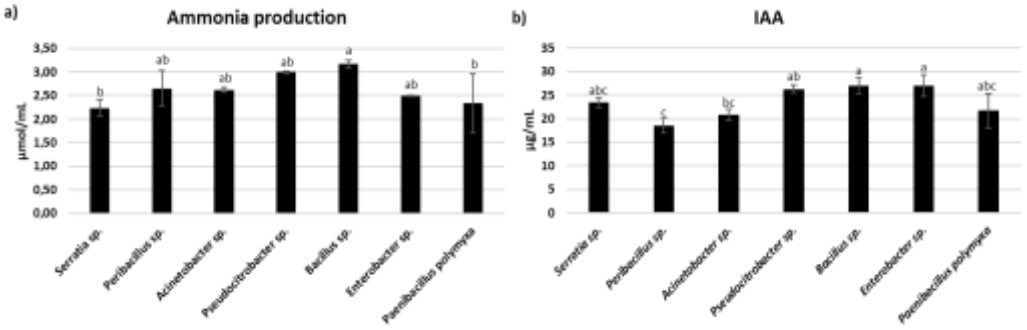
bacteria *Acinetobacter* sp. and *Bacillus* sp. had the broadest activity within the evaluated parameters. *Serratia* sp., *Peribacillus* sp., and *Enterobacter* sp. are unable to produce gibberellins but demonstrate high compatibility with humic acids and the ability to solubilize phosphate. Conversely, *Pseudocitrobacter* sp. lacks phosphate-solubilizing capacity but is capable of producing gibberellins and growing in the presence of humic acids. The control strain *P. polymyxa* did not exhibit phosphate-solubilizing ability, which differs from what has been previously reported in the literature.

Table 2. The six selected bacterial strains, chosen based on their high performance in preliminary screening for plant growth-promoting traits. Compatibility with humic acids: (-) Not able to grow in the presence of humic acids; (+) Able to grow in the presence of humic acids. Phosphate solubilization: (-) Absence of solubilization halo; (+) Presence of solubilization halo. Gibberellin production: (-) Not able to produce gibberellins; (+) Able to produce gibberellins. Data are results of three independent experiments.

Bacteria	Compatibility with humic acids	Phosphate solubilization	Gibberellin production
Serratia sp.	+	+	-
Peribacillus sp.	+	+	-
Acinetobacter sp.	+	+	+
Pseudocitrobacter sp.	+	-	+
Bacillus sp.	+	+	+
Enterobacter sp.	+	+	-
Paenibacillus polymyxa	+	-	-

Next, the initial screening for ammonia and auxin production was assayed to evaluate variability for these six isolates (Figure 4). In absolute terms, *Bacillus* sp. exhibited the highest ammonia production (Figure 4a), showing a significant difference compared to *P. polymyxa* and *Serratia* sp. However, no significant differences were observed among the other selected bacterial strains. The *Bacillus* sp. also exhibited the highest IAA production, together with the *Enterobacter* sp. (Figure 4b), showing a significant difference compared to *Peribacillus* sp. and *Acinetobacter* sp.

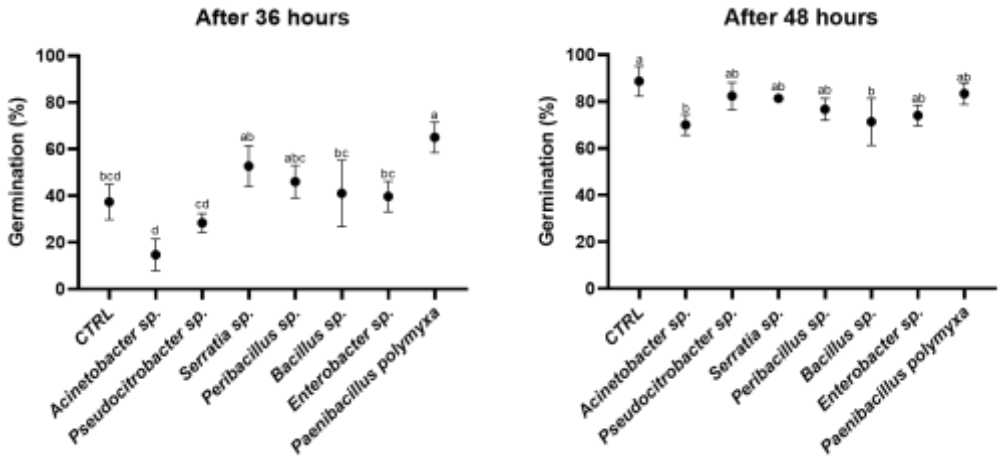
Figure 4. Ammonia (a) and indole-3-acetic acid (IAA) production for the six selected bacterial strains (b). Different letters designate significantly different values. Data are presented as the mean of replicates (N=3) ± SD (Standard Deviation) significantly different according to one-way ANOVA followed by Tukey post-hoc test ($p < 0.05$)



3.5 Effects of on germination and early plant growth of *Arabidopsis thaliana*

To evaluate whether these *in vitro* tests accurately predict an effect on plant growth, the impact of the selected isolates on the germination process of *Arabidopsis thaliana* seeds was explored (Supplementary Figure 4), with germination assessments conducted every 12 hours after seed placement on the plates. Differences in seed germination were evident after 36 and 48 hours. After that period, no differences between treatments were observed anymore. Seeds treated with *P. polymyxa* exhibited one of the highest germination percentages after 36 hours (Figure 5). However, this was only statistically significant when compared to the CTRL and the other isolates, with the exception of *Serratia* sp. and *Peribacillus* sp., which showed comparable germination rates. After 48 hours, no significant enhancement was observed compared to the CTRL; nevertheless, *P. polymyxa* still maintained a significantly higher germination rate compared to *Acinetobacter* sp. and *Bacillus* sp.

Figure 5. Effect of germination of seeds inoculated with select strains after 36 hours and 48 hours. Different letters designate significantly different values. Data are presented as the mean of replicates (N=3) ± SD (Standard Deviation) significantly different according to one-way ANOVA followed by Tukey post-hoc test ($p < 0.05$)



Not only germination was assessed, after five days, three more parameters were evaluated: stem length, root length, and the number of root hairs per mm of root length (Figure 6a, 6b and 7 respectively). Notably, for the stem length (Figure 6a) the control treatment (bacterial growth medium) only showed a significant difference compared to the *P. polymyxa* treatment, while no significant differences were observed in comparison to all other treatments. For the root length on the other hand, a significant reduction in root elongation was observed in plants treated with four bacterial isolates: *Acinetobacter sp., Pseudocitrobacter sp., Enterobacter sp., and P. polymyxa*, when compared to the untreated control. This suggests that these strains may exert inhibitory effects on early root development under the tested conditions. In contrast, no statistically significant differences in root length were found between the control and the other bacterial treatments, indicating that the remaining isolates did not negatively impact root elongation. These results highlight the variable influence of different bacterial strains on root growth dynamics and emphasize the importance of strain-specific screening when evaluating plant-microbe interactions.

Finally, interesting differences were observed in the number of root hairs per mm of root length (Figure 7). To illustrate this, a microscopic image of the control condition versus the *Acinetobacter sp*. inoculated treatment is depicted in Figure 8. Treatments that resulted in reduced root length tended to have a higher number of

root hairs, compared to the control treatment. This was the case for *Acinetobacter* sp. and *Pseudocitrobacter* sp., which, despite not promoting root length, induced a notable increase in root hair formation. Similarly, *Enterobacter* sp. and *Paenibacillus polymyxa* also showed high root hair density. These findings suggest that isolates promoting root hair development regardless of their effect on elongation may be promising candidates for enhancing nutrient and water uptake in plants and thus hold agronomic potential in the long term. On the other hand, two bacteria that did not reduce root length had an increased number of hair roots compared to the control, being *Peribacillus* sp. and *Bacillus* sp. Indeed, the control exhibited a significantly lower number of root hairs compared to all treatments except for *Serratia* sp. The treatments that showed the highest number of root hairs in absolute value were *P. polymyxa* and *Enterobacter* sp.

Figure 6. Stem length (a) and root length (b)measured after 5 days. Different letters designate significantly different values. Data are presented as the mean of replicates (N=10) ± SD (Standard Deviation) significantly different according to one-way ANOVA followed by Tukey post-hoc test ($p < 0.05$).

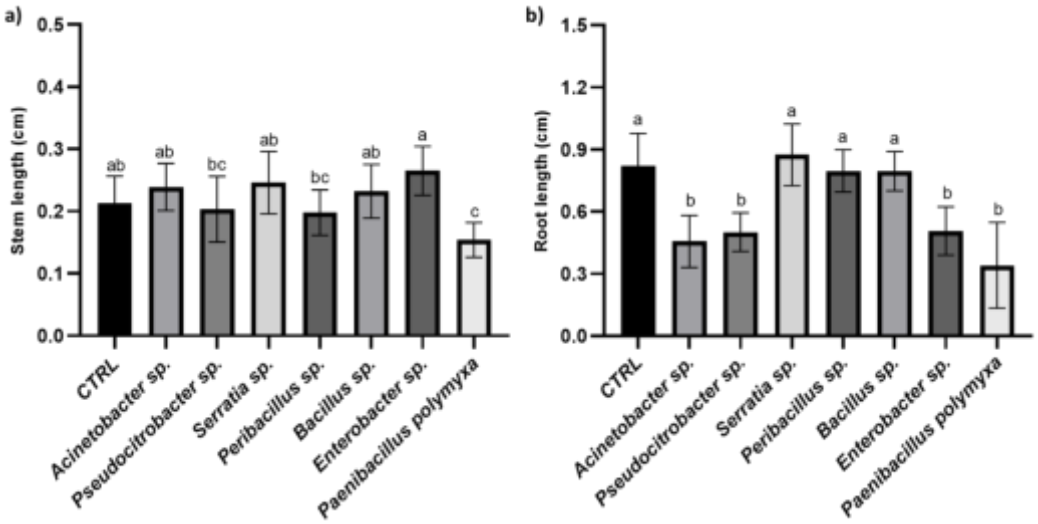


Figure 7. N° hairs root/mm. Different letters designate significantly different values. Data are presented as the mean of replicates (N=10) ± SD (Standard Deviation) significantly different according to one-way ANOVA followed by Tukey post-hoc test ($p < 0.05$).

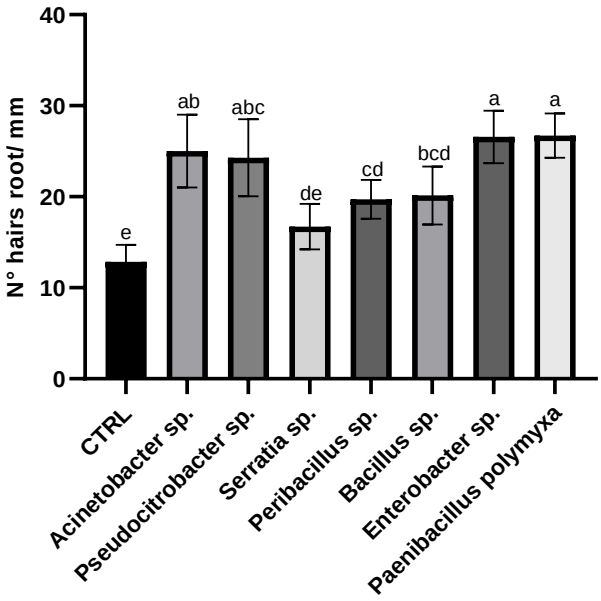
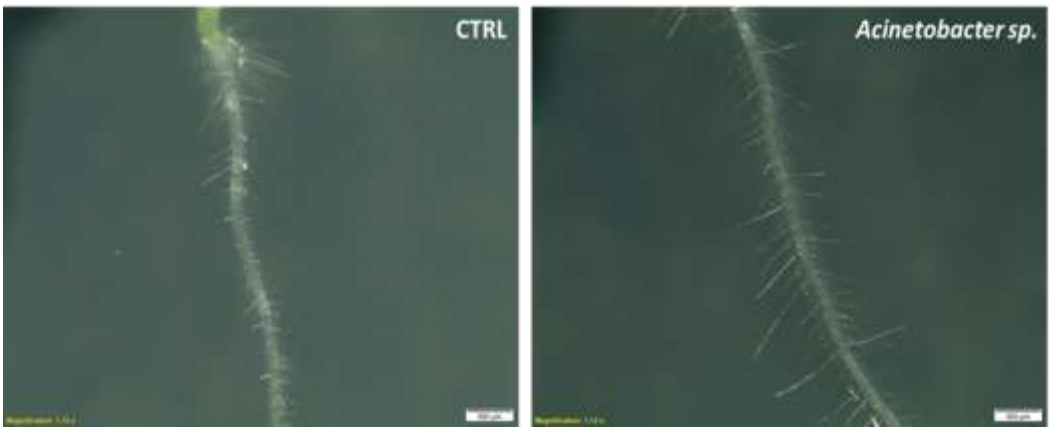


Figure 8. Microscopic photo of root hairs. Comparison of the Control (CTRL) and *Acinetobacter sp.*



3.6 Determining the presence/abundance of the six most promising isolates in the different frass types

To get a sense of how abundant and widespread these promising isolates are across the frass originating from the different feeds, the complete bacterial community of the ten frass types was analysed through sequencing of the V4 region of the 16S rRNA gene. PERMANOVA analysis revealed significant differences in bacterial community composition among frass types ($R^2 = 0.980$, $p = 0.001$), although some frass types exhibited greater similarity than others (Figure 9). For example, frass types E and F exhibited relatively low species richness and evenness (Figure 10) and were dominated by zOTU3 (28.9% and 23.1%) and zOTU5 (13.22% and 10.0%), respectively (Figure 11, Table 3). On contrast, frass types DS and SB displayed high species diversity and were characterized by the presence of zOTU18 (7.8% and 3.4%) and zOTU19 (8.4% and 2.6%), which were absent in the other frass types. Finally, frass types B and T exhibited similar bacterial composition, with zOTU1 comprising the largest proportion of the microbiome (26.6% and 24.8%), followed by zOTU2 (16.3% and 15.0%, respectively). Next, the short reads zOTU were identified using a NCBI nucleotide BLAST analysis, using the generated full 16S rRNA gene sequences of the six high performing plant-promoting bacteria as references (section 3.2). For five zOTUs, the short reads of the V4 had a 100% match with one of the reference bacteria, and it can therefore be assumed that these zOTUs represent the six selected bacterial strains of interest in the frass samples (Supplementary Table 2). zOTU673 had the highest match with the *Peribacillus* sp. strain (98.4%) match, however this zOTU673 contained nearly no reads and was removed during the filtering step. The relative abundance of the five remaining zOTUs for each of the three repetitions in the ten different types of frass is shown in Figure 12. Interestingly, *Enterobacter* sp. (zOTU2) was found in most frass types (A, B, E, F, OP and T), although the relative abundance was different in each group. Four of the high-performance bacteria (*Enterobacter* sp., *Acinetobacter* sp. (zOTU34), *Serratia* sp. (zOTU46) and *Pseudocitrobacter* sp. (zOTU162)) were found in all B frass samples. Frass types P and WS did not contain any of the bacteria of interest, and only one type of beneficial bacteria was found in the DS, SB and F frass.

Figure 9. Ordination plot of the ten different frass types. Non-metric multidimensional scaling (NMDS) of the 30 frass samples is based on the Bray-Curtis dissimilarities of the Hellinger-transformed relative abundances data. Colours indicate the different frass types and the stress value of the NMDS ordination is shown in the upper-right corner.

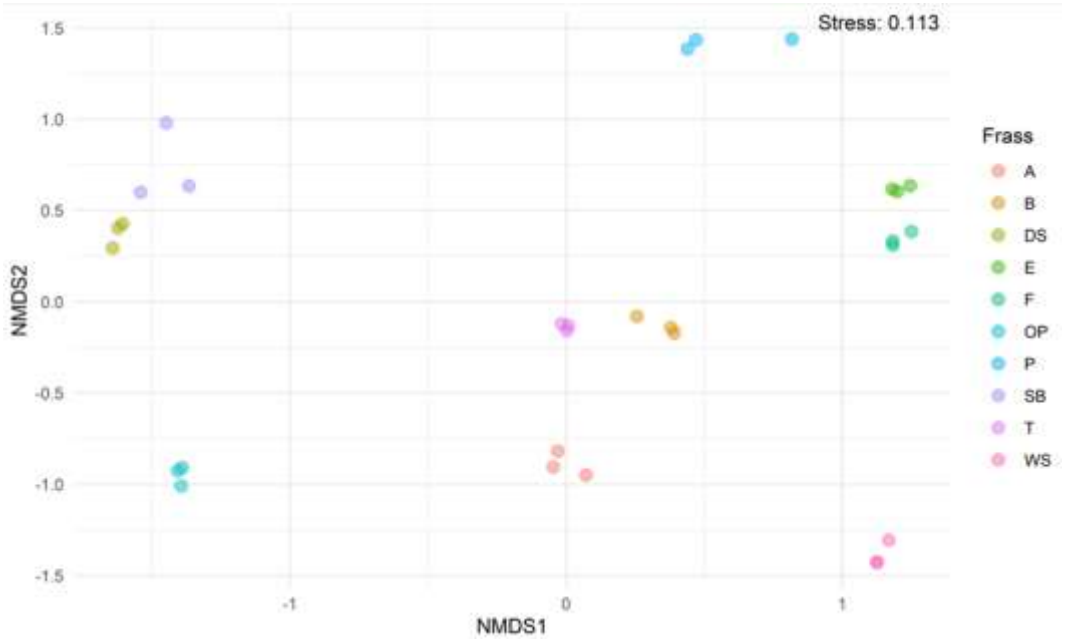


Figure 10. Alpha diversity matrices Chao1 and Shannon Diversity index for the ten different frass types. Colours indicate the different frass types.

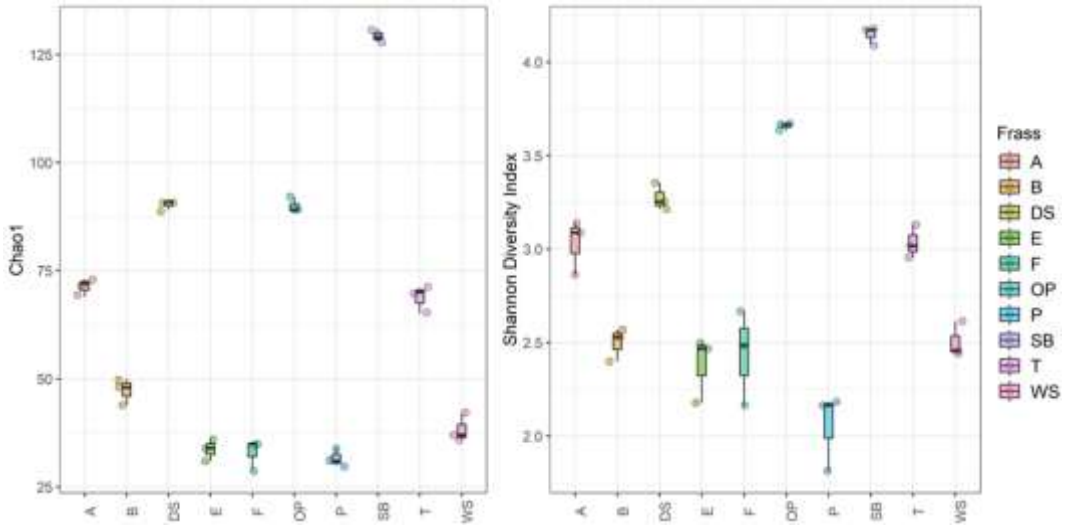


Figure 11. Mean relative abundance of the zOTUs per frass type. Size of the dots represent the relative abundance of each of the three frass sample replicates, for each different frass type. Taxa were identified at the genus level or, when not possible, at the highest resolved taxonomic level. Only taxa with a relative abundance >5% in at least one frass type are shown (n = 28). All remaining zOTUs with <5% relative abundance were grouped under 'Other'.

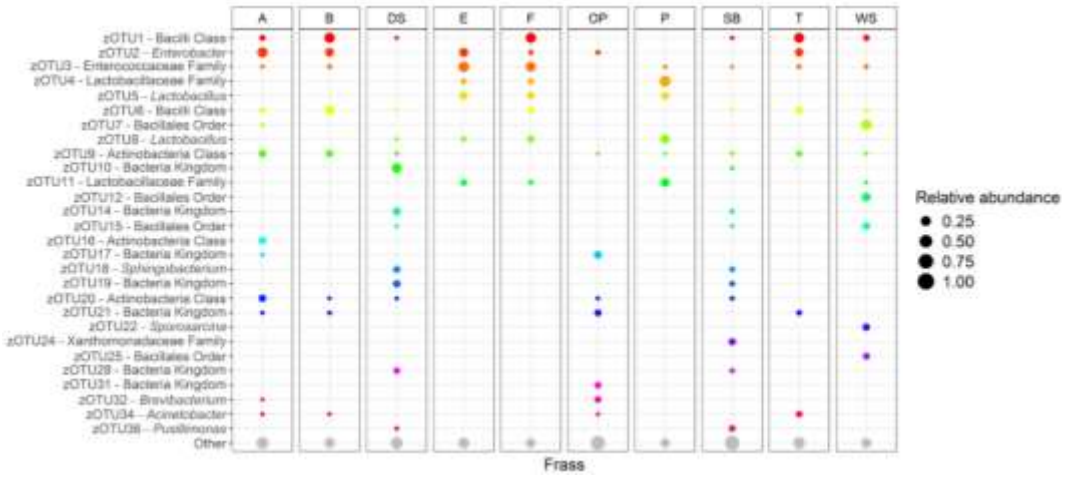


Figure 12. Relative abundance of the zOTUs corresponding to the bacterial isolates with plant-promoting growth. Size of the dots represent the relative abundance of each of the three frass sample replicates, for each different frass type.

