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Metagenomics and functionality of microbial communities involved in Smartcomp composting and in the agronomic quality of the final product

Settore scientifico disciplinare

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Abstract

The increasing world population poses great challenges in waste management, with global municipal waste production estimated to reach 3.4 billion tonnes per year by 2050. In Europe, since 1990 the European Union issued several Directives to promote sustainable waste management. Bio-waste constitutes the largest part of the municipal solid waste and represents a valuable source to produce energy, fertilizers and biogas. However, most of this waste is still largely managed through unsustainable ways like landfilling and incineration, contributing to groundwater contamination, heavy metal exposure and greenhouse gas emissions.

Currently, among more sustainable options, composting is the most used method. Composting is a simple technology and does not require complex equipment, nonetheless, it presents some challenges such as long processing times and insufficient quality that does not compete with chemical fertilizers. To increase composting efficiency, it is important to optimize physiochemical parameters such as pH, C/N ratio, moisture and aeration. Because bio-waste does not have alone the optimal physiochemical characteristics for composting, bulking agents like sawdust, woodchips and straw are added to adjust starting conditions. These materials are not only influencing the physiochemical properties of compost but also the microbial community that transforms the organic matter.

Microbial communities are central to the composting process. Fungi and bacteria are the most important microorganisms involved and usually include saprophytes capable of degrading several types of substrates, including the recalcitrant ones like lignin. Bacteria typically dominate during the thermophilic phase, where temperatures can reach 60°C, while fungi are more active during the starting and final phase. The study of microbial dynamics during composting is fundamental to optimizing the composting process. Technologies like high-throughput sequencing (HTS) enabled complete microbial profiling without the limitation of culture-based approach, that remains fundamental for isolating novel strains with potential biotechnological applications. Some isolated microorganisms have been tested as inoculants to increase the efficiency of compost. This methodology, known as bioaugmentation, has gained attention but is still debated in the scientific community because of contrasting results and difficulties in replication and results comparison. Similar obstacles exist for the assessment of compost maturity, because many parameters are context dependent and are not standardized at international level.

The research carried out during this PhD aimed to contribute to the implementation of the composting process, from the formulation of compost mixtures to the quality evaluation of the final product.

An initial study tested three different proportions of woodchips as bulking agent for bio-waste composting, measuring both physical (e.g. temperature and moisture) and chemical (e.g. pH, N content) parameters. At the same time, microbial community analysis was carried out using both culture-dependent methods and HTS. The results obtained enabled the determination of correct bulking agent ratio, the isolation of microorganisms with potential use for bioaugmentation, the investigation of microbial dynamics and the identification of taxa associated with specific mixtures.

Building on this, a more complete analysis of the fungal community was performed. Four distinct mixtures (two designed to represent optimal composting conditions and two under suboptimal conditions) were designed using woodchips or a perlite and cardboard blend. HTS identified a core fungal community shared across all mixtures and its succession through composting phases. Moreover, it determined indicator taxa linked to specific mixtures, especially during the late stages, offering cues for their use as biomarkers for compost quality assessment.

Subsequently, the possibility of reusing phytopathogen-infected woody material as a bulking agent under optimal composting conditions was explored. Using *Phytophthora cinnamomi* as a model plant pathogen, its persistence was investigated using three different diagnostic techniques: baiting, qPCR and HTS. By the end of the composting process, no *P. cinnamomi* was found, confirming that a

composting process under optimal conditions can effectively eradicate this pathogen. Moreover, with HTS it was possible to identify the decline of the DNA of other plant pathogens which were present in the starting material. This study demonstrated the possibility to produce a pathogen free compost from recycled horticultural and nursery waste without the need for complex composting infrastructure.

In a final experiment, the bacterium *Bacillus velezensis*, isolated during the first study, was selected for compost bioaugmentation. This experiment was carried out with the collaboration of Dr. Paolo Roberto di Palma and Dr. Giulio Gazzola of the Laboratory of Technologies for the Reuse, Recycling, Recovery and Valorization of Waste and Materials (USER Division, SSPT Department, ENEA Casaccia, Italy), that provided bioreactors for composting and sensors for monitoring process parameters. The use of *B. velezensis* increased microbial activity, as demonstrated by higher temperatures during the thermophilic phase, CO2 emissions and carbon degradation. Unfortunately, this did not reflect in changes in the chemical properties of the final product in comparison with the non-bioaugmented treatments. To obtain more marked differences, further research is needed to optimize the concentration of inoculum to be added.

The results of this PhD research contribute significantly to composting process optimization and to the understanding of its microbial ecology and demonstrate how molecular diagnostics can serve to improve compost efficiency, safety and quality. These contributions will help with the development of more suited waste management practices.

Keywords

Compost, metabarcoding, HTS, microbial community, bio-waste

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CHAPTER I – State of the art

Bio-waste represents a significant portion of municipal and industrial waste in Europe, setting environmental challenges but also opportunities for resource recovery and job creation. The following chapter will introduce the concept of bio-waste, investigate its production at European level, its policy frameworks and the effects of its unsustainable management. After that, it will be discussed how composting can contribute to sustainable bio-waste management, its technical aspects and the role of microorganisms in the process. This initial part will provide a starting point for understanding the issues and opportunities for the sustainable management of bio-waste, which this thesis aims to address.

1.1. Bio-waste

1.1.1. Definition

Bio-waste is a general term used for different types of organic waste such as household waste, food waste and green waste. The European Commission defines bio-waste as "biodegradable garden and park waste, food and kitchen waste from households, restaurants, caterers and retail premises, and comparable waste from food processing plants". From this classification other types of organic materials like agricultural residues, manure and sewage sludge are not included. In this thesis the term bio-waste will be referred to the abovementioned definition of the European Commission. Other wastes were not considered in this PhD project because the focus was on the treatment, at small and medium scale, of bio-waste originating from municipal waste.

1.1.2. Bio-waste production at European level

Currently, an estimated 71 million tonnes of bio-waste from municipal waste (47 million tonnes) and commercial/industrial activities (24 million tonnes) are collected and treated every year in the European Union. Circa 42 million tonnes per annum (tpa) (59%) are composted while 28 million tpa (41%) are anaerobically digested (AD). The amount of bio-waste separately collected and treated per capita greatly varies among European countries, from less than 28 kg/capita/year in eastern countries like Poland and the Baltics to more than 166 kg/capita/year in France, Austria and Belgium (Figure 1.1). Green and food waste are the main feedstocks that are composted, whilst still food waste and other unspecified wastes are dominating at AD. When excluding commercial and industrial activities, 47 million tpa of bio-waste are collected and treated at municipal level with most of it composted (70%) and the rest sent for AD (30%). This amount is equivalent to only 17% of the total municipal solid waste fraction (Gilbert & Siebert, 2022). Capturing bio-waste from municipal sources is