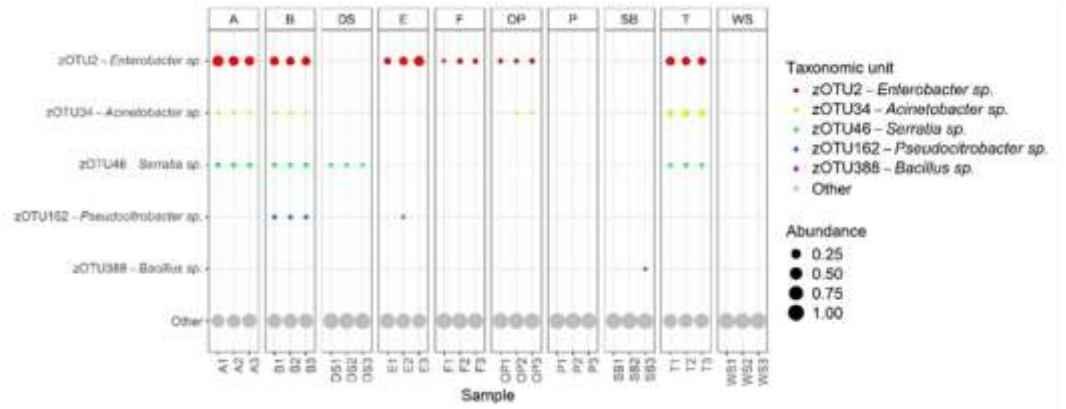


Table 3 Mean relative abundance per frass type. For each frass type (column 2 to 11), the relative abundance of three replicate samples (n = 3) was averaged. Taxa were identified at the genus level or, when not possible, at the highest resolved taxonomic level. Only taxa with a relative abundance >5% in at least one frass type are shown (n = 28). All remaining zOTUs with <5% relative abundance were grouped under 'Others'.

Bacteria	A	B	DS	E	F	OP	P	SB	T	WS
zOTU1 - Bacilli Class	4.0%	26.6%	0.1%	0.0%	25.9%	0.0%	0.0%	0.5%	24.8%	4.6%
zOTU2 - *Enterobacter*	25.6%	16.3%	0.0%	17.6%	1.7%	1.6%	0.0%	0.0%	15.0%	0.0%
zOTU3 - Enterococcaceae Family	0.3%	1.4%	0.0%	28.9%	23.1%	0.0%	0.1%	0.0%	1.3%	1.4%
zOTU4 - Lactobacillaceae Family	0.0%	0.0%	0.0%	1.9%	2.7%	0.0%	32.6%	0.0%	0.0%	0.0%
zOTU5 - *Lactobacillus*	0.0%	0.0%	0.0%	13.2%	10.0%	0.0%	12.8%	0.0%	0.0%	0.0%
zOTU6 - Bacilli Class	1.8%	18.8%	0.0%	0.0%	4.7%	0.0%	0.0%	0.0%	7.8%	0.4%
zOTU7 - Bacillales Order	0.1%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	27.4%
zOTU8 - *Lactobacillus*	0.0%	0.0%	0.2%	2.2%	7.8%	0.0%	17.1%	0.0%	0.0%	0.0%
zOTU9 - Actinobacteria Class	9.1%	9.0%	0.5%	0.0%	0.0%	0.0%	0.0%	0.2%	4.1%	0.0%
zOTU10 - Bacteria Kingdom	0.0%	0.0%	22.7%	0.0%	0.0%	0.0%	0.0%	0.3%	0.0%	0.0%
zOTU11 - Lactobacillaceae Family	0.0%	0.0%	0.0%	5.7%	1.6%	0.0%	18.1%	0.0%	0.0%	0.0%
zOTU12 - Bacillales Order	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	16.4%
zOTU14 - Bacteria Kingdom	0.0%	0.0%	11.5%	0.0%	0.0%	0.0%	0.0%	1.5%	0.0%	0.0%
zOTU15 - Bacillales Order	0.0%	0.0%	0.4%	0.0%	0.0%	0.0%	0.0%	0.7%	0.0%	9.8%
zOTU16 - Actinobacteria Class	10.8%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
zOTU17 - Bacteria Kingdom	0.1%	0.0%	0.0%	0.0%	0.0%	11.5%	0.0%	0.0%	0.0%	0.0%
zOTU18 - Sphingobacterium	0.0%	0.0%	7.8%	0.0%	0.0%	0.0%	0.0%	3.4%	0.0%	0.0%
zOTU19 - Bacteria Kingdom	0.0%	0.0%	8.4%	0.0%	0.0%	0.0%	0.0%	2.6%	0.0%	0.0%
zOTU20 - Actinobacteria Class	8.8%	0.2%	0.4%	0.0%	0.0%	0.5%	0.0%	1.0%	0.0%	0.0%
zOTU21 - Bacteria Kingdom	0.3%	0.7%	0.0%	0.0%	0.0%	6.9%	0.0%	0.0%	2.5%	0.0%
zOTU22 - *Sporosarcina*	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	8.6%
zOTU24 - Xanthomonadaceae Family	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	8.3%	0.0%	0.0%
zOTU25 - Bacillales Order	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	6.9%
zOTU28 - Bacteria Kingdom	0.0%	0.0%	5.3%	0.0%	0.0%	0.0%	0.0%	2.0%	0.0%	0.0%
zOTU31 - Bacteria Kingdom	0.0%	0.0%	0.0%	0.0%	0.0%	7.0%	0.0%	0.0%	0.0%	0.0%
zOTU32 - *Brevibacterium*	0.2%	0.0%	0.0%	0.0%	0.0%	6.3%	0.0%	0.0%	0.0%	0.0%
zOTU34 - *Acinetobacter*	0.4%	0.2%	0.0%	0.0%	0.0%	0.2%	0.0%	0.0%	5.9%	0.0%
zOTU36 - *Pusillimonas*	0.0%	0.0%	0.6%	0.0%	0.0%	0.0%	0.0%	5.4%	0.0%	0.0%
Other (< 5%)	38.5%	26.6%	42.0%	30.5%	22.6%	66.0%	19.4%	74.1%	38.6%	24.4%

4. Discussion

Frass is not a homogeneous product; its composition and quality are strongly influenced by the feed substrates utilized throughout the rearing (Klammsteiner *et al.*, 2020a), as well as by post-processing treatments applied for hygienic purposes (Lopes *et al.*, 2020), which can significantly alter its characteristics (Anyega *et al.*, 2021). Since BSFL can consume a variety of feeding substrates, the composition of frass can also vary. In a previous review paper (Lomonaco *et al.*, 2024), a comparison among different macro- and micro-nutrient composition of different frass types was performed, showing how these differences can depend on the feeding substrate. This work investigates the microbial composition of different types of frass, with a specific focus on the presence of bacteria exhibiting plant growth-promoting characteristics. Plant-rhizobacteria interactions can enhance both growth and protection of economically important crops. The presence of PGPM in frass therefore offers a potential agronomic and commercial advantage.

4.1 A screening revealed a high number of isolates with at least one plant-growth promoting activity

A total of 149 bacterial isolates were collected and subjected to a preliminary screening phase to identify plant-growth promoting activities. As illustrated in Figure 1, bacteria isolated from different types of frass display dissimilar characteristics; this confirms the variability between frass from different diets which also has been reported by Wynants *et al.* (2015) and Osimani *et al.* (2021). Bacterial isolates recovered from heat-treated frass exhibited variability across the evaluated parameters, in some cases showing either reduced or enhanced activity. For instance, in the case of bacteria isolated from frass type A, notable differences were observed: isolates from untreated frass demonstrated higher performance in ammonia and gibberellin production, whereas those from heat-treated frass showed greater phosphate solubilization capacity. Similar trends in functional variability were observed across all frass types, as illustrated in Figure 1. To narrow down the list of bacterial isolates, the top 73 strains were selected based on their overall performance across the evaluated PGP traits. Strains exhibiting the highest activity in one or more of these assays were retained for subsequent molecular identification using full length16S rRNA gene sequencing to reveal the different bacterial genera, with plant-growth promoting activities, associated with the various types of frass. As stated in the results the genera most commonly found in NT frass were also

present in HT frass, indicating that heat treatment did not reduce the presence of bacteria with beneficial traits for plant growth. While the survival of *Bacillus* can be attributed to its ability to form heat resistant spores, the persistence of the other non-spore forming genera is particularly remarkable and suggests a notable tolerance to thermal stress. This is further confirmed by dedicated testing of the ability of isolates to survive a thermal treatment of 1 h at 70°C. This suggests there is a potential for their inclusion in integrated biofertilizer formulations where frass acts both as a microbial inoculum and organic matrix. This finding is particularly relevant from an application standpoint, as it suggests that increasing their initial concentration prior to heat treatment may be a viable strategy to enhance the microbial load of the final product, without compromising bacterial viability. Such an approach could help ensure both the sanitary safety and biological effectiveness of frass-based biofertilizers. Though more research is needed on the effect of processing on one hand and the potential of these isolates to boost growth of other plant species on the other hand.

4.2 Most identified genera with *in vitro* PGP activity are frequently reported to be associated with BSF frass

Overall, the literature on the BSFL microbiota has revealed clear correlations between the gut and frass microbial community, where microbes related to the larvae become more dominant in the frass compared to the initial diet (Vandeweyer *et al.*, 2023). Our study aligns with such results, the bacterial genera *Serratia, Peribacillus, Acinetobacter, Pseudocitrobacter, Bacillus and Enterobacter*, which we isolated from frass, have already been reported in previous analyses of the microbiota associated with *H. illucens*. Regarding spore-forming bacteria, several studies have consistently reported *Bacillus spp.* as one of the most predominant in the gut microbiota of *H. illucens*, underscoring its central role in the digestive ecology of the species (Jeon *et al.*, 2011; Callegari *et al.*, 2020; Cifuentes *et al.*, 2020; Tegtmeier *et al.*, 2021). In particular, Callegari *et al.* (2020) reported a notably high abundance of *Bacillus spp.* in the larval gut, suggesting that members of this genus are actively involved in the degradation of polysaccharides such as cellulose and starch. The genus *Enterobacter* has been frequently identified in the larval gut of *H. illucens* across several studies, including those by Callegari *et al.* (2020), Gorrens *et al.* (2021), and Cifuentes *et al.* (2020). Although less dominant, *Serratia* species were also detected in the gut of larvae which suggests their stable

presence, albeit in low amounts, in the microbial community (Cifuentes *et al.*, 2020). *Acinetobacter* is another genus commonly reported in the gut of larvae (Callegari *et al.*, 2020). The detection of these bacterial genera in the larval gut of *H. illucens*, as documented in various studies, along with their presence in the larval frass analysed in this study, also supports a transfer of bacteria from the gut to the frass during the digestive process. In contrast, a literature review revealed no studies reporting the presence of the genera *Peribacillus* and *Pseudocitrobacter* in the gut microbiota of *H. illucens*. Therefore, the occurrence of these genera in the frass could be attributed to their presence in the initial feeding substrate.

4.3 The six most potent isolates belong to genera with known PGP-activity, confirming the suitability of our screen

Serratia sp., Peribacillus sp., Acinetobacter sp., Pseudocitrobacter sp., Bacillus sp., and *Enterobacter sp.* were the six studied bacteria that showed the best traits and were explored in more detail. Their potential is also confirmed to a large extent from literature. Numerous bacteria in the *Serratia* genus have been reported to increase plant growth by generating phytohormones, increase nutrient availability, and offer defense against biotic and abiotic stress (Trinh *et al.*, 2024). *Peribacillus* have already reported among PGPM showing a high potential under stress condition, particularly due to features such as nutrient solubilization an phytohormone production (Senko *et al.*, 2024). Members of the *Acinetobacter* genus exhibit plant growth–promoting characteristics, particularly phosphate solubilization, which contributes to improved plant performance under metal stress (Ren *et al.*, 2013). Similarly, *Pseudocitrobacter* has been shown to promote plant growth by reducing heavy metal accumulation and enhancing phytohormone production and antioxidant activity in plants (Husna *et al.*, 2020). Numerous *Bacillus* species have been shown to be beneficial for plants by increasing biomass, growth, and yield. They also aid in the synthesis of phytohormones, nitrogen fixation, phosphate solubilization, and enhanced nutrient availability (Sun *et al.*, 2025). The genus *Enterobacter* also has been reported to exhibit excellent plant growth-promoting characteristics (Wang *et al.*, 2023). *Peribacillus polymyxa* was used as a control, but it did not solubilize phosphate and produce gibberellins as reported by Weselowski *et al.* (2016).

4.4 Root length and root hair formation are differentially influenced in Arabidopsis seedlings after isolate supplementation

The effect of these isolates on plant growth was then observed in *Arabidopsis* seedlings. When comparing root length (Figure 6b) with the number of root hairs (Figure 7) across the different treatments in these trials, a noteworthy observation was the negative correlation between these two parameters. Treatments which exhibited a greater root length generally showed a lower number of root hairs. For example, treatments with *Acinetobacter sp., Pseudocitrobacter sp., Enterobacter sp*., and *Paenibacillus polymyxa* showed shorter root lengths compared to the other treatments and the control. However, these same treatments led to a higher production of root hairs. Conversely, other treatments, such as those with *Serratia sp., Peribacillus sp*., and *Bacillus sp*., exhibited the opposite trend. Root formation of *A. thaliana* has been extensively studied, and the different steps of root hair development have been described (Schiefelbein and Somerville, 1990; Dolan *et al.*, 1993; Galway *et al.*, 1994). Hairs originate from selected epidermal cells influenced by genetic factors and local conditions affecting the loosening of cell walls to promote hair initiation and outgrowth (Galway *et al.*, 1994; Singh *et al.*, 2008). These processes can be influenced by the involvement of hormones (e.g. auxin, cytokinin, ethylene), local variations in nutrient or toxic element, redox conditions or pH (Sanchez-Fernandez *et al.*,1997; Bibikova *et al.*, 1998). The results of our study highlight the potential of specific bacterial isolates associated with *H. illucens* frass to promote root hair formation. Notably, strains such as *Serratia sp., Peribacillus sp., and Bacillus sp*. not only demonstrated PGP traits—including phosphate solubilization and gibberellin production but also led to a significant increase in both root length and the density of root hairs per unit root length in controlled assays. These findings are consistent with literature indicating that bacterial production of phytohormones and solubilization of nutrients can lead to a modulated root system architecture (Spaepen *et al.*, 2009; Vacheron *et al.*, 2013). Interestingly, isolates such as *Acinetobacter sp., Pseudocitrobacter sp., Enterobacter sp.*, and *Paenibacillus polymyxa*, while also exhibiting certain PGP features, were associated with shorter root lengths but a higher density of root hairs per mm of root. This inverse relationship suggests that specific microbial metabolites may preferentially stimulate lateral root structures over elongation. For instance, gibberellin production has been linked to modulation of cell elongation

and differentiation in roots, but its combined effect with other substances (e.g., IAA, ammonia) could result in diverse morphological outcomes depending on concentration and interaction (Glick, 2012). Regarding root hair formation, while the role of IAA-producing bacteria in enhancing root hair density has been documented (Patten *et al.*, 2002), less is known about the synergistic role of ammonia and gibberellins in this process. In our assay, *Acinetobacter sp. and Pseudocitrobacter sp*. both capable of gibberellin production induced the highest number of root hairs, even though their overall root length was limited. This observation may point to a mechanism where energy is diverted toward lateral differentiation rather than elongation. From the perspective of plant–microbe interactions, this could be particularly advantageous: root elongation combined with an increased number of root hairs enhances the absorptive surface area, thereby improving the efficiency of water and nutrient uptake. Therefore, bacterial strains that promote the formation of a higher density of root hairs, even when associated with a slight reduction in overall root elongation, may represent desirable candidates for the development of bioinoculants under resource-limited conditions.

4.5 Abundance of the six most potent isolates is substrate dependent, explaining to some extent observed variability in the effect of frass treatments in literature

Identification of the bacterial communities through 16S rRNA gene amplicon sequencing revealed clear differences among frass types. These variations likely reflect the ability of specific microbes to utilise specific nutrients in the diet, producing metabolites that enable them to thrive and dominate the microbiome. The diet dependency is evident from the absence of any single bacterium with both a high abundance and a wide prevalence across all samples. For example, the most abundant bacterium, the unidentified Bacilli zOTU1, accounts for approximately 25% of reads in frass types B, F and T, yet occurs at much lower levels in four other frass types (A, DS, SB and WS), while being completely absent in three others (E, OP and P). In most frass types, 60–80% of the bacterial community consists of fewer than 30 dominant microorganisms. A microorganism is defined as dominant if it is present in >5% in that specific frass type. In contrast, approximately 75% of the relative abundance in SB frass consists of species outside the 28 dominant taxa, highlighting the substantial variability in microbiome composition among frass types. These differences in the relative abundance were also evident among the six

selected bacterial isolates across frass types. Notably, *Enterobacter* was detected in several frass types, specifically A, B, E, F, OP, and T, and consistently exhibited the highest relative abundance among the targeted genera. *Acinetobacter* and *Serratia* were also detected in multiple frass types, including A, B, and T, with *Serratia* additionally present in DS and *Acinetobacter* also found in OP. However, the abundance of these two genera was clearly lower than that of *Enterobacter*. *Pseudocitrobacter* was exclusively detected in frass B and F, while *Bacillus* was found only in SB. Notably, frass B contained four different plant growth-promoting genera: *Enterobacter*, *Acinetobacter*, *Serratia*, and *Pseudocitrobacter*, *which indicates it might have* the most diverse composition in terms of beneficial microbes. Overall, frass A, B, and T originating from larvae fed with artichokes, broccoli, and turnip greens, respectively, showed the highest potential in terms of content of the six characterised PGPM. In addition, these frass types were also associated with high loads overall of bacteria capable of growth on RMA (> 6 log CFU/g). The increased presence of (potential) PGPM in frass derived from artichokes, broccoli, and turnip greens may be attributed, at least in part, to the biochemical composition of these substrates. Artichoke is characterized by high levels of soluble fiber (notably inulin) (Ayuso *et al.*, 2024), whereas Brassicaceae vegetables (e.g., broccoli and turnip greens) accumulate significant amounts of sulfur-rich glucosinolates (Wu *et al.*, 2021). These biochemical traits make these substrates chemically distinctive compared with other vegetable by-products tested, and could selectively favor microbial taxa adapted to metabolize or tolerate such compounds. The observed differences underscore the influence of larval diet on the microbial profile of the resulting frass. By strategically selecting feeding substrates for *H. illucens*, it may be possible to enhance the abundance of specific PGPM in the frass. Such an approach could be leveraged to develop functionally enriched frass-based biofertilizers tailored to agronomic needs, provided that the economic costs of substrate optimization remain justifiable. This study is among the firsts to provide direct evidence of microbes exhibiting plant growth-promoting traits in frass derived from BSFL reared on various dietary substrates. Therefore, this study provides evidence supporting the hypothesis that plant growth-promoting microbes present in frass may partially explain the beneficial effects on plant growth and health reported in previous literature. Our findings also highlight that the composition of the larval diet significantly influences the diversity and abundance of plant growth-promoting microbes present in BSF frass. This could explain the

large variability between studies when testing the effect of frass supplementation to crops. Interestingly, most of the PGPM isolated in this study demonstrated an unexpected ability to survive the applied heat treatment. This suggests that frass could retain beneficial microbial activity even after mandatory heat treatments. Further studies are needed to deeply understand the molecular mechanisms involved and to determine whether co-application of such strains could lead to additive or synergistic benefits in plant nutrient uptake, particularly under water or nutrient-limited conditions.

5. Conclusion

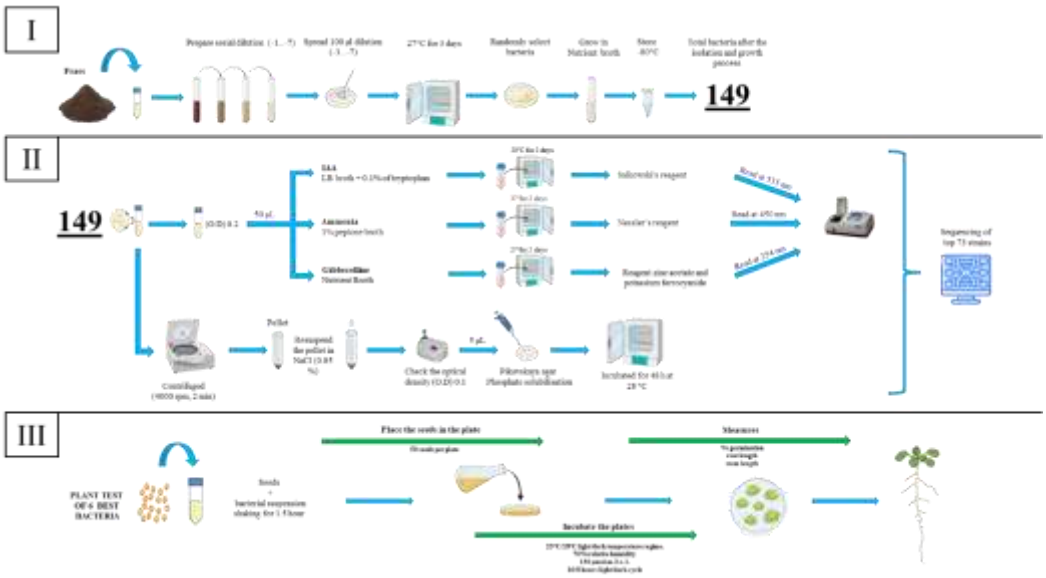
This study is among the firsts to provide direct evidence of microbes exhibiting plant growth-promoting traits in frass derived from BSFL reared on various dietary substrates. Therefore, this study provides evidence supporting the hypothesis that plant growth-promoting microbes present in frass may partially explain the beneficial effects on plant growth and health reported in previous literature. Our findings also highlight that the composition of the larval diet significantly influences the diversity and abundance of plant growth-promoting microbes present in BSF frass. This could explain the large variability between studies when testing the effect of frass supplementation to crops. Interestingly, most of the PGPM isolated in this study demonstrated an unexpected ability to survive the applied heat treatment. This suggests that frass could retain beneficial microbial activity even after mandatory heat treatments. Further studies are needed to deeply understand the molecular mechanisms involved and to determine whether co-application of such strains could lead to additive or synergistic benefits in plant nutrient uptake, particularly under water or nutrient-limited conditions.

6. Supplementary

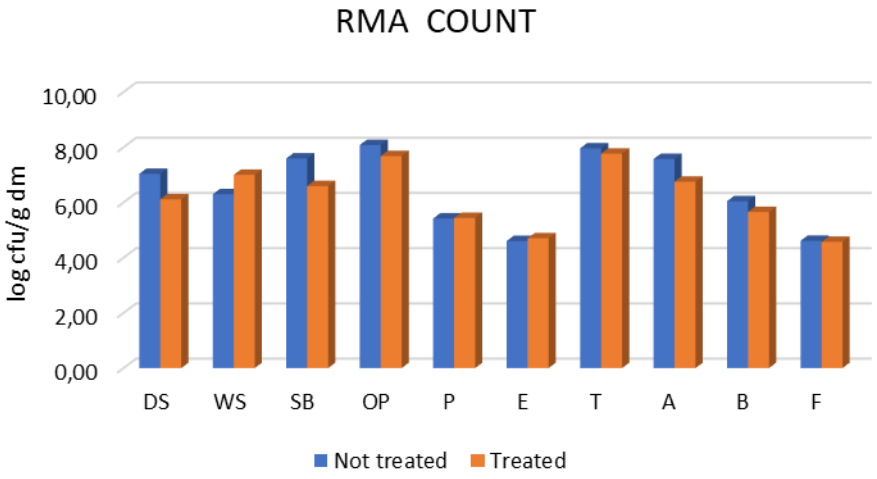
Supplementary Table 1. Composition of Rhizosphere Mimicking Agar (Brescia *et al.*, 2020).

Type	Ingredients	Concentration (g/l)
Synthetic root exudates	Citric acid	0.010
	Fructoseb	0.039
	Glucose	0.039
	Glutamic acid	0.019
	L-Alanine	0.019
	L-Serine	0.023
	Lactic acid	0.001
	Succinic acid	0.010
	Sucrose	0.037
Recalcitrant organic carbon sources	Cellulose	0.079
	Humic acids	0.033
	Lignin	0.065
	Starch	0.017
Salts	CuSO4 5H2O	0.005
	KCl	0.499
	KH2PO4	0.680
	Fe2(SO4)3	0.031
	MgSO4 7H2O	0.493
	MnSO4	0.001
	Na2MoO4 2H2O	0.008
	(NH4)2SO4	0.002
Cycloheximide		0.1

Supplementary Figure 1. A schematic and simplified overview of the experimental procedures, which are described in full detail in the Materials and Methods section. I: isolation of bacteria from frass; II: screening for evaluation of PGPM trait from bacteria selected from frass; III: *in vivo* test with Arabidopsis.



Supplementary Figure2. Total bacterial counts on RMA (expressed as log CFU/g dry matter (dm)) from ten different types of frass, either untreated (blue bars) or heat-treated (orange bars). Frass types include: DS = Gainesville diet, WS = whey + seeds, SB = spent barley, OP = olive pomace, P = peppers, E = eggplant, T = turnip greens, A = artichokes, B = broccoli, F = fennel. Each bar represents the count value of a single replicate. Heat treatment resulted in a general reduction of bacterial load, although variability among substrates was observed.



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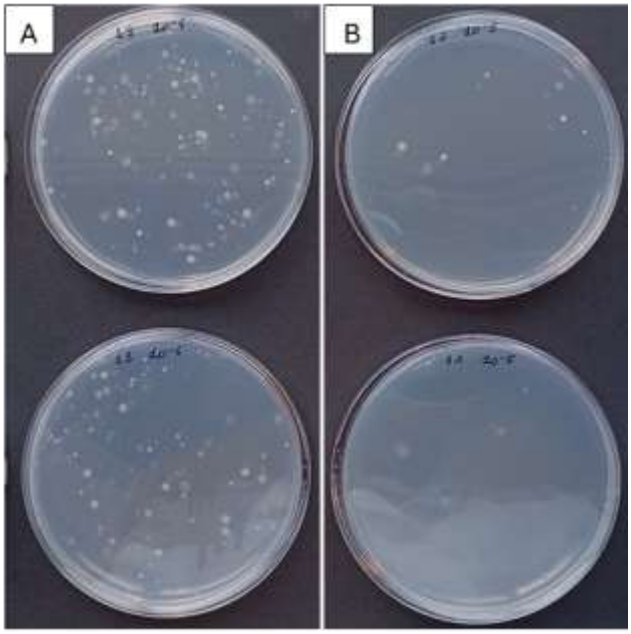
The data shown in the graph are presented for descriptive purposes only for the bacterial count on RMA and were not subjected to statistical testing. Heat treatment at 70 °C generally reduced the presence of bacteria grown on RMA.

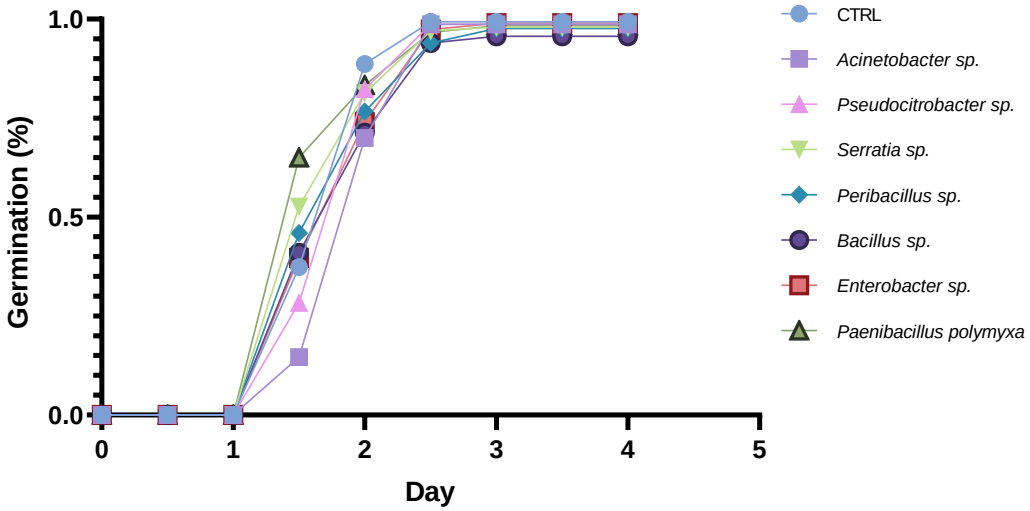
Supplementary Figure 3. The figure illustrates the RMA plates displaying the growth of bacterial colonies. Different letters represent different dilution of frass extract (A: 10^{-4}; B: 10^{-5})



Supplementary Table 2. Taxonomic units found in the 16S rRNA gene amplicon analysis of the frass samples were compared with the full 16S sequences of the six bacterial isolates of interest. Names of the bacterial isolates and the best matching sequences from the amplicon sequencing analysis of the frass are shown in column 1 and 2, respectively. Column 3 shows the percentage that the short reads matched with the bacteria isolate full 16S rRNA gene sequences.

Bacteria	**Short read ID frass**	**Percentage identification match (%)**
Enterobacter sp.	zOTU2	100.000
Acinetobacter sp.	zOTU34	100.000
Serratia sp.	zOTU46	100.000
Pseudocitrobacter sp.	zOTU162	100.000
Bacillus sp.	zOTU388	100.000
Peribacillus sp.	zOTU673	98.400

Supplementary Figure 4. Germination percentage of Arabidopsis thaliana seeds treated with different plant growth-promoting bacteria (PGPB), compared to the control (CTRL). Data are presented as mean of 3 replications with 50 seeds per plate.



CHAPTER 5

Effects of Black Soldier Fly larval frass on seed germination, growth and functional traits of Romaine lettuce

Chapter 5 examines lettuce (*Lactuca sativa*) and evaluates frass BSFL produced from a standard diet and heat-treated at 70 °C for: (i) effects on plant growth, (ii) potential phytotoxicity, and (iii) suitability as a partial peat substitute in the substrate. The optimal frass dose to improve growth parameters is identified, and functional quality traits including total polyphenols and antioxidant capacity are analyzed. *(Editorial status: original, unpublished work conducted as part of this thesis; not submitted for publication at time of deposit.)*

1. Introduction

Consistent with the biological and regulatory framework detailed in the previous chapters (Chapters 1-4), *Hermetia illucens* (BSF) larvae efficiently convert organic waste into nutrient-rich biomass and a residual by-product known as larval frass. As previously established regarding EU Regulation 2021/1925, this material requires specific heat treatment to ensure microbiological safety before agricultural use (Houben *et al.*, 2020). Building on the characterization of frass provided in Chapters 2 and 3, it is well known that the significant variation in its nutrient content is directly related to the initial feeding substrate (Klammsteiner *et al.*, 2020b; Osimani *et al.*, 2018; Lopes *et al.*, 2022). The significant variation in the macro- and micronutrient content of various types of frass is well established (Lomonaco *et al.*, 2024), demonstrating how these variations are directly related to the initial feeding substrate. The use of frass as a partial substitute for peat is one of the main topics of interest for the agricultural sector (Setti *et al.*, 2019). Several studies have already explored the potential of insect frass as an input for horticultural crop production, particularly on leafy vegetables such as lettuce (*Lactuca sativa*). Chiam *et al.* (2021) demonstrated that frass obtained from larvae fed on okara could support lettuce growth at low application rates (10% v/v), while higher concentrations negatively affected plant development, likely due to the low C:N ratio and rapid nutrient mineralization. This can lead to excessive ammonium (NH_4^+) release and high electrical conductivity (EC), inducing ammonia toxicity and osmotic stress (Setti *et al.*, 2019). Setti *et al.* (2019) similarly assessed the potential of BSF frass, produced using the Gainesville Diet as feeding substrate, as a partial alternative to commercial peat in soilless cultivation. Their results

indicated that up to 20% substitution improved growth parameters in lettuce. The different optimal application rates observed in these studies are likely attributable to the distinct chemical and biological properties of the frass, which are strongly influenced by the rearing substrates. The use of okara in Chiam *et al.* (2021) and the Gainesville Diet in Setti *et al.* (2019) resulted in frass with different nutrient profiles, microbial loads, and possibly phytotoxic compounds, underlining the importance of substrate selection when evaluating frass performance in horticultural systems.

In this work, we investigated how frass obtained from *H. illucens* rearing can affect plant yield and chemical parameters. This study was conducted on Romaine lettuce (*Lactuca sativa* L. var. *longifolia*), one of the most important and widely cultivated vegetable crops globally (Veronica *et al.*, 2025). Member of the Asteraceae family, lettuce is naturally adapted to temperate climates but is now grown under a range of environmental conditions, both in open fields and greenhouse systems. Its short growth cycle and relatively simple agronomic management make it a model species frequently used in agricultural research (Thomas *et al.*, 2021). In addition to evaluating the effects of frass on plant growth, potential phytotoxicity, as well as its suitability as a partial substitute for peat in the cultivation substrate were assessed. The study also aimed to determine the optimal percentage of frass that can be incorporated into a growing medium to enhance key growth parameters in lettuce, also assessing its content of some functional compounds, such as polyphenols, and its antioxidant capacity. This integrated approach allows for a comprehensive evaluation of BSF frass as a sustainable input in horticultural production systems.

2. Materials and Methods

2.1 Black Soldier Fly rearing, frass production and frass extract preparation

The frass tested in the present study was produced by Xflies srl (Potenza, Italy). Six-day old BSFL were reared on a standard Gainesville diet provided by the feed manufacturer Mangimi Losasso srl (Balvano, Potenza, Italy), composed of 20% corn, 30% alfalfa meal, and 50% wheat bran (Hogsette, 1992), with a moisture content of 70%. Larval growth conditions were as follows: temperature of 27.0 ± 1.0 °C, 70.0% relative humidity, and a photoperiod of 0:24 (light:dark). After approximately 10 days of bioconversion, larvae were separated from the frass using a vibrating sieve (Guangzhou Flysource Biotechnology Co. Ltd., China) with a 5

mm mesh size. The frass was pasteurized through thermal treatment at 70 °C for 1–2 hours, in accordance with Commission Regulation (EU) No. 2021/1925 (Reg. UE 2021/1925). It was then stored in a sealed plastic bag until use. The chemical and microbiological properties of the frass, presented in Table 1, were analyzed by an external laboratory (IRSAQ Srl – Tito, Italy) With a few modest adjustments, frass extract was produced in accordance with Arabzadeh *et al.* 2022. In short, 100 mL of sterile physiological saline solution (0.5% NaCl) was mixed with 10 g of pasteurized frass and stirred for 1 hour at 27°C. The final frass extract was obtained by recovering the supernatant using a double layer of sterile gauze following a 15-minute ultracentrifugation at 6000 × g.

2.2 Salad seed germination test with frass extract

Extract of diluted heat-treated frass (100%, 50%, 25%, 12.5% and 6.25%) was used to evaluate the phytotoxicity of frass; the model species used in the trial was Romaine lettuce (*Lactuca sativa* L. var. *longifolia*). The experiment was performed by placing 6 lettuce seeds in 6 mm petri plates with sterile filter paper at the bottom. 2 ml of each dilution was then added per plate and incubated at 21°C. After 96 hours, the germinated seeds were counted, the root length and stem length were measured. Seed germination index (SGI) was determined according to the following formula:

$$SGI\ (\%) = (RSG\% \times RRG\%)/100$$

RSG (%), the relative seed germination, and RRG (%), the relative root growth, were calculated according to the following formulas:

RSG (%) = (Number of germinated seeds (sample)/ Number of germinated seeds (control)) *100

RRG (%) = (Total radicle length of germinated seeds (sample)/ Total radicle length of germinated seeds (control)) * 100

The SGI value is used to assess the phytotoxicity of frass; a value of less than 50% is regarded as extremely phytotoxic, a value of 50% to 80% as moderately phytotoxic, and a value of more than 80% as non-phytotoxic. The frass can be regarded as a phytonutrient or phytostimulant when the value is greater than 100% (Beesigamukama, D. *et al.*, 2020a).

2.3 Germination test

To assess the suitability of frass as a growing medium, ten different germination substrates were prepared with varying proportions of soil and frass, expressed as volume percentages: 100% soil; 100% soil + NPK fertilizer; 90% soil + frass 10%; 85% soil + frass 15%; 80% soil + frass 20%. The frass inclusion rates were selected based on preliminary trials (data not shown) conducted to identify suitable application levels, that allowed a measurable response. A commercial universal soil (Compo - Naturasol Universal) was used as soil and commercial mineral fertilizer (Compo - BLU universal fertilizer) was used as NPK fertilizer. Seed germination was performed in a growth chamber under controlled conditions, in order to ensure uniform and reproducible seed emergence: 21 °C, 70 % relative humidity, and a 10 h light / 14 h dark photoperiod, which fall within the optimal range commonly used for Romaine lettuce (*Lactuca sativa* L. var. *longifolia*) germination trials (Reynolds *et al.*, 2024). Styrofoam pots filled with the different germination substrates were used. For each treatment, 12 alveoli (each containing three seeds) were prepared, with each individual alveolus considered an experimental unit (n = 12). Seedling emergence was monitored at 7 and 14 days after sowing. The Germination Speed Index (GSI) was calculated for each experimental unit according to the formula described by Hojjat and Kamyab (2017):

$$GSI = \left(\frac{G_7}{7}\right) + \left(\frac{G_{14} - G_7}{14}\right)$$

where G_7 is the number of germinated seeds at day 7, and $(G_{14} - G_7)$ is the number of new seeds germinated between day 7 and day 14. Data were expressed as the mean GSI per treatment ± standard deviation. Following the final count, thinning was performed to retain one seedling per alveolus for subsequent growth analysis.

2.4 Agronomic performance of seedlings

The experiment on agronomic performance of lettuce treated with frass was conducted under different environmental conditions than those described for the germination test. Specifically, it took place in a greenhouse under controlled range temperature (e.g. 20-30°C) and natural photoperiod (experiment conducted between June and July) to simulate realistic commercial growing environments and to evaluate plant performance under practical cultivation conditions. The objective of the study was to evaluate the effects of increasing concentrations of frass on the

post-transplant growth stages of Romaine lettuce. The effects of the same frass inclusion rates previously tested in the germination trial were evaluated, using lettuce seedlings ready for transplanting. The experimental design included five substrate formulations: 100% soil; 100% soil + NPK fertilizer; 90% soil + frass 10% w/w; 85% soil + frass 15% w/w; 80% soil + frass 20% w/w. A commercial universal soil (Compo - Naturasol Universal) was used as soil and commercial mineral fertilizer (Compo - BLU universal fertilizer) was used as NPK fertilizer.
Each treatment consisted of 15 plants arranged in a completely randomized design. 35 days after transplanting, the following growth parameters were measured: plant height, root length, fresh and dry weight of plants, root dry mass, crown width and SPAD. The SPAD index was determined using a portable chlorophyll meter (CHL PLUS, atLEAF), which provides a non-destructive estimate of leaf chlorophyll content by measuring the absorbance of the leaf in the red and near-infrared regions.

2.5 Quantitative analysis on lattice extracts

2.5.1 Preparation of plants' methanolic extracts

Dried lettuce powder samples (1g) were extracted with 25 ml of methanol/water (80% v/v) (Gomes *et al.*, 2013). The mixture was rapidly vortexed and sonicated in an ultrasonic bath for 10 minutes at room temperature; then the mixture was stirred for an hour at room temperature. Extraction was carried out in dark conditions to prevent light degradation during extraction. The extracts were filtered and stored at 4 °C. The resulting lettuce extracts were used for the determination of the total phenolic and flavonoid content, and total antioxidant activity.

2.5.2 Total phenolic and total flavonoid content evaluation

The total phenolic content (TPC) of the samples was assessed according to the Folin-Ciocalteu method (Khosravi *et al.*, 2024). An amount of 0.1 mL of the methanolic extract, 2.5 mL of distilled water, and 0.1 mL of Folin-Ciocalteu's reagent were combined. After 6 min, 0.15 mL of 20% w/v sodium carbonate was added to the mixture, and after 30 min incubation at room temperature, the absorbance was determined spectrophotometrically at 760 nm. The results were expressed as mg of gallic acid equivalent (GAE) per gram of dry weight (DW) using a calibration curve of gallic acid standard (12.5– 200 mg L-1).
Total flavonoid content (TFC) was determined by the aluminium chloride colorimetric method, adapted from the procedure reported by Ordonez *et al.* (2006).

Each extract was added to 2% AlCl3 methanolic solution. After 60 min at room temperature, the absorbance was measured at 415 nm using a scanning multiwell Varioskan LUX spectrophotometer (Thermo Scientific) and the results were expressed as mg quercetin equivalent (QE) g-1 DW using a calibration curve of quercetin standard (0–125 mg L-1).

2.5.3 Determination of antioxidant activity

The ABTS assay was used to evaluate the total antioxidant activity (TAA) of the lettuce methanolic extract. As described by Triunfo *et al.* (2023a), the ABTS+• solution was produced, diluted to an absorbance of 0.7 at 734 nm and mixed with each sample. The absorbance was measured at 734 nm (Thermo Scientific Varioskan LUX) after 30 min of incubation at room temperature. The results were expressed as mg of Trolox equivalent (TE) g-1 DW, using a calibration curve of Trolox standard (0-500 mg L-1).

2.6 Statistical Analysis

Results are expressed as means ± standard deviation. The normality of the data was assessed using the Shapiro-Wilks test. Data were analyzed by one-way Anova and Tukey's *post-hoc* test at $P = 0.05$. Statistical analyses were performed using a GraphPad Prism version 6.0.0 for Windows (GraphPad Software, San Diego, California USA).

3. Results

3.1 Chemical and microbiological composition of BSF frass

The analysis of the chemical and microbiological composition of larval frass, obtained from the standard diet and after heat treatment at 70 °C, is shown in Table 1. The frass showed a total nitrogen content of 25.23 g/kg, a phosphorus concentration of 14894.67 mg/kg, and a potassium content of 306.00 mg/kg, with a C:N ratio of 16.94. The pH was slightly alkaline (8.31). Microbiological analysis confirmed the absence of *Salmonella spp*. and *Escherichia coli* levels were below the legal limits.

Table 1. Chemical and microbiological composition of frass treated at 70 °C

Parameter	Frass treated at 70°C for 1 hour
Dry Matter %	88.43 ± 0.15
Water Content %	11.57 ± 0.15
Ashes (%)	86.57 ± 0.59
pH	8.31 ± 0.13
Conductivity (µS/cm)	61553.33 ± 2664.31
Organic carbon (%)	42.73 ± 2.56
Total N (g/Kg)	25.23 ± 0.84
C/N	16.90 ± 0.92
P_2O_5 (mg/kg)	14894.67 ± 2880.11
K (mg/kg)	306.00 ± 54.74
Ca (mg/kg)	82.83 ± 17.95
Mg (mg/kg)	58.47 ± 10.78
Zn (mg/kg)	149.33 ± 8.5
Cu (mg/kg)	29.93 ± 1.11
Fe (mg/kg)	777.00 ± 57.66
$N\text{-}NH4^+$ and $N\text{-}NO3^-$ (%)	1.24 ± 0.14
Protein (g/100g)	15.77 ± 0.55
Salmonella spp.	Not found
E. coli	2.51±0.23

Mean values ± standard deviation (n = 3)

3.2 Evaluation of phytotoxicity of frass extract

The results of the seed germination test using different dilutions of the frass extract are reported in Table 2. According to Luo *et al.* (2018), a seed germination index (SGI) below 80% indicates phytotoxicity, whereas values significantly above 100% suggest a biostimulant effect. The 12.5% frass dilution showed a numerical trend toward a biostimulant effect, with an SGI of 122.72%. Although this value was higher than the control (100%), the difference was not statistically significant. While root length (3.75 cm) also showed only a numerical increase, a statistically significant enhancement was observed in stem length (4.76 cm) compared to the control. A biostimulant effect was also observed, though less pronounced, at the 25% dilution (SGI: 86.81%), particularly for shoot elongation (5.12 cm), despite a decrease in the germination rate. Phytotoxic effects were evident at the highest concentrations tested. The 100% frass extract treatment significantly inhibited germination (41.67%) and resulted in the lowest SGI (22.19%), accompanied by marked reductions in both root (1.56 cm) and stem length (2.27 cm). Similarly, the 50% dilution also exhibited phytotoxic effects (SGI: 63.66%), although less severe.

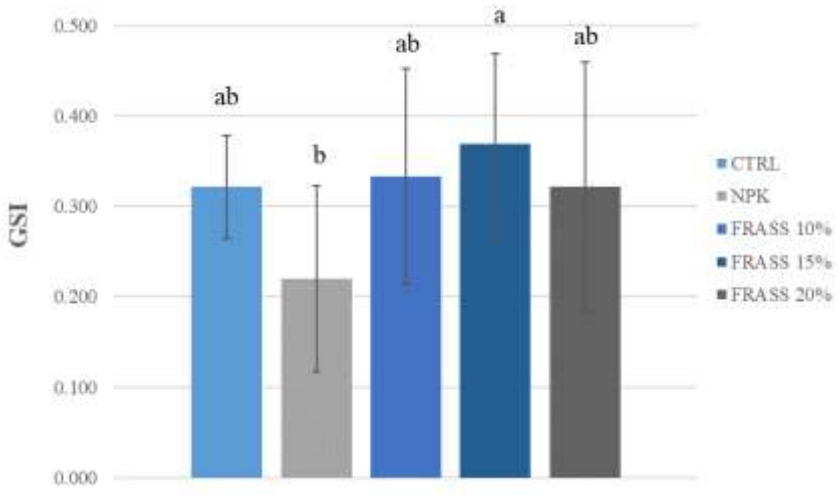
Table 2. Effect of frass extract as such and at the different dilutions (100%, 50%, 25%, 12.5%, 6.25%) on seed germination, seed germination index, root and stem length of lettuce. CTRL indicates seeds treated with distilled water. Different letters in the same column per each considered parameter indicate value significantly different. Data are presented as the mean of replicates (N=4) ± SD (Standard Deviation) significantly different according to one-way ANOVA followed by Tukey *post-hoc* test ($p < 0.05$).

% frass extract	Germinated seeds	Seed germination index	Root length (cm)	Stem length (cm)
CTRL	83.33± 23.57 ^{ab}	100.00 ± 28.28 ^{ac}	3.52 ± 0.35 ^{a}	3.65 ± 0.36 ^{bcd}
6.25	74.17 ± 21.15 ^{ab}	51.52 ± 15.25 ^{be}	2.13 ± 1.07 ^{bc}	3.28 ± 1.85 ^{b}
12.5	100.00 ± 0.001 ^{a}	122.72 ± 10.67 ^{a}	3.75 ± 0.77 ^{a}	4.76 ± 0.81 ^{ac}
25	75.00 ± 9.62 ^{ab}	86.81 ± 11.14 ^{ade}	3.39 ± 0.57 ^{a}	5.12 ± 0.53 ^{a}
50	62.5 ± 25.00 ^{ab}	63.66 ± 25.46 ^{bcd}	2.99 ± 0.40 ^{ac}	4.62 ± 0.66 ^{ad}
100	41.67 ± 28.87 ^{b}	22.19 ± 15.37 ^{b}	1.56 ± 0.66 ^{b}	2.27 ± 0.82 ^{b}

3.3 Germination test in cell trays

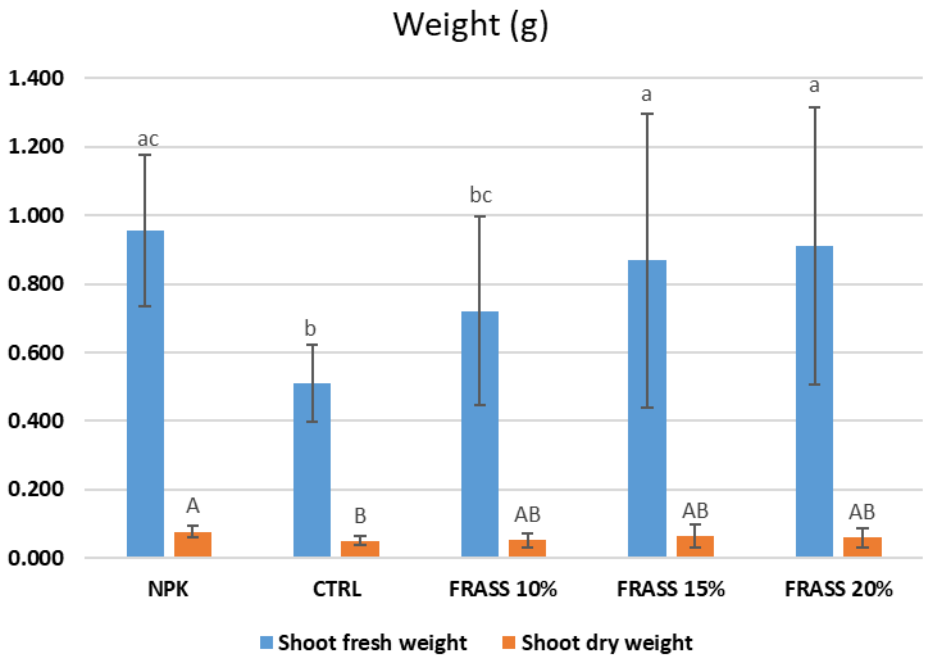
The seedling emergence was monitored daily, and the resulting Germination Speed Index (GSI) was calculated for each alveolus based on the counts at 7 and 14 days after sowing. The one-way ANOVA revealed that the type of germination substrate significant influenced the germination speed ($p = 0.0186$). As shown in the Figure 1, the 15% frass inclusion achieved the highest GSI mean value (0.369). This treatment performed significantly better than the NPK treatment (0.220), which exhibited the lowest mean GSI. No significant differences were found between the control (soil only) and any of the frass inclusion rates (10%, 15%, and 20%).

Figure 1. Germination Speed Index (GSI) of Romaine lettuce seeds sown in different substrates. Bars represent mean values of 12 replicates per treatment (n = 12). Error bars represent the standard deviation (SD). Means with different letters are significantly different according to Tukey's post-hoc test ($p < 0.05$).



At the end of the experiment, two parameters were evaluated: fresh shoot weight and dry shoot weight (Figure 2). For fresh weight, all treatments with different frass inclusion rates showed significantly higher values compared to the control, except for the 10% treatment. In contrast, dry shoot weight did not differ significantly among any of the frass treatments, the control, or the NPK treatment.

Figure 2. Fresh weight and dry weight of shoots. Lowercase letters above the bars denote significant differences among treatments for shoot fresh weight; uppercase letters denote significant differences for shoot dry weight. Different letters designate significantly different values. Data are presented as the mean of replicates (N=12) ± SD (Standard Deviation) significantly different according to one-way ANOVA followed by Tukey post-hoc test ($p < 0.05$)

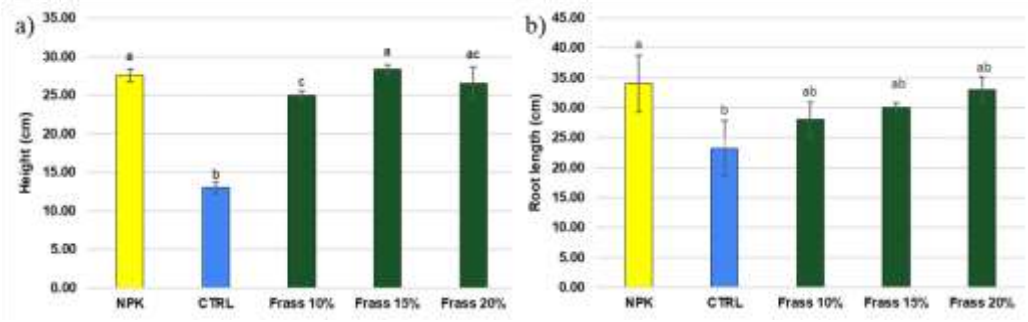


3.4 Agronomic response of lettuce to BSFL Frass Treatments

All frass treatments resulted in significantly greater plant height compared to the control, indicating a positive effect of frass amendments on aboveground growth (Figure 3). Figure 4a shows the plant height measured at the end of the trial. Regarding root length (Figure 4b), no significant differences were observed between the control and the frass treatments. However, the NPK treatment produced a significantly longer root system than the control, although it did not differ significantly from the frass treatments. These findings suggest that while frass contributes notably to shoot development, its effect on root elongation may be less pronounced than that of conventional mineral fertilization.

Figure 3. Agronomic response of lettuce to different inclusion rates of BSFL frass.

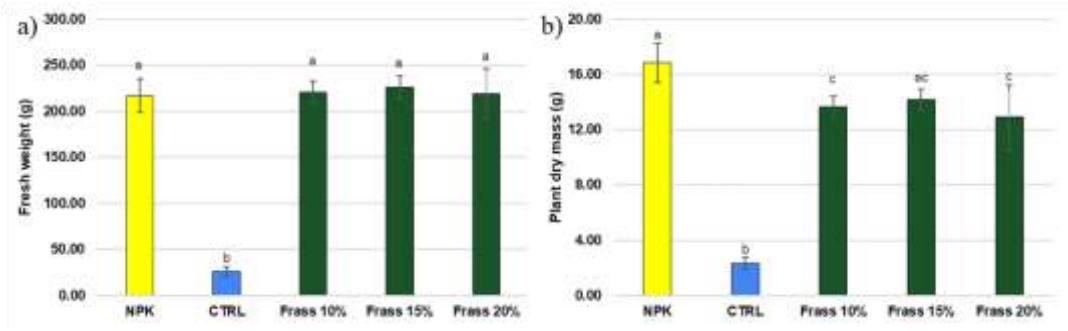
Figure 4. Effect of different frass inclusion rates and NPK treatment on lettuce growth in greenhouse conditions. (a) Plant height and (b) root length measured at the end of the trial. Different letters designate significantly different values. Data are presented as the mean of replicates (N=4) ± SD (Standard Deviation) significantly different according to one-way ANOVA followed by Tukey post-hoc test ($p < 0.05$)



Additional parameters evaluated at the end of the experiment included plant fresh weight and dry weight. Fresh weight (Figure 5a), a key indicator of plant productivity and water content, showed significant differences among treatments. The control group exhibited a significantly lower fresh weight compared to all other treatments, including those with frass and the NPK treatment, highlighting the positive impact of nutrient addition on biomass accumulation. Dry weight results are presented in Figure 5b. This parameter showed even more marked differences across treatments. As observed for fresh weight, the control had a significantly lower dry weight than all other treatments. Among the frass treatments, no significant differences were detected across the different inclusion rates. However, the NPK treatment resulted in significantly higher dry weight compared to the 10%

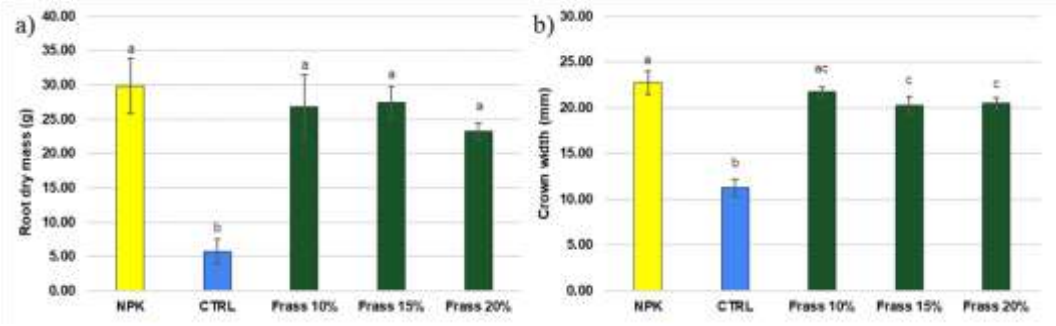
and 20% frass treatments, but did not differ significantly from the 15% frass treatment. These results suggest that while frass can enhance biomass production compared to untreated controls, and that its effect at certain inclusion rates may be comparable to that of conventional fertilization.

Figure 5. Effects of different frass inclusion rates and NPK treatment on lettuce biomass production. (a) Fresh weight and (b) dry weight of plants at the end of the greenhouse trial. Different letters designate significantly different values. Data are presented as the mean of replicates (N=4) ± SD (Standard Deviation) significantly different according to one-way ANOVA followed by Tukey post-hoc test ($p < 0.05$).



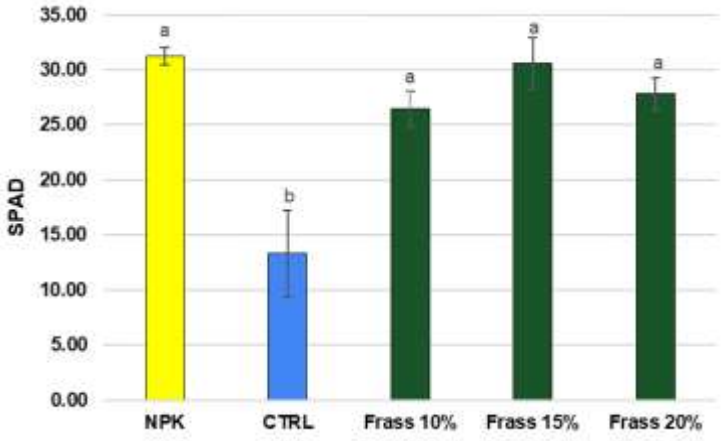
In addition to root length measurements (Figure 4b), root dry matter content was evaluated and is presented in Figure 6a. Root dry matter in the control group was significantly lower compared to all other treatments, indicating improved root biomass accumulation following nutrient supplementation. The NPK treatment did not differ significantly from the frass treatments, suggesting a comparable effect on root tissue development. Crown width was also assessed, and the results revealed significant differences among treatments (Figure 6b). The control showed significantly lower crown width values compared to all frass and NPK treatments. Furthermore, the NPK treatment exhibited significantly higher crown width than the 15% and 20% frass treatments, although it did not differ significantly from the 15% frass treatment. These findings suggest that while both frass and mineral fertilization improve shoot architecture, the effect of NPK may be slightly more pronounced at higher inclusion rates.

Figure 6. Effects of different frass inclusion rates and NPK treatment on lettuce biomass production. (a) Root dry mass and (b) Crown width of plants at the end of the greenhouse trial. Different letters designate significantly different values. Data are presented as the mean of replicates (N=4) ± SD (Standard Deviation) significantly different according to one-way ANOVA followed by Tukey post-hoc test ($p < 0.05$).



The SPAD values, indicative of leaf chlorophyll content, showed significant differences among treatments (Figure 7). The control treatment exhibited significantly lower SPAD readings compared to all other treatments, confirming a lower chlorophyll content likely associated with limited nitrogen availability. In contrast, no significant differences were observed among the remaining treatments, suggesting that all nutrient-based treatments maintained comparable chlorophyll levels in lettuce leaves. These results indicate that while nutrient supplementation improved leaf greenness relative to the control, increasing nutrient input beyond a certain level did not lead to further increases in chlorophyll content, as detected by SPAD measurements.

Figure 7. Effects of different frass inclusion rates and NPK treatment on lettuce SPAD. Different letters designate significantly different values. Data are presented as the mean of replicates (N=3) ± SD (Standard Deviation) significantly different according to one-way ANOVA followed by Tukey post-hoc test ($p < 0.05$)



3.5 Quantitative analysis on lettuce extracts

The effects of different fertilization treatments on the total phenolic content (TPC), total flavonoid content (TFC), and antioxidant activity (ABTS assay) of lettuce methanolic extracts are presented in Table 4.

Table 4. Results of quantitative analysis on lettuce extracts, respectively: TPC (expressed as mg of GAE g^{-1} lettuce DM), TFC (expressed as mg of QE g^{-1} lettuce DM), ABTS (expressed as mg of TE g^{-1} lettuce DM). Different letters designate significantly different values. Data are presented as the mean of replicates (N=3) ± SD (Standard Deviation) significantly different according to one-way ANOVA followed by Tukey post-hoc test ($p < 0.05$).

	TPC	**TFC**	**ABTS**
	mg GAE/g DM	**mg QE /g DM**	**mg TE/g DM**
CTRL	6.48 ± 2.33 ^{d}	24.23 ± 6.96 ^{a}	749.37 ± 142.98 ^{a}
NPK	16.15 ± 1.53 ^{b}	19.1 ± 0.35 ^{ab}	549.71 ± 43.12 ^{b}
10%	15.64 ± 2.33 ^{b}	13.23 ± 0.35 ^{b}	466.13 ± 14.00 ^{bc}
15%	22.77 ± 0.89 ^{c}	14.10 ± 1.92 ^{b}	404.90 ± 34.45 ^{bd}
20%	28.87 ± 2.33 ^{a}	12.73 ± 0.92 ^{b}	321.92 ± 16.28 ^{cd}

As shown in Table 4, frass application brought a significant increase in TPC compared to the control ($p < 0.05$), for which was recorded the lowest TPC value. The highest TPC value was observed in the 20% treatment (28.87 ± 2.33 mg GAE g^{-1} DW), followed by 15% (22.77 ± 0.89 mg GAE g^{-1} DW), whereas the 10% treatment provided a TPC value comparable to the NPK positive control. These results suggested that frass application, as alternative fertilizer, at rates higher than 10% can enhance total polyphenol content compared to results obtained with conventional mineral fertilization.

In contrast, TFC showed an opposite trend. The control treatment exhibited the highest flavonoid content (24.23 ± 6.96 mg QE g^{-1} DW), while all frass-treated groups had significantly lower values ($p < 0.05$), ranging from 12.73 ± 0.92 mg QE g^{-1} DW in the 20% treatment to 14.10 ± 1.92 mg QE g^{-1} DW in the 15% treatment. In a similar way, also antioxidant activity, measured by the ABTS assay, decreased with increasing fertilizer levels. All frass treatments led to low values of TE g^{-1} lettuce (DM) compared to the control ($p < 0.05$), which had the highest value (749.37 ± 142.98 mg TE g^{-1} DW).

4. Discussion

4.1 Chemical and microbiological composition of BSFL frass

The frass employed in the present study, obtained from larvae reared on a standard diet and subsequently subjected to thermal treatment, exhibited an alkaline pH of 8.31. This value is consistent with the findings of González *et al.* (2024), whereas other studies have reported different pH levels, suggesting that frass pH is highly variable depending on the initial substrate provided to the larvae. In a previous study conducted by our group (Lomonaco *et al.*, 2025), frass derived from larvae reared on the same standard diet used to assess the optimal dosage for barley growth displayed a pH of 7.5. Similarly, Labella *et al.* (2024) reported a pH of 7.04 for thermally treated frass obtained from BSFL reared on a standard diet. This variability in frass pH, even when originating from the same initial substrate, may be attributed to rearing conditions such as larval density (Parra Paz *et al.*, 2015), as well as the size and height of the rearing containers and the amount of feed supplied, which may all influence pH (Meneguz *et al.*, 2018). The chemical composition of the frass analyzed in the present study confirms its potential value as an organic fertilizer, due to its relatively high nitrogen content (25.23 g kg^{-1}), and substantial phosphorus concentration (14.89 g kg^{-1}). Similar nutrient profiles have been reported for BSF frass produced from plant-based diets, which are generally characterized by high levels of readily available nitrogen and phosphorus (Setti *et al.*, 2019; Beesigamukama *et al.*, 2020b; Anyega *et al.*, 2021). The C:N ratio observed in the present study (16.9) falls within the range commonly reported for black soldier fly frass and is considered suitable for soil amendment and plant nutrition. Gärttling and Schulz (2022), in a comprehensive compilation of frass chemical analyses, reported C:N ratios typically ranging between 10 and 25 depending on the larval feeding substrate and rearing conditions.

The high electrical conductivity recorded in the present work may pose a challenge for seed germination and subsequent plant development, particularly at high inclusion rates. Elevated salinity levels have been reported as a potential limiting factor for seed germination and early seedling development, especially for salt-sensitive species such as lettuce (Wang G. *et al.,* 2022).

The thermal treatment applied in accordance with Commission Regulation (EU) No. 2021/1925 proved effective in ensuring microbiological safety, as confirmed

by the absence of *Salmonella spp.* and the low levels of *Escherichia coli* detected, in agreement with those reported by Labella *et al.* (2024).

Overall, the chemical and microbiological characteristics of the frass analyzed in the present study confirm its suitability as a safe and nutrient-rich organic fertilizer.

4.2 Evaluation of phytotoxicity of frass extract

The frass extract exhibited a dose-dependent effect on lettuce seed germination. The extract of frass obtained from the standard diet exhibited a biostimulant effect on the seed germination index (SGI) of lettuce at concentrations of 25% and 12.5%, as shown in Table 2. However, both concentrations did not differ significantly from the control. Similar stimulatory responses at low concentrations have been reported for natural biostimulants and organic extracts (Canellas *et al.*, 2015; Lucini *et al.*, 2015). Conversely, concentrations higher than 25% induced phytotoxic effects, causing a negative effect on SGI. This inhibitory response is likely related to the high electrical conductivity and salinity of the frass extract, which may generate osmotic stress during germination (Dawd and Abdulla, 2020; Yildirim *et al.*, 2022). Overall, these results confirm that frass extract can act as a seed biostimulant at appropriate dilutions, whereas excessive concentrations may impair germination due to salinity-related stress.

4.3 Germination test in cell trays

Another The suitability of frass as a growing medium component was further confirmed by the germination trials. Our results indicated that the inclusion of 15% frass represented the optimal dose, achieving the highest Germination Speed Index (GSI) among all treatments. The significantly lower GSI observed in the NPK-fertilized soil (0.220 ± 0.103) compared to the 15% frass treatment (0.369 ± 0.1) suggests that mineral fertilization at sowing may induce a high salt concentration in the rhizosphere. As reported by Machado *et al.* (2023), such conditions can lead to osmotic stress, which hinders water imbibition by the seeds and slows down the biochemical processes required for embryo activation, ultimately reducing seedling vigor. Fresh weight in the frass treatments was significantly higher than in the control, indicating that frass derived from the standard diet improved plant growth parameters (Lomonaco *et al.*, 2025). However, evidence from other studies highlights that increasing frass doses can negatively impact plant growth (Chiam *et al.*, 2021; Song *et al.*, 2021; Lomonaco *et al.*, 2025), with the threshold dose being strongly influenced by both the type of frass and the plant species used (Lomonaco

et al., 2024). When comparing seed emergence percentage and fresh seedling weight, the 15% inclusion level provided the best overall response.

4.4 Agronomic response of lettuce to BSFL Frass Treatments

The application of frass as a fertilizer for lettuce plants yielded promising results across the evaluated parameters (SPAD index, fresh shoot weight, shoot dry weight, root dry weight, plant height, root length, and crown width). The application rates of 10%, 15%, and 20% frass proved to be optimal when compared to the control. These findings are consistent with those reported by Setti *et al.* (2019), where the two tested dosages that most effectively enhanced lettuce growth were 10% and 20%. The study by Setti *et al.* (2019) provides an excellent point of comparison, as the initial diet administered to the BSFL was the same as the one used in our experiment. However, unlike Setti *et al.* (2019), in our study the frass was subjected to a heat-treatment (70 °C for 1 h) in accordance with current European regulations. The comparable results suggest that thermal treatment does not impair the agronomic effectiveness of frass at application rates of 10–20%.

Similar positive effects of black soldier fly frass on plant growth and biomass production have been reported in other studies. Anyega *et al.* (2021) demonstrated that frass-based fertilization significantly increased biomass, leaf chlorophyll content and nitrogen use efficiency in leafy vegetables, while Beesigamukama *et al.* (2020b) reported growth responses comparable to those obtained with mineral fertilizers.

The outcomes obtained at the end of the trial are particularly noteworthy, as frass supplementation produced results comparable to chemical fertilization (NPK) especially in terms of fresh and dry biomass, SPAD values, and root development. This finding is in agreement with previous studies reporting that insect frass can act as an effective biofertilizer and a sustainable alternative to conventional mineral fertilizers (Quilliam *et al.*, 2020; Beesigamukama *et al.*, 2022). Overall, the present results further support the agronomic potential of BSFL frass as viable alternative to conventional chemical fertilizers for lettuce cultivation.

4.5 Quantitative analysis on lettuce extracts

Plants can detect and respond to different environmental stimuli, such as agronomic practices and fertilization, changing synthesis and/or accumulation of polyphenols. In this work, lettuce extracts were prepared after 35 days of cultivation, which resulted as the optimal time for harvesting mature lettuce under greenhouse

conditions. This could give adequate time for the plants to fully respond to nutrient stress. The results demonstrated that the application of different fertilization treatments markedly influenced the phytochemical composition and antioxidant potential of lettuce extracts. Particularly the total phenolic content (TPC) increased significantly with higher frass percentage, reaching the maximum at 20% (28.87 mg GAE g^{-1} DW), suggesting that overall phenolic biosynthesis was enhanced under these conditions. The significant increase in TPC with higher frass rates is in line with previous reports showing that organic fertilization can enhance phenolic compound accumulation in lettuce, potentially due to a combination of nutrient availability and plant stress responses (Alkaabi *et al.*, 2025).

Conversely, total flavonoid content (TFC) exhibited a declining trend compared to the control, with values dropping from 24.23 mg QE g^{-1} DW in untreated plants to 12.73 mg QE g^{-1} DW at 20%, indicating a possible trade-off between phenolic and flavonoid pathways. Interestingly, also antioxidant activity (ABTS assay) decreased progressively with increasing frass percentage, with the highest value recorded in the control (749.37 mg TE g^{-1} DW). This apparent discrepancy between results can be explained by the fact that the Folin–Ciocalteu assay quantifies total phenolics but does not discriminate among individual phenolic subtypes (Platzer *et al.*, 2021). As reported by Cavalheiro *et al.* (2020) and Amarowicz *et al.* (2024), fertilization and nutrient-rich conditions may prioritize growth and promote the synthesis of phenolics with structural rather, while reducing the accumulation of secondary metabolites with antioxidant activity. Therefore, not flavonoids, but other phenolic compounds may have contributed to the increase in TPC, but this did not necessarily translate into higher antioxidant capacity. Lower TFC and ABTS values observed in samples of plants fertilized with frass may reflect reduced oxidative stress and better conditions during growth. Overall, these findings underscore the potential of BSFL frass and the importance of balancing nutrient input to optimize both yield and nutritional quality.

5. Conclusion

This study demonstrates a positive impact of BSFL frass, when applied at appropriate dosages, to growth parameters of Romaine lettuce. Optimal responses were observed at 15% inclusion, demonstrating frass as a good organic alternative to commercial NPK fertilizer for lettuce cultivation. The integration of frass allows to reduce the use and environmental impact of peat, conventionally used for the

preparation of commercial soils (Chavez and Uchanski, 2021). Moreover, frass application modulated secondary metabolism of lettuce, increasing total phenolic content while reducing flavonoid levels and antioxidant activity, highlighting a trade-off between yield and nutritional quality. Overall, these findings support the potential of BSFL frass as a sustainable biofertilizer, though careful optimization of dosage is essential to maximize agronomic benefits without compromising functional properties of the crop.

CHAPTER 6

Production and characterization of protein-based bioplastic films from Black Soldier Fly larvae and their preliminary evaluation as biodegradable mulching materials

Chapter 6 evaluates BSFL proteins as raw material for bioplastic applications. The chapter focuses on: (i) lipid removal and protein extraction from BSFL biomass, (ii) formulation and characterization of self-standing protein films, and (iii) preliminary assessment of film behavior under soil mulching conditions, including water evaporation control and macroscopic degradation. *(Editorial status: original, unpublished work conducted as part of this thesis; not submitted for publication at time of deposit.)*

1. Introduction

The unstoppable expansion of plastic need and production raises significant concerns regarding the handling and disposal of post-consumer waste, especially for single-use plastics that are difficult to collect and recycle. Complying with historical growth trends, global plastic manufacturing is projected to hit 1.1 billion tons by 2050 (Geyer, 2020). Alarmingly, around 85% of these plastics are discarded in landfills or unmanaged environments. Additionally, nearly 98% of conventional plastic products are manufactured from fossil-based raw materials (Houssini *et al.*, 2025). This scenario underscores the urgent need for alternative strategies to reduce the environmental impact of conventional plastic materials. Transitioning towards more sustainable solutions, such as bioplastics, has gained increasing attention in recent years. The term "bioplastics" includes bio-based, biodegradable or both materials (Shlush & Davidovich-Pinhas, 2022). These alternatives aim not only to reduce dependency on fossil resources but also to mitigate the accumulation of persistent plastic waste in terrestrial and marine ecosystems. However, despite their potential, bio-based plastics present challenges in terms of scalability, performance, cost, and end-of-life management. Moreover, plants are generally used as raw material in bioplastic manufacturing, leading to a possible competition with agricultural resources for food production (Abbate *et al.*, 2023). Proteins are increasingly being investigated as promising biopolymeric matrices for the development of biodegradable films and coatings (Pirsa & Aghbolagh Sharifi, 2020). Owing to their inherent ability to form cohesive networks through intermolecular interactions such as hydrogen bonding, disulfide bridges, and

hydrophobic interactions, proteins can generate self-standing films with tunable properties. Traditionally, proteins from animal (e.g., gelatin, casein) or plant (e.g., soy, wheat gluten, zein) sources have been employed for this purpose (Chen H. *et al.*, 2019). However, the growing need for circular and sustainable feedstocks is driving research toward unconventional protein sources. In this context, as extensively discussed in the previous chapters regarding the bioconversion efficiency and metabolic flexibility of *Hermetia illucens*, insect-derived proteins represent a renewable and resource-efficient alternative. Building on the nutritional characterization provided in Chapters 1 and 2, the resulting BSFL biomass is particularly rich in lipids and high biological value proteins, averaging 41% of dry matter (Lu *et al.*, 2022), making it an ideal biopolymeric matrix for material science applications.

Several studies have demonstrated the feasibility of producing biopolymer films using insect proteins, either alone or blended with other biopolymers. For example, Qoirinisa *et al.* (2022) produced edible films from grasshopper gelatin, while Zhang *et al.* (2022) developed protein-based films extracted from the migratory locust. Conversely, Kiiru *et al.* (2020) blended cricket flour with soy protein, and Chandran *et al.* (2021) incorporated Black Soldier Fly prepupae flour into composite bio-packaging films, showing how mixtures with other biopolymers can further enhance material properties. However, research on the optimization of processing parameters, film formulation, and functional properties is still in its early stages. In this paper, we explored the feasibility of producing bioplastic through insect-mediated bioconversion. Particularly, we propose the use of proteins extracted from BSFL as alternative and more sustainable raw material for the realization of self-standing bioplastic films.

In addition to packaging applications, protein-based bioplastic films may be exploited as biodegradable mulching materials in agriculture. Plastic mulches are extensively used to improve soil water conservation and microclimatic conditions; however, their widespread use contributes to plastic accumulation in soils due to incomplete removal and limited recyclability (Salama *et al.*, 2023). Biodegradable mulches produced from renewable and circular feedstocks represent a viable alternative, potentially minimizing environmental impacts while offering comparable agronomic benefits (Abbate *et al.*, 2023). In this study, the self-standing bioplastic films produced from BSFL proteins were investigated for their application as biodegradable mulching materials. By coupling insect-derived

biopolymers with a mulching application, this work addresses a biotechnological use of protein-based films that extends beyond material production and sustainability challenges in soil management practices.

2. Materials and methods

2.1 Insect rearing

Hermetia illucens larvae were purchased from Xflies s.r.l. (Potenza, Italy). BSFL were reared at 27 °C, 70% RH, 24 h dark on standard Gainesville diet (30% alfalfa, 50% wheat bran, 20% corn meal) (Hogsette, 1992) and were collected in the last larval stage. BSFL samples were washed by distilled water, stored at −20 °C for 1 h and then dried in a microwave (MAXINDUSTRIAL—Microwave Dryer MAXB-18B). The methodology of larval treatment after feeding refers to specific regulations of IPIFF (International Platform of Insects for Food and Feed), reported in the Guide on Good Hygiene Practices for European Union (EU), producers of insects as food and feed - November 2022.

2.2 Lipid extraction

BSFL contain around 30-35% lipid on dry matter (Fitriana *et al.*, 2022). High fat contents may interfere and be unfavourable for the next steps, so dried larvae were subjected to lipid extraction. Lipids were extracted by a two-step method. To reduce time and chemical usage, a mechanical extraction by benchtop oil press machine was used as first and efficient step to remove most of the fats. The oil press operates at 105 °C, squeezes the fat out of the larvae and produces a press cake. The press cake was easily manually grinded and subjected to a further refining step to remove the remaining fats. A cellulose extraction thimble was filled with two parts of mechanical defatted BSFL biomass and dipped in one part of petroleum ether (40–60 °C boiling point fraction) (weight/volume) for 4 hours. The thimble was removed from the solvent, the defatted pellet was recovered and dried at room temperature under chemical fume hood to remove the residual solvent.

2.3 Protein extraction

Proteins were extracted following the method reported by (Caligiani *et al.*, 2018) with some modifications. Defatted BSFL pellet was treated under stirring with 1 M sodium hydroxide (NaOH) (ratio 1:10 w/v) at 40 °C for 1 h. The supernatant containing proteins was recovered by centrifugation for 5 min at 4000 rpm and neutralized. Proteins were recovered by precipitation with 10% trichloroacetic acid

solution in acetone (ratio 1:1, v/v), previously prepared and stored at −20 °C. Sample was incubated overnight at −20 °C for the protein precipitation. Then the protein pellet was recovered by centrifugation at 4 °C (8000 rpm for 15 min), washed three times with acetone and dried in oven at 90 °C for 3 h.

2.4 Protein-based biofilm production

Proteins extracted from BSFL were used to test the realization of plastic biofilms using the Wet Casting technique. An appropriate protein solution was prepared and poured into Petri dishes, as molds. For the preparation, the BSFL proteins were finely ground with a mortar and dissolved at a concentration of 10% w/v in a 0,05M sodium dodecyl sulfate (SDS) solution in distilled water. To enhance the solubilisation of proteins, 1M NaOH was added to reach pH 10 and the prepared protein solution was kept under stirring at 70 °C for three hours. Subsequently, the solution was allowed to cool at room temperature, the pH value was rechecked and glycerol (Gly) was added as a plasticizing agent in different w/w percentages compared to the proteins (Table 1).

Sample	pH	SDS (M)	Glycerol (% w/w)
T1	10	0.05	10
T2	10	0.05	15
T3	10	0.05	17
T4	10	0.05	20

Table 1. Composition of the samples prepared at pH 10 with 0.05 M SDS and varying glycerol concentrations.

The solution thus prepared was finally poured into Petri dishes (10 cm diameter) used as molds for the drying phase. The films were dried overnight in an incubator (Innova 42R – New Brunswick, Eppendorf) at 25 °C and then stabilized at room temperature before being removed from the mold. Thickness of the samples was measured using a digital caliper.

2.5 Fourier transform infrared spectroscopy with Attenuated total reflection (ATR-FTIR) analysis

Chemical characterization of BSFL proteins and corresponding biofilms was obtained by Fourier transform infrared spectroscopy with Attenuated total

reflection (ATR-FTIR) analysis on a Spectrum Two FTIR Spectrometer (PerkinElmer) with a resolution of 2 cm−1 and with scanning from 4000 to 400 cm−1. A number of 16 scans were averaged; background spectra were acquired against air, and spectra baseline subtraction and ATR correction were performed using the instrument software. All IR-ATR experiments were performed in triplicate. Specifically, for bioplastic films, ATR-FTIR spectra were collected in triplicate on both sides of each sample, namely the surface in contact with the Petri dish during casting (DOWN) and the air-exposed surface (UP), in order to assess chemical homogeneity. The spectra reported in the Results section correspond to the average spectra of the three replicates for each surface.

2.6 Thermogravimetric analysis (TGA)

The water content and decomposition temperature of the samples were investigated through thermogravimetric analysis (TGA) using a Pyris 1 TGA (Perkin-Elmer Thermogravimetric Analyzer). The data were acquired using Pyris™ software and analyzed with Matlab 2024b (The MathWorks Inc.). The samples were heated at a rate of 30 °C/min over a temperature range of 25–600 °C under a continuous nitrogen flow (20 ml/min). All TGA experiments were performed in triplicate. The differential thermogravimetric (DTG) curves were obtained as the first derivative of the TGA data and smoothed using a centered moving average (7-point window, corresponding to approximately 3 °C).

2.7 Dynamic mechanical analysis (DMA)

The protein biofilm samples listed in Table 1 were analyzed through tensile dynamic mechanical analysis (DMA) measurements using a single motor stress-controlled rheometer (Anton Paar MCR 702e, Anton Paar). The specimen dimensions were, on average, 40 ± 1 mm length and 10 ± 1 mm width for all samples. The experiments were performed keeping the instrument room and the samples at 25 °C. Uniaxial tensile tests were performed with the bottom clamp fixed and the upper clamp moving at a speed of 0.05 mm/min, keeping the films in tension with an initial normal force of 0.15 N. This normal force was then converted into stress, σ, while the strain, γ, was derived from the elongation. From the stress-strain diagram, the Young's modulus, E* was calculated by linear regression of the elastic region in the strain range 0–0.3%, according to the following: $\sigma = E^* \gamma$. Moreover, deformation at break, γ_r, and tensile strength or breaking stress, σ_r, were also obtained from the stress-strain curve. All tensile experiments were performed in

triplicate. All tensile and torsional data were acquired using the Anton Paar RheoCompass software 1.32 (Anton Paar, Austria) and analyzed with Matlab 2024b (The MathWorks Inc., USA). Statistical analysis was performed using GraphPad Prism 8.4.2 (GraphPad Software, USA). Data are reported as mean ± standard deviation, and comparisons between samples T2 and T3 were carried out using an unpaired Student's t-test with Welch's correction ($p < 0.05$).

2.8 Mulching preliminary test

A preliminary pot experiment was carried out to investigate the potential mulching effect of BSFL protein-based bioplastic films, with particular emphasis on their ability to reduce soil water evaporation under controlled environmental conditions. In addition, film behavior during soil contact was visually monitored to assess early-stage biodegradation, while soil electrical conductivity (EC) was measured at the end of the experiment as a complementary parameter to evaluate possible material–soil interactions.

After the chemical, thermal, and mechanical characterization of all film formulations described above, sample T2 was selected for the mulching experiment. This selection was based on its adequate mechanical integrity and handling properties, as T1 was too brittle and T4 excessively sticky. Although T3 exhibited properties comparable to those of T2, T2 was preferred due to its lower glycerol content.

2.8.1 Soil water evaporation assessment

Soil water evaporation was assessed using a gravimetric approach in a controlled pot experiment. Square plastic pots (10 × 10 cm) were filled with approximately 950 g of substrate, consisting either of commercial potting soil alone or commercial potting soil mixed with washed white quartz sand at a 1:1 (w/w) ratio. After filling, all pots were irrigated with distilled water until field capacity was reached and allowed to equilibrate overnight to ensure uniform water distribution within the substrate. The exact weight of each pot at field capacity was recorded and used as the reference value for subsequent gravimetric calculations. Protein-based bioplastic films (sample T2) were cut to match the pot surface area (10 × 10 cm) and applied so as to completely cover the substrate surface, avoiding uncovered areas that could allow direct water evaporation. Three experimental conditions were tested: unmulched pots used as controls (CTRL), pots mulched with the film placed on the soil surface (A), and pots mulched with the film buried at a depth of

approximately 1 cm below the substrate surface (B), leaving the soil surface uncovered while limiting direct exposure of the film to air and light. Each treatment was applied to both substrate types. Treatments involving the soil–sand mixture were identified by the suffix "–S" (i.e., CTRL-S, A-S, and B-S).

All pots were maintained in a controlled-environment growth chamber (MRL352H, PHCbi), programmed to simulate a 24 h summer day–night cycle. Temperature, relative humidity conditions and light conditions were set according to the daily cycle reported in Table 2 and kept constant throughout the experimental period.

Period	**12 h**	**4 h**	**4h**	**4h**
Temperature	20 °C	25 °C	30 °C	25 °C
Relative humidity	50	50	50	50
Lux	0	5000	22000	5000

Table 2. Daily temperature, relative humidity, and light intensity settings used in the controlled-environment chamber to simulate a 24 h summer day–night cycle during the pot experiment.

Pots were weighed daily at the same time of day for a total experimental duration of 20 days using a digital balance with a sensitivity of 1 g. Measurements were performed on a daily basis. Gravimetric data were processed to quantify soil water loss over time. Cumulative water loss was calculated according to the following equation:

$$Water\ loss\ (\%) = \frac{W_o - W_t}{W_o - W_{dry}} \times 100$$

Where W_0 is the pot weight at field capacity, W_t is the pot weight at time t, and W_{dry} is the average dry weight of the corresponding substrate. Water loss was thus expressed as percentage of the initial water content. To estimate the dry weight of the substrates, three replicates of each substrate type were dried in a ventilated oven at 80 °C for 24 h until constant weight.

Water loss data were analyzed using GraphPad Prism 8.4.2 (GraphPad Software, USA). Cumulative water loss values were analyzed by two-way analysis of variance (ANOVA) considering substrate type and mulching treatment as independent factors. When significant effects were detected ($p < 0.05$), multiple

comparisons were performed using Tukey's *post hoc* test. Data are reported as mean ± standard deviation.

2.8.2 Macroscopic assessment of film behavior

The macroscopic behavior of the BSFL protein-based bioplastic films during soil contact was qualitatively evaluated by visual inspection and photographic documentation to assess early-stage biodegradation phenomena. The qualitative assessment focused on observable macroscopic changes, including loss of structural integrity, surface alterations, fragmentation, and overall film continuity. Visual monitoring was performed only for films applied on the soil surface (treatment A), as these films remained directly exposed and accessible throughout the experiment. In contrast, films buried below the soil surface (treatment B) could not be inspected during the experimental period and were visually evaluated only at the end of the test, after removal from the substrate.

2.8.3 Soil electrical conductivity assessment

Soil electrical conductivity (EC) was determined at the end of the mulching experiment as a complementary parameter to evaluate possible interactions between the protein-based bioplastic films and the soil matrix. EC was determined on aqueous soil extracts following the FAO standard operating procedure for soil electrical conductivity (soil/water ratio 1:5, w/v) (FAO, 2021). At the end of the experimental period, soil samples were collected from each pot as composite samples obtained from multiple subsamples taken at different depths to ensure representativeness. For each sample, 40 g of fresh soil were mixed with 200 mL of distilled water and mechanically agitated for 30 min. The suspension was then allowed to settle, and EC was measured in the supernatant. EC measurements were performed using a multi-parameter RS485 soil sensor (DFRobot), operating in the range 0–20,000 µS cm^{-1} with a resolution of 1 µS cm^{-1} and automatic temperature compensation. Prior to measurements, the sensor was externally calibrated using a potassium chloride (KCl) standard solution. All measurements were conducted at approximately 25 °C to minimize temperature-related variability. EC values were expressed as decisiemens per meter (dS m^{-1}) after conversion from µS cm^{-1} (1 dS m^{-1} = 1000 µS cm^{-1}). Statistical analysis was performed using GraphPad Prism 8.4.2 (GraphPad Software, USA). EC data were analyzed by two-way analysis of variance (ANOVA), considering substrate type and mulching treatment as independent factors. When significant effects were detected ($p < 0.05$), Tukey's

multiple comparisons test was applied. Results are reported as mean ± standard deviation.

3. Results

3.1 Lipid removal and protein extraction yield

The lipid content of *Hermetia illucens* larvae was determined on a dry matter (DM) basis, and the efficiency of the two-step defatting procedure is reported in Table 3. The initial mechanical extraction performed using the oil press resulted in the removal of 21.5 g lipid per 100 g of larvae DM. An additional 1.6 g of lipid per 100 g of larvae press cake was recovered by subsequent solvent extraction with petroleum ether. Overall, the combined extraction process led to a total lipid removal of 23.1 g per 100 g of larvae DM.

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Extraction step	Lipid (g/100g DM)
Mechanical extraction	21.5
Solvent extraction	1.6
Total lipid extracted	23.1

Table 3. Lipid extraction results: yields from mechanical extraction and subsequent maceration of BSFL on a dry matter basis.

Protein extraction from defatted BSFL powder, carried out according to the modified protocol proposed by Caligiani *et al.* (2018), resulted in an average protein recovery yield of 40.9 ± 3.3%. The extracted proteins were successfully employed for the production of self-standing bioplastic films by the wet casting technique.

3.2 Film formation and macroscopic appearance

After drying and detachment from the molds, the obtained bioplastic films exhibited distinct macroscopic characteristics depending on the glycerol content (Figure 1). Sample T1, containing 10% (w/w) glycerol relative to protein content, resulted in rigid and highly brittle films. Conversely, sample T4, formulated with 20% glycerol, appeared markedly sticky and difficult to handle due to the high plasticizer concentration. Among the tested formulations, sample T2 (15% glycerol) exhibited

the most suitable balance between flexibility and workability, allowing easy handling without mechanical failure.

All films appeared opaque and displayed a light brownish coloration. The average thickness of the films was 0.10 ± 0.02 mm, with minor local variations across the samples.

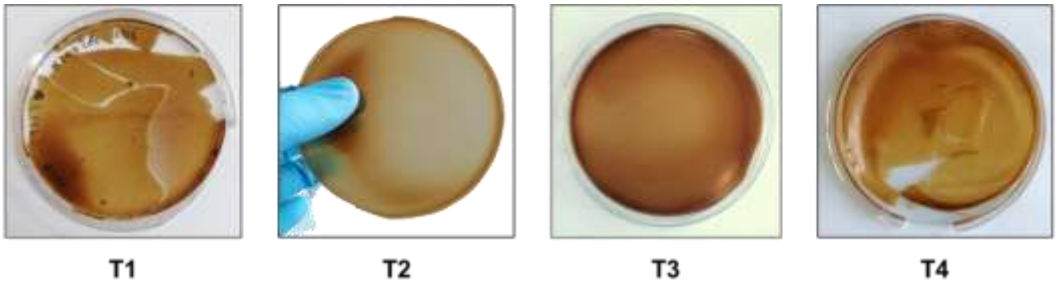

Figure 1. Representative photographs of insect protein-based bioplastic films prepared with increasing glycerol concentrations: T1 (10%), T2 (15%), T3 (17%), and T4 (20%).

3.3 ATR-FTIR characterization of BSFL protein powder and biofilms

The ATR-FTIR spectrum of the protein powder extracted from *Hermetia illucens* larvae is reported in Figure 2. The spectrum exhibits the characteristic absorption bands of protein-based materials. A broad band centered at approximately 3300 cm^{-1} was observed and is attributed to O–H and N–H stretching vibrations, associated with hydrogen-bonded hydroxyl groups and amide A of proteins. In the region between 3000 and 2800 cm^{-1}, weak absorption bands corresponding to C–H stretching vibrations of aliphatic groups were detected. The most prominent feature of the spectrum is the intense absorption band located at around 1650 cm^{-1}, assigned to the amide I band, mainly arising from C=O stretching vibrations of the peptide backbone. A second well-defined band at approximately 1540 cm^{-1} corresponds to the amide II band, associated with N–H bending coupled with C–N stretching vibrations. Additional absorption bands were observed in the range between 1450 and 1400 cm^{-1}, which can be attributed to CH_2 and CH_3 bending vibrations, as well as to contributions from side-chain functional groups. The amide III region, extending between 1300 and 1200 cm^{-1}, showed multiple overlapping bands related to C–N stretching and N–H bending vibrations. Furthermore, absorption features below 1200 cm^{-1} were detected, associated with C–O and C–C stretching vibrations of amino acid side chains. Overall, the ATR-FTIR spectrum confirms the protein

nature of the extracted material and indicates the preservation of the characteristic functional groups of proteins after the extraction process.

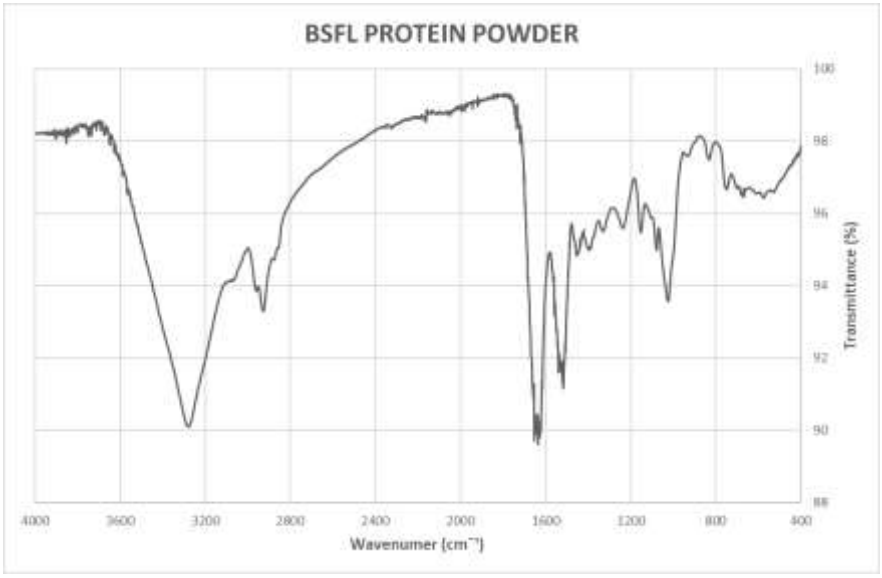

Figure 2. ATR-FTIR spectrum of protein powder extracted from *Hermetia illucens* larvae after lipid removal and alkaline extraction.

The ATR-FTIR spectra of BSFL protein-based bioplastic films prepared with different glycerol concentrations (T1–T4) are reported in Figure 3. The figure shows the average spectra collected from the surface in contact with the Petri dish during casting (DOWN, Figure 3a) and from the air-exposed surface during drying (UP, Figure 3b). For all formulations, spectra recorded on both sides of the films were highly reproducible and displayed comparable spectral profiles, indicating a homogeneous chemical composition across the film thickness. Minor differences in spectral variability between the two surfaces might be attributed to the casting process; the surface in contact with the Petri dish (DOWN) may experience slight local fluctuations in plasticizer distribution or molecular orientation due to interfacial interactions with the support, whereas the air-exposed surface (UP) undergoes more uniform solvent evaporation during the drying phase. All samples exhibited the characteristic absorption bands of protein-based materials. A broad absorption band centered around 3300 cm^{-1}, attributed to overlapping O–H and N–H stretching vibrations, was observed in all films. The intensity of this band showed a slight increase with increasing glycerol content, while its position remained unchanged. The amide I band, located at approximately 1650 cm^{-1}, and the amide II band, around 1540 cm^{-1}, were clearly detected in all spectra, confirming the preservation of the protein backbone after film formation. Additional absorption

bands in the range between 1450 and 1400 cm^{-1} were associated with CH_2 and CH_3 bending vibrations, whereas the amide III region (1300–1200 cm^{-1}) displayed multiple contributions related to C–N stretching and N–H bending modes. No new absorption bands or significant peak shifts were observed among films prepared with different glycerol concentrations on either surface. Overall, the ATR-FTIR results indicate that glycerol acted as a physical plasticizer, influencing hydrogen-bond-related interactions without altering the chemical structure of the protein matrix.

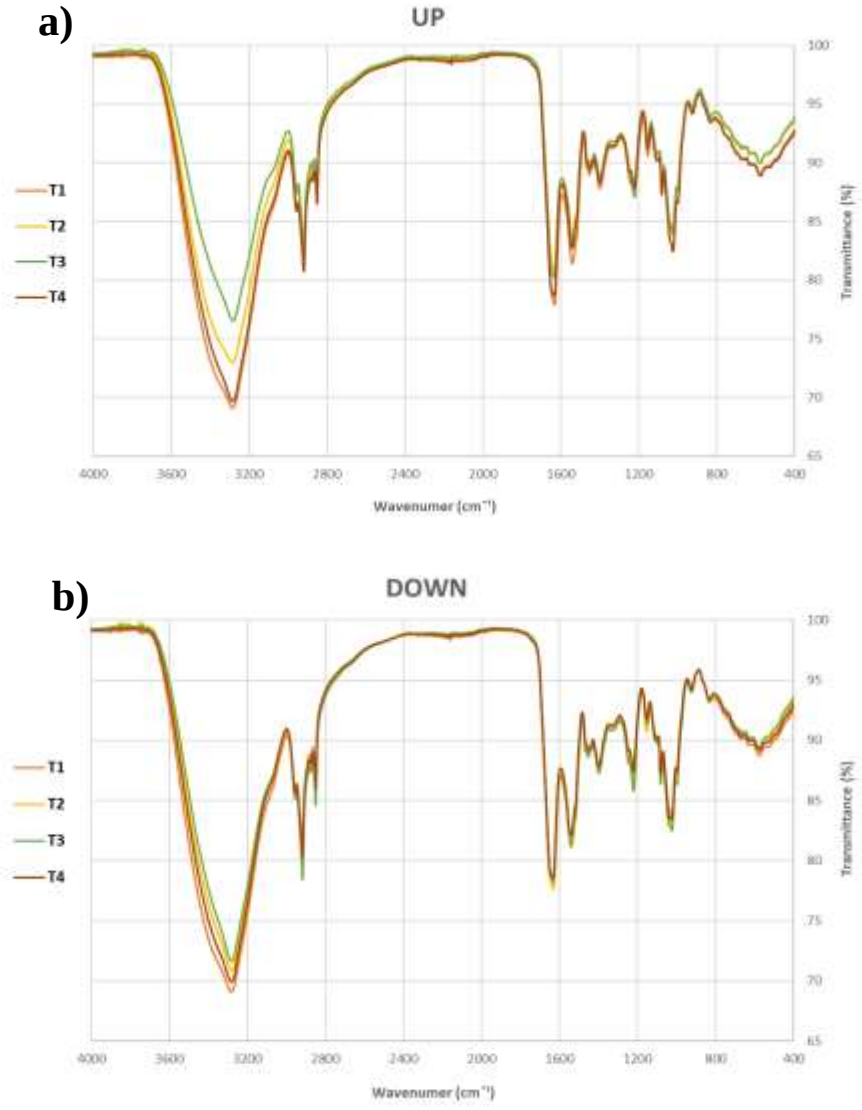

Figure 3. ATR-FTIR spectra of BSFL protein-based bioplastic films prepared with different glycerol concentrations (T1: 10%, T2: 15%, T3: 17%, T4: 20%). Spectra are shown as averages of three measurements collected from **a)** the surface in contact with the Petri dish during casting (DOWN) and **b)** the air-exposed surface during drying (UP).

3.4 Thermogravimetric analysis

The TGA profile of the BSFL protein powder shows a multi-step thermal degradation behavior (Fig. 4a). A first mass loss is observed at low temperatures, below approximately 150 °C, corresponding to a minor decrease in sample mass. This initial event is followed by a major degradation step occurring between approximately 250 °C and 400 °C, where a sharp mass reduction is recorded. The most pronounced weight loss takes place in this temperature interval, after which the degradation rate progressively decreases. At the end of the thermal treatment, a residual mass of approximately 30% is retained at 600 °C. Differential thermogravimetric (DTG) analysis of the raw protein powder was performed to allow a clearer identification of the individual degradation events observed in the TGA curve. Minor DTG peaks are detected at approximately 72 and 145 °C, followed by an additional event centered around 257 °C, while the main degradation process is associated with a pronounced DTG minimum at approximately 366 °C (Fig. 4b).

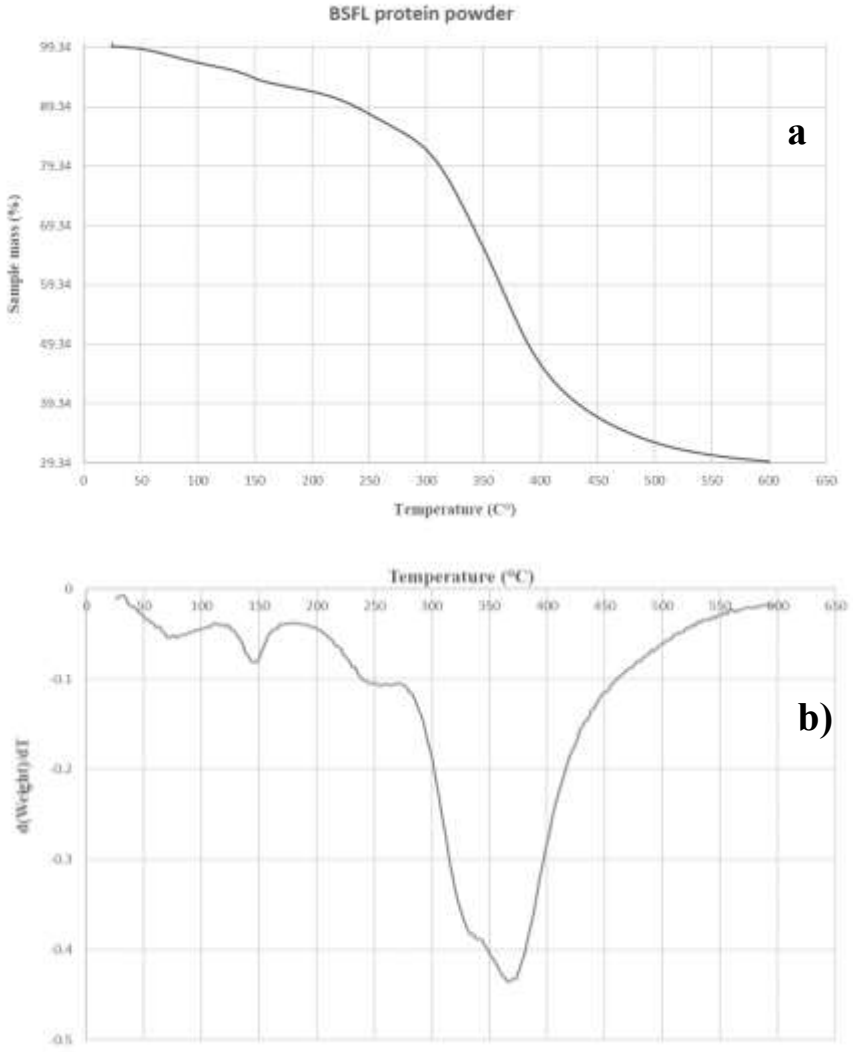

Figure 4. Thermal degradation behavior of the BSFL protein powder: **a)** TGA curve of the analyzed sample and **b)** relative DTG curve

The TGA curves of samples T1–T4, reported together in Fig. 5a, display comparable thermal degradation patterns, characterized by an initial low-temperature mass loss followed by a main decomposition stage at higher temperatures. All samples exhibit their primary degradation event within the temperature range of approximately 250–380 °C. Despite the similar overall trends, differences among the samples are evident in terms of mass retention throughout the heating process. Sample T1 consistently retains a higher percentage of mass compared to the other samples, while T2, T3, and T4 show progressively lower residual masses as the temperature increases. At 600 °C, the residual mass differs among the samples, indicating variations in thermal stability under identical experimental conditions. The DTG curves of the protein-based films (Fig. 5b) further confirm these observations, revealing three main degradation events

common to all formulations. An initial DTG minimum is observed at approximately 100–107 °C, followed by a dominant degradation peak centered in the range of 332–338 °C. A third degradation event is detected at higher temperatures, between approximately 572 and 593 °C. Only the main DTG peaks were considered for the film samples, as minor features did not correspond to clearly distinguishable changes in the slope of the corresponding TGA curves. The temperatures of the DTG minima are summarized in Table 4.

	T1	**T2**	**T3**	**T4**
First Peak	107	103	100	100
Second Peak	332	334	338	334
Third Peak	572	593	588	

Table 4. Temperatures of the main differential thermogravimetric (DTG) minima for protein-based films T1–T4, expressed in degrees Celsius (°C), as determined from the relative curves.

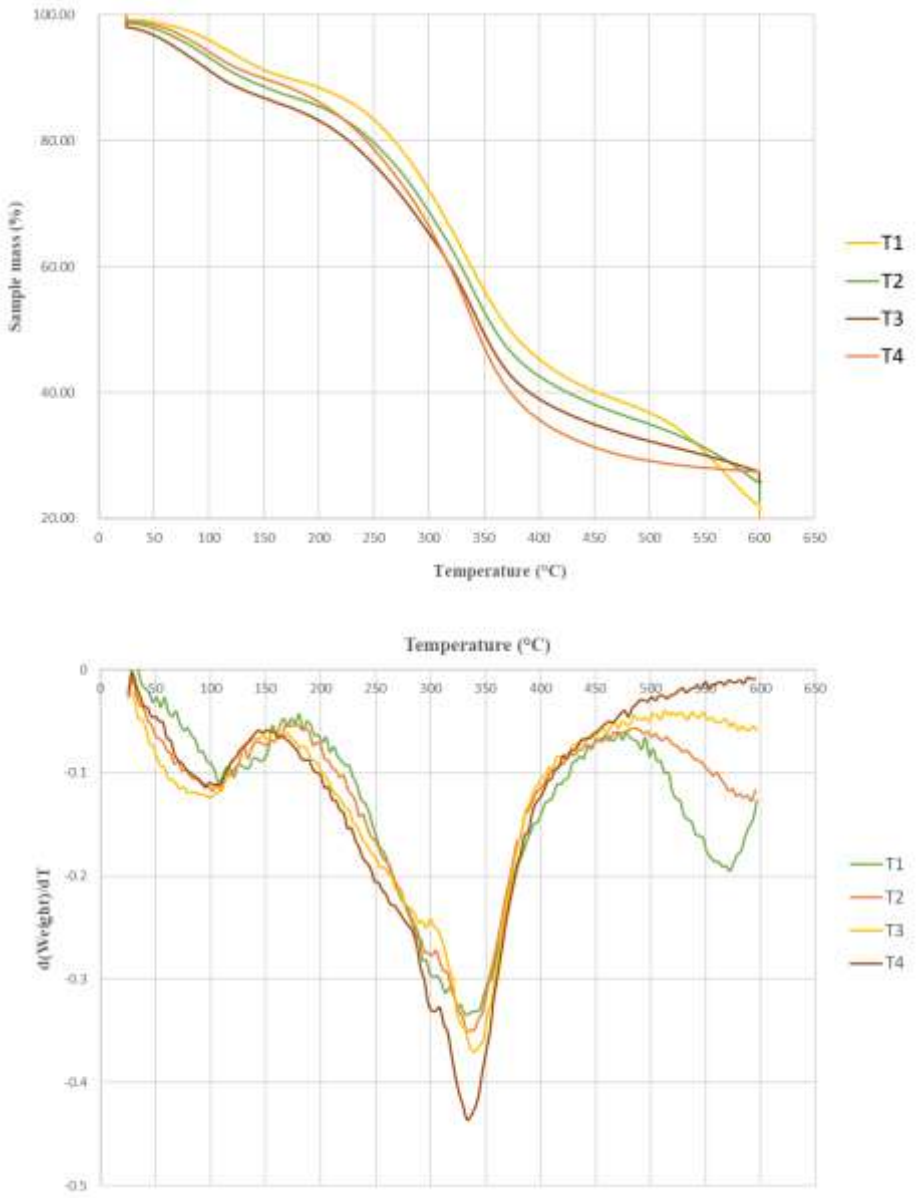

Figure 5. Thermal degradation behavior of the analyzed samples: **a)** TGA curves of samples T1–T4 and **b)** relative DTG curves.

3.5 Tensile dynamic mechanical analysis

Tensile dynamic mechanical analysis was performed to evaluate the mechanical response of the protein-based films. Sample T1 was not analyzed due to its rigid and highly brittle behavior, which prevented reliable tensile testing, while sample T4 could not be consistently characterized because of its marked stickiness and poor handling, allowing only a single, non-reproducible measurement. Therefore, quantitative tensile properties were determined only for samples T2 and T3. The stress–strain curves of samples T2 and T3 exhibit a non-linear mechanical response, characterized by an initial elastic region followed by progressive strain hardening

until rupture (Fig. 6). Both samples show a smooth increase in extensional stress with increasing strain, indicating a ductile deformation behavior. Differences in deformation at break are evident depending on the sampling position within the films (border, intermediate, and center), with the central regions generally exhibiting higher elongation before failure. Sample T3 displays higher extensibility, with strain at break values reaching up to approximately 7% in the central region, while the border and intermediate areas fail at lower strains. In contrast, sample T2 shows reduced elongation at break, particularly in the border and intermediate regions, whereas the central region sustains deformation up to approximately 5.5% strain before rupture. The maximum extensional stress achieved prior to failure is comparable between the two samples, with values in the range of approximately 2–3 MPa. The Young's modulus (E*), calculated from the linear fit of the elastic region in the strain range 0–0.3%, is similar for the two samples (Table 5), with values of 225.7 ± 58.1 MPa for T2 and 208.1 ± 44.9 MPa for T3. Statistical analysis showed no statistically significant differences ($p > 0.05$) between samples T2 and T3 for Young's modulus, extensional stress at break, and extensional strain at break. For sample T4, the single tensile test yielded an extensional stress at break of 2.861 MPa, an extensional strain at break of 1.749%, and a Young's modulus of 321.1 MPa; however, these values are reported for descriptive purposes only due to the lack of replicates.

Biofilm	Extensional Stress (MPa)	Extensional Strain %	Young's module
T2	1,973 ± 0,375	2,659 ± 2,448	225,7 ± 58,1
T3	1,865 ± 0,225	5,477 ± 1,637	208,1 ± 44,9
T4	2,861	1,749	321,1

Table 5. Tensile mechanical properties of protein-based biofilms obtained from DMA. Values for T2 and T3 are reported as mean ± standard deviation (n = 3); no statistically significant differences were observed between T2 and T3 ($p > 0.05$). Data for T4 are reported for completeness only and were not included in the statistical analysis.

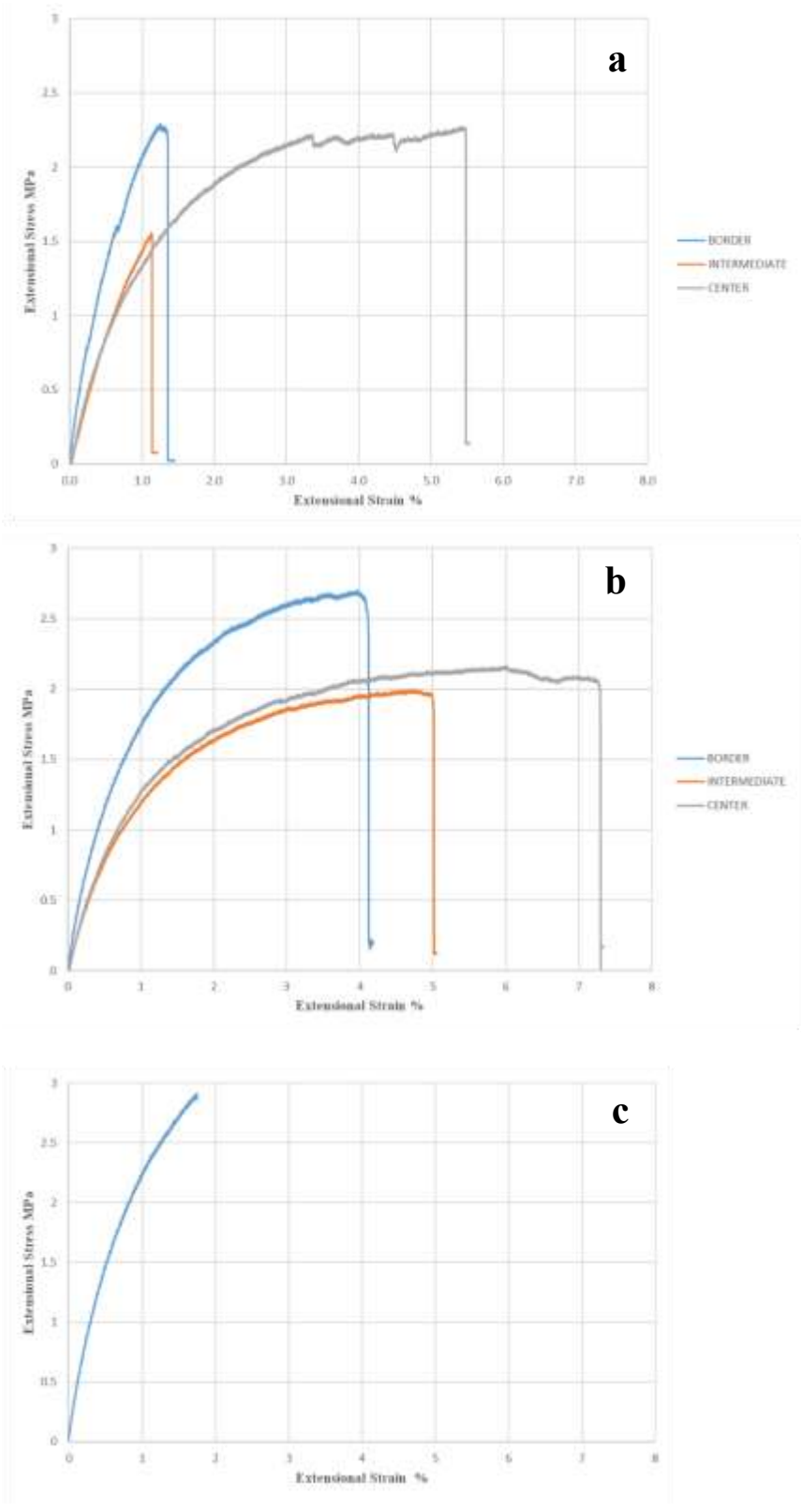

Figure 6. Extensional stress–strain curves obtained from tensile dynamic mechanical analysis of protein-based films T2 (**a**), T3 (**b**), and T4 (**c**). For samples T2 and T3, curves correspond to measurements performed at the border, intermediate, and central regions of the films, while the curve for sample T4 refers to a single measurement due to handling limitations.

3.6 Mulching preliminary test

3.6.1 Soil water evaporation assessment

Soil water evaporation was evaluated by monitoring cumulative water loss over time under controlled environmental conditions. After the first week (Fig. 7a), cumulative water loss was significantly affected by the mulching treatment, whereas no significant effect of substrate type or substrate × treatment interaction was detected. In commercial soil, cumulative water loss decreased from 59.2 ± 4.2% in unmulched controls (CTRL) to 52.1 ± 3.7% and 46.8 ± 7.0% for surface-applied (A) and buried films (B), respectively. A similar trend was observed in the soil–sand mixture, where water loss decreased from 66.1 ± 6.0% in CTRL-S to 57.9 ± 4.6% in A-S and 45.8 ± 6.9% in B-S. Statistical analysis indicated that buried films (B) resulted in significantly lower water loss compared to unmulched controls, while surface-applied films (A) showed intermediate values and did not differ significantly from CTRL.

After two weeks of incubation (Fig. 7b), cumulative water loss increased in all treatments. Statistical analysis revealed significant main effects of both mulching treatment and substrate type, whereas no significant interaction between these factors was detected. In commercial soil, water loss reached 78.2 ± 3.0% in CTRL, compared to 77.5 ± 2.4% in A and 70.6 ± 2.9% in B. Higher overall losses were recorded in the soil–sand mixture, with values of 86.4 ± 2.4% for CTRL-S, 80.9 ± 1.1% for A-S, and 73.7 ± 5.2% for B-S. Overall, buried films (B) consistently resulted in the lowest water loss, whereas surface-applied films (A) exhibited intermediate behavior between buried films and unmulched controls. Independently of the mulching treatment, the soil–sand mixture showed significantly higher cumulative water loss than commercial soil.

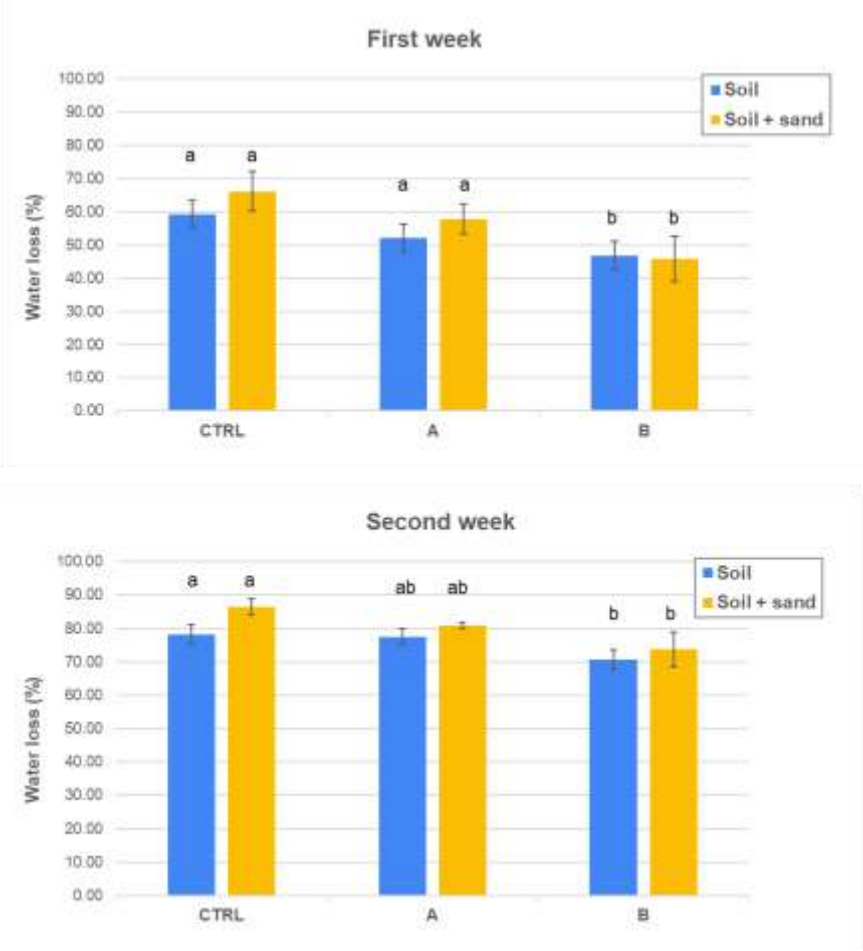

Figure 7. Cumulative soil water loss (%) after one week (a) and two weeks (b) of incubation under controlled environmental conditions for unmulched pots (CTRL), surface-applied bioplastic film (A), and buried bioplastic film (B) in commercial soil and in a soil–sand mixture. Data are reported as mean ± standard deviation (n = 5). Different letters indicate statistically significant differences among mulching treatments (two-way ANOVA followed by Tukey's *post hoc* test, $p < 0.05$). No significant substrate × treatment interaction was detected at either time point.

3.6.2 Macroscopic assessment of film behavior

Macroscopic changes in film integrity were visually monitored throughout the experiment to qualitatively assess early-stage degradation phenomena (Figure 8). Surface-applied films (treatment A) showed evident structural alterations already after the first day of soil contact. These initial changes consisted of localized swelling and surface wrinkling, while the films maintained their original square shape and continued to fully cover the pot surface without leaving uncovered areas along the edges. As the experiment progressed, these alterations became increasingly pronounced. By mid-experiment, the films exhibited the appearance of the first visible pores and partial loss of adhesion along the pot borders, resulting

in small uncovered areas of the substrate (Figure 8.2). At the end of the experimental period, surface-applied films still retained their original macroscopic shape but appeared highly heterogeneous, characterized by widespread porosity, fractures, and loss of structural continuity. Once removed from the substrate, the films showed a complete loss of the initial elastic behavior, appearing brittle and glassy to the touch, with soil particles firmly embedded within the polymer matrix (Figure 8.3). These macroscopic degradation features were more evident in films applied on the soil + sand mixture, where fragmentation and vitrification phenomena appeared more extensive (Figure 8.4). In contrast, buried films (treatment B) could only be assessed at the end of the experiment. After removing the superficial substrate layer, no recognizable film structure could be recovered in any replicate. Instead, a thin, highly fragile, vitrified substrate layer was observed at the expected film position. This material showed no distinguishable film shape, color, or continuity, preventing photographic documentation. The absence of intact film remnants suggests a more advanced macroscopic alteration under buried conditions compared to surface application.

Quantitative mass loss of the films was not determined, as extensive substrate incorporation and vitrification prevented reliable separation of the film from the soil matrix at the end of the experiment.

This assessment was intended as a qualitative, preliminary evaluation of macroscopic changes only and does not aim to quantify biodegradation rates or identify underlying degradation mechanisms

Figure 8. Macroscopic evolution of the BSFL protein-based bioplastic film (T2) during the mulching experiment for treatment A (surface application). 1) Film at day 0; 2) film at mid-experiment; 3) film replicates recovered from commercial soil at the end of the experiment; 4) film replicates recovered from the soil + sand (1:1, w/w) substrate at the end of the experiment. Progressive structural alteration and substrate incorporation are evident over time.

3.6.3 Soil electrical conductivity assessment

Soil electrical conductivity (EC), measured at the end of the experiment using the soil–water extract method (1:5, w/v), is reported in Figure 9. EC values were generally low for all treatments, remaining below 0.5 dS m^{-1}, indicating non-saline conditions for both substrates. Statistical analysis revealed a significant interaction between substrate type and mulching treatment ($p = 0.0009$), indicating that the effect of the bioplastic film on EC depended on the substrate composition. Consequently, comparisons were conducted separately for each substrate. For the commercial soil, EC increased progressively from the unmulched control (0.097 ± 0.011 dS m^{-1}) to surface mulching (A, 0.190 ± 0.005 dS m^{-1}) and buried film application (B, 0.365 ± 0.021 dS m^{-1}), with all pairwise differences being statistically significant ($p < 0.05$). A similar trend was observed for the soil–sand

mixture, where EC values increased from CTRL-S (≈0 dS m^{-1}) to A-S (0.113 ± 0.039 dS m^{-1}) and further to B-S (0.481 ± 0.085 dS m^{-1}), with significant differences among all treatments ($p < 0.05$). Within each substrate, EC values were highest in buried-film treatments (B and B-S), followed by surface-applied films and unmulched controls. These results suggest that film placement and soil contact conditions influenced solute availability at the end of the test, likely as a consequence of film–soil interactions occurring during the experimental period.

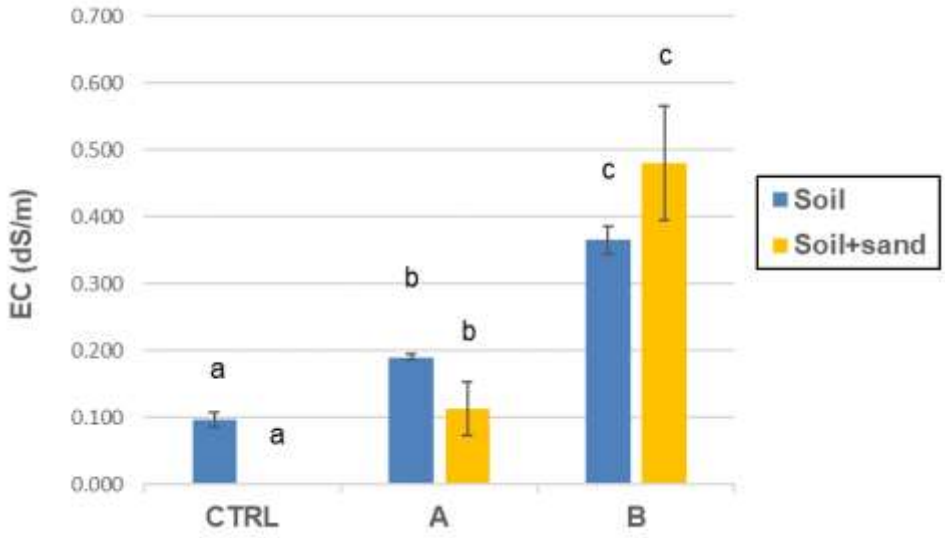

Figure 9. Soil electrical conductivity (EC) at the end of the mulching experiment. Data are expressed as mean ± SD (n = 3). Different letters indicate statistically significant differences among treatments within the same substrate (two-way ANOVA and Tukey's *post hoc* test, $p < 0.05$).

4. Discussion

4.1 Lipid removal and protein extraction yield

Mechanical pressing recovered the majority of BSFL lipids, with only a small fraction extracted by petroleum ether, likely representing lipids tightly associated with structural components. This aligns with previous reports showing that mechanical extraction captures most accessible fat, while solvent extraction targets the residual fraction (Franco *et al.*, 2021; Srisuksai *et al.*, 2024). Combining mechanical pressing with a mild solvent step represents a balanced approach that solvent use and associated environmental concerns while maintaining sufficient defatting efficiency for downstream protein extraction and material processing (Marasca *et al.*, 2025). The subsequent alkaline-based protein extraction yielded

approximately 41% protein, in line with reported extraction efficiencies for BSFL and other insects using similar solubilization–precipitation methods. Alkaline treatments are widely used given their capacity to increase protein solubility and promote partial unfolding; this partial denaturation can be advantageous for material applications, as exposed functional groups facilitate intermolecular interactions and network formation during film casting (Peydayesh *et al.*, 2022). These results support the suitability of an integrated extraction strategy to obtain BSFL protein fractions for film production, in line with insect biorefinery approaches aimed at maximizing resource valorization through sequential fractionation (Caligiani *et al.*, 2018).

4.2 Film formation and macroscopic appearance

The macroscopic properties of the BSFL protein films were strongly dependent on glycerol content, confirming the critical role of plasticizer concentration in tailoring protein film performance. Low glycerol content (T1) resulted in rigid, brittle films, consistent with reduced chain mobility and limited plasticization observed in other protein-based materials (Lyu *et al.*, 2022). In contrast, high plasticizer levels (T4) produced overly sticky films, reflecting excessive disruption of protein–protein interactions, as also reported for glycerol-plasticized protein and polysaccharide films (Kowalczyk *et al.*, 2024). The intermediate glycerol formulation (T2) achieved the best balance between flexibility and handling, indicating an optimal plasticizer window, a common observation in biodegradable protein films where excessive or insufficient plasticizer impairs mechanical performance (Vieira *et al.*, 2011). The opaque appearance and light brown coloration of all films are attributable to the intrinsic color of insect-derived proteins and to thermal treatment during alkaline solubilization and casting, in agreement with previous reports on insect- and protein-based bioplastics (Zhang *et al.*, 2022; Chandran *et al.*, 2021). The films exhibited an average thickness of 0.10 ± 0.02 mm, although noticeable spatial variations were detected across their surface. The observed film morphology is consistent with heterogeneous drying dynamics and the well-documented coffee-ring effect, in which differential evaporation rates induce capillary flows that drive suspended particles toward the droplet perimeter during drying (Routh, 2013).

4.3 ATR-FTIR characterization of BSFL protein powder and biofilms

ATR-FTIR analysis confirmed the proteic nature of the extracted BSFL material and demonstrated the preservation of the main functional groups after film formation. The presence of well-defined amide I (~1650 cm^{-1}) and amide II (~1540 cm^{-1}) bands in both the protein powder and the bioplastic films indicates that the peptide backbone remained chemically intact throughout alkaline extraction and wet casting, in agreement with previous reports on insect- and protein-based biopolymer systems (Zhang *et al.*, 2022). Across all films, the broad absorption band centered around 3300 cm^{-1} (O–H and N–H stretching vibrations), showed a slight increase in intensity with increasing glycerol content. Importantly, no significant peak shifts were observed, suggesting that glycerol primarily interacted with the protein matrix through hydrogen bonding without inducing chemical modifications, acting as a physical plasticizer. Similar FTIR responses have been reported for glycerol-plasticized protein films, where increased hydrogen bonding is reflected by enhanced band intensity rather than the appearance of new absorption features (Kowalczyk *et al.*, 2024). Despite the non-uniform drying dynamics discussed in the macroscopic analysis, comparable ATR-FTIR profiles on both sides of cast protein films (UP and DOWN) are generally interpreted as evidence of molecular homogeneity and organization across the film (Dordevic *et al.*, 2021).

4.4 Thermogravimetric analysis

TGA analysis revealed a multi-step degradation behavior typical of protein-based materials and their plasticized films, consistent with the presence of water, plasticizer, surfactant residues, and the protein matrix. The initial mass loss observed below ~120 °C is attributable to the evaporation of free and weakly bound water, a common feature in protein-based materials and biopolymer films due to their hygroscopic nature (Fernández-Sánchez *et al.*, 2024). The main degradation step was detected between ~200 and 380 °C. The first part of this event around ~180–230 °C can be associated with the thermal decomposition of glycerol used as plasticizer. Glycerol is known to undergo dehydration and fragmentation reactions in this temperature range (Almazrouei *et al.*, 2022). The increased mass loss observed for films with higher glycerol content is consistent with this behavior. DTG minima centered around 330–370 °C, correspond to the thermal decomposition of the protein backbone. Protein-based films exhibited comparable degradation profiles to the raw BSFL protein powder, indicating that alkaline

extraction and wet casting did not compromise the intrinsic thermal stability of the protein matrix. This also aligns well with reported degradation temperatures for insect-derived proteins and protein-based edible films (Fernández-Sánchez *et al.*, 2024). Contributions from residual surfactant (SDS), which is known to decompose in a similar temperature window, may also partially overlap with this event (Ramimoghadam *et al.*, 2012). The residual mass at 600 °C for the samples is indicative of char formation and inorganic residues, commonly observed in nitrogen-rich biopolymers (Ricciardi *et al.*, 2025).

4.5 Tensile dynamic mechanical analysis

DMA measurements of protein-based bioplastic films revealed that the incorporation of an optimal amount of glycerol (sample T2, 15% w/w) resulted in ductile deformation behavior with appreciable strain at break and tensile strength. formulations with too little (T1) or too much glycerol (T4) impaired reliable tensile testing. Protein-based films exhibited a non-linear tensile response, consisting of an initial elastic region followed by progressive strain hardening and eventual failure, consistent with trends reported for other protein-derived biopolymers in the literature (Shah *et al.*, 2023). The Young's modulus values obtained for T2 and T3 (225.7 ± 58.1 MPa and 208.1 ± 44.9 MPa, respectively) fall within the range typically observed for edible films derived from plant and animal proteins, which are often in the order of tens to hundreds of MPa depending on formulation and testing conditions (Chen X. *et al.*, 2019; Abdalrazeq *et al.*, 2019). This similarity suggests that the protein network in BSFL films maintain mechanical integrity through extraction and wet casting process. The absence of statistically significant differences between T2 and T3 within DMA indicates that increasing glycerol content beyond 15% does not provide additional mechanical benefits under static loading conditions, consistent with previous observations of diminishing plasticization effects once sufficient mobility is achieved (Lyu *et al.*, 2022). In this context, the selection of formulation T2, requiring a lower amount of glycerol, appears preferable for further testing and potential applications. This choice is also supported by sustainability considerations, since glycerol production is associated with a non-negligible environmental footprint (Bansod *et al.*, 2024).

4.6 Mulching preliminary test

The preliminary mulching test demonstrated that the BSFL protein-based bioplastic film (T2) was effective in reducing soil water evaporation under controlled

conditions, while simultaneously undergoing rapid macroscopic alteration upon soil contact. Although sodium dodecyl sulfate (SDS) was used as a surfactant to solubilize proteins during film preparation, it was applied at a low concentration (0.05 M), corresponding to approximately 288 mg of SDS per film. SDS is known to be readily biodegradable under aerobic conditions, with studies reporting rapid degradation by mixed microbial communities within 48 h even at concentrations substantially higher than those used here (Najim *et al.*, 2022). Therefore, the limited amount of SDS employed is unlikely to persist in soil or pose long-term environmental concerns.

Across both substrates, mulching treatments reduced cumulative water loss compared to un-mulched controls, with the effect becoming more pronounced over time and clearly dependent on film placement. In particular, buried treatments consistently resulted in significantly lower water losses than other samples, especially in substrates with reduced water-holding capacity such as soil–sand mixtures, in agreement with previous studies (Gao *et al.*, 2023; Ramos *et al.*, 2024). Visual inspection revealed that the BSFL protein-based films underwent rapid and progressive macroscopic changes upon soil contact, with swelling, wrinkling, and porosity formation. These observations are consistent with the intrinsic hydrophilicity of protein-based polymers, which readily absorb water and undergo structural rearrangements in moist environments (Qazanfarzadeh and Kumaravel, 2023). The vitrified appearance of treatments A observed at the end of the experiment should not be interpreted as an experimental artifact, but rather as an expected outcome. Several studies have shown that water contact promotes glycerol migration or leaching, leading to an increase in the effective glass transition temperature and a consequent transition of the polymer matrix from a rubbery to a glassy state (Paramita *et al.*, 2025). Similar vitrification phenomena have been consistently reported during soil exposure and storage of protein- and polysaccharide-based biodegradable films (Nuvoli *et al.*, 2021; Zhang *et al.*, 2021). Regarding treatments B, these could not be recovered as recognizable structures at the end of the experiment, suggesting a more advanced level of macroscopic alteration. Although quantitative biodegradation was not assessed, the disappearance of intact film remnants under buried conditions, which are known to accelerate hydrolytic and microbially mediated degradation, aligns with previous reports (Sintim *et al.*, 2020; Convertino *et al.*, 2024).

Soil EC measurements, while not the primary endpoint, provided complementary insights. Within each substrate, higher EC values were observed in buried-film treatments compared to surface-applied films and unmulched controls, suggesting an increased availability of soluble compounds in the soil, potentially arising from partial film solubilization, plasticizer release, or enhanced microbial activity at the film–soil interface. Similar trends in soil chemical properties have been reported during the early stages of biodegradable mulch degradation (Sintim *et al.*, 2019). Importantly, EC values remained well below salinity thresholds and the starting substrates were characterized by very low soluble ion contents, particularly the soil–sand mixture, which exhibited EC values close to zero following irrigation to field capacity (Montanaro *et al.*, 2024). This suggests that the observed EC increases are more likely associated with film–soil interactions than with pre-existing substrate salinity.

5. Conclusions

This study demonstrates the feasibility of producing self-standing bioplastic films from proteins extracted from Black Soldier Fly larvae, supporting the potential of insect-derived biomasses as sustainable and circular feedstocks for biodegradable materials. The adopted extraction strategy enabled the recovery of protein fractions suitable for film formation, while glycerol content was identified as a key parameter governing film processability and mechanical performance. Among the tested formulations, the film containing 15% glycerol (T2) provided the best compromise between mechanical integrity, handling properties, and reduced plasticizer demand, making it suitable for further functional evaluation. Structural, thermal, and mechanical analyses confirmed that the extraction and casting processes preserved the proteic nature and stability of the material, yielding properties comparable to those reported for conventional protein-based films. The preliminary mulching test highlighted the functional potential of the BSFL protein-based films in reducing soil water evaporation under controlled conditions, particularly when applied in buried configurations, while simultaneously undergoing rapid macroscopic alteration in soil. These results indicate that BSFL protein-based films may represent a promising biodegradable alternative to conventional plastic mulches, combining short-term functionality with limited persistence in soil. Although further investigations are required to assess long-term degradation behavior, agronomic performance, and field-scale applicability, this work provides a proof of

concept for the valorization of insect-derived proteins in sustainable bioplastic and agricultural applications.

CONCLUSION AND FUTURE PERSPECTIVES

This PhD thesis provides an integrated evaluation of Black Soldier Fly larvae (BSFL)–derived products, with particular emphasis on their direct agricultural and material applications. Overall, the results confirm that BSFL frass possesses real agronomic potential, but that its effectiveness is strongly context-dependent and modulated by frass type, application rate, and plant species. In lettuce, intermediate inclusion levels (around 15%) consistently improved seed emergence and biomass, achieving performances comparable to mineral fertilization, whereas higher doses induced phytotoxic effects, mainly associated with increased electrical conductivity. These findings identify BSFL frass as a viable organic alternative to chemical fertilizers when applied within well-defined operational windows.

A central contribution of this thesis is the demonstration of how frass application affects not only crop performance but also product quality. Frass-based fertilization significantly modulated lettuce secondary metabolism, enhancing total phenolic content while reducing flavonoid levels and antioxidant activity. This highlights a potential trade-off between yield and nutritional quality and underscores the importance of tailoring fertilization strategies to specific agronomic and market-oriented objectives. Together, these results provide practical guidance for the agronomic use of frass in horticultural systems.

The work also clarifies the role of the microbial component of BSFL frass. Plant growth–promoting microorganisms were detected and functionally validated, and their composition was shown to depend on larval diet. Importantly, part of this beneficial microbiota survived thermal sanitization, while hygienic–sanitary indicators were consistently reduced to levels compliant with EU regulations. This supports a risk-based approach in which frass processing is calibrated to ensure safety while preserving functional potential.

In parallel, this thesis advances the valorization of BSFL biomass through protein extraction and bioplastic film production as a concrete material application. Protein-based films exhibited mechanical and thermal properties comparable to other protein-derived bioplastics, with glycerol content emerging as a key parameter for tuning performance. Preliminary mulching tests demonstrated that BSFL protein films can effectively reduce soil water evaporation and undergo rapid macroscopic degradation in soil, indicating their suitability for short-term, biodegradable agricultural applications.

Overall, the findings support an operational framework for BSFL-derived products based on: (I) standardized chemical and microbiological characterization; (II) crop-

specific optimization of application rates; (III) sanitization protocols tailored to actual risk; and (IV) integration of frass and protein-based materials within circular-economy strategies. Future research should focus on long-term field validation, deeper investigation of frass–microbiome–plant interactions, and optimization of BSFL-based biodegradable materials. In conclusion, BSFL-derived frass and proteins emerge as complementary resources which, when managed through evidence-based and application-specific approaches, can contribute to more sustainable and resilient agri-food systems.

SCIENTIFIC PUBLICATIONS

1. Anna Guarnieri, Rosanna Mallamaci, Giuseppe Trapani, Dolores Ianniciello, Carmen Scieuzo, **Francesco Iannielli**, Luigi Capasso, Maria Chiara Sportelli, Alessandra Barbanente, Michela Marsico, Angela De Bonis, Stefano Castellani, Patrizia Falabella and Adriana Trapani (2025). Physicochemical and biological properties of quercetin-loaded low-molecular-weight chitosan nanoparticles derived from *Hermetia illucens* larvae and crustacean sources: a comparative study. *Pharmaceutics*, *17*(8), 1016. https://doi.org/10.3390/pharmaceutics17081016

2. Antonella Vitti, Leonardo Coviello, Patrizia Falabella, Stefania Mirela Mang, Carmen Scieuzo, **Francesco Iannielli**, Domenico Ronga & Maria Nuzzaci. (2025). Exploring the agronomic traits, antioxidant and antifungal properties of *Hermetia illucens* frass extract in durum wheat (*Triticum durum* Desf.). *BMC Plant Biology*, *25*(1), 1075. https://doi.org/10.1186/s12870-025-07086-5

3. Antonio Franco, **Francesco Iannielli**, Giuliana Parisi, Nicola Francesco Addeo, Andrea Boschi, Giovanni Lomonaco, Giulia Secci, Patrizia Falabella and Fulvia Bovera. (2026). The role of the growing substrate. In *The Black Soldier Fly (Hermetia illucens)* (pp. 79-100). Academic Press. https://doi.org/10.1016/B978-0-443-29896-7.00003-2

4. **Francesco Iannielli**, Antonio Dolce, Federica De Stefano, Jesus D. Fernandez-Bayo, Carmen Scieuzo, Patrizia Falabella (2025). Transformative potential of insect bioconversion and its role in circular economy. *Journal of Environmental Management*, *396*, 128091. https://doi.org/10.1016/j.jenvman.2025.128091

5. Giovanni Lomonaco, Jeroen De Smet, Freek IJdema, Johan Ceusters, **Francesco Iannielli**, Rosanna Salvia, Mariana Amato, Carmen Scieuzo, Patrizia Falabella (2026). Selection and *In Vitro* Assessment of Plant Growth-Promoting Bacteria from Black Soldier Fly (*Hermetia illucens*) Frass. *ACS Agricultural Science & Technology*. https://doi.org/10.1021/acsagscitech.5c00811

CONFERENCE CONTRIBUTIONS

1. CNiE 2023 Palermo. Franco A., Scieuzo C., Salvia R., Pucciarelli V., **Iannielli F.**, Ouazri S., Borrelli L., Bovera F., Schmitt E., Patrizia Falabella. Evaluation of antimicrobial activity of lipids extracted from Yellow Mealworm *Tenebrio molito*r (Coleoptera: Tenebrionidae) (POSTER)

2. CNiE 2023 Palermo. D. Ianniciello, R. Salvia, C. Scieuzo, M. Triunfo, A. Guarnieri, A. Boschi, A. Franco, G. Lomonaco, **F. Iannielli**, A. Dolce, M. Ventura, A. De Bonis, Patrizia Falabella. Usage of insect-based chitosan for the preservation of fresh fruits (POSTER)

3. ECE 2023 Creta. A. Franco, C. Scieuzo, R. Salvia, V. Pucciarelli, M. Rubino, E. Derin, S. Ouazri, **F. Iannielli**, F. L. Borrelli, F. Bovera, E. Schmitt, P. Falabella. Evaluation of antimicrobial activity of lipids extracted from *Hermetia illucens* reared on different feeding substrates (POSTER)

4. ECE 2023 Creta. A. Franco, C. Scieuzo, R. Salvia, V. Pucciarelli, F. Giglio, R. Rinaldi, **F. Iannielli**, F. De Stefano, S. Ouazri, L. Borrelli, F. Bovera, E. Schmitt, P. Falabella. Evaluation of antimicrobial activity of lipids extracted from *Tenebrio molitor*. (POSTER)

5. ECE 2023 Creta. R. Salvia, C. Scieuzo, M. Triunfo, A. Guarnieri, D. Ianniciello, A. Franco, G. Lomonaco, **F. Iannielli**, A. Dolce, M. Ventura, A. De Bonis, P. Falabella. Usage of insect-based chitosan for the preservation of fresh fruits (POSTER)

6. 4th MS NatMedDay 2024 Salerno. Antonio Franco, Carmen Scieuzo, Rosanna Salvia, Valentina Pucciarelli, **Francesco Iannielli**, Sofia Ouazri, Ilaria Caivano, Luca Borrelli, Fulvia Bovera, Eric Schmitt, Patrizia Falabella. Antimicrobial activity of lipids extracted from *Hermetia illucens* larvae reared on different substrates. (POSTER)

7. INSECTA 2024 POTSDAM. Antonio Franco, Carmen Scieuzo, Rosanna Salvia, Valentina Pucciarelli, Sofia Ouazri, **Francesco Iannielli**, Miriam Viola, Ilaria Caivano, Luca Borrelli, Fulvia Bovera, Eric Schmitt, Patrizia Falabella. Lipids extracted from *Hermetia illucens* reared on different substrates: evaluation of antimicrobial activity. (Oral Communication)

8. INSECTA 2024 POTSDAM. Carmen Scieuzo, Antonio Franco, Rosanna Salvia, Micaela Triunfo, **Francesco Iannielli**, Andrea Boschi, Valentina Pucciarelli, Fulvia Bovera, Ambrogio Laginestra, Eric Schmitt, Patrizia Falabella. Valorization of fruit byproducts through bioconversion by *Hermetia illucens* (Diptera: Stratiomyidae). (POSTER)

9. ICE 2024 Kyoto. Franco A., Scieuzo C., Salvia R., Pucciarelli V., **Iannielli F.**, Caivano I., Triunfo M., Guarnieri A., Borrelli L., Bovera F., Schmitt E., Falabella P. Evaluation of antimicrobial activity of lipids extracted from *Hermetia illucens* reared on different feeding substrates. (Oral Communication)

10. 5th API Congress. **Francesco Iannielli**, Margherita Izzi, Carmen Scieuzo, Andrea Brattelli, Rosanna Salvia, Maria Chiara Sportelli, Luigi Gentile,

Rosaria Anna Picca, Nicola Cioffi, Patrizia Falabella. INSECT PROTEIN-BASED BIOPLASTICS. (POSTER)

11. Agriworld EXPO 2025. **Francesco Iannielli**, Carmen Scieuzo, Luigi Gentile, Nicola Cioffi, Antonio Carlomagno, Giuseppe Montanaro, Patrizia Falabella. Insect Protein-Based Bioplastics and potential application as a mulching material. (Oral communication)
12. Agriworld EXPO 2025. Valentina Pucciarelli, Giovanni Lomonaco, **Francesco Iannielli**, Carmen Scieuzo, Patrizia Falabella. *Hermetia illucens*: un modello sostenibile per la valorizzazione dei sottoprodotti vegetali e la produzione di biodiesel. (Oral communication)
13. 4th Coatings and Interfaces Online Conference. **Francesco Iannielli**, Margherita Izzi, Carmen Scieuzo, Andrea Brattelli, Rosanna Salvia, Maria Chiara Sportelli, Luigi Gentile, Rosaria Anna Picca, Nicola Cioffi, Patrizia Falabella. Novel Composite Films Starting from Black Soldier Fly Protein Extract. (Oral communication)
14. Future Foods 2025. **Francesco Iannielli**. Alternative foods and sustainability. (Oral communication)
15. CNIE 2025 Siena. Valentina Pucciarelli, Giovanni Lomonaco, **Francesco Iannielli**, Carmen Scieuzo, Patrizia Falabella. *Hermetia illucens*: a sustainable model for vegetable by-products valorization and biodiesel production. (Oral communication)

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Chapter 2

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Chapter 3

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Chapter 5

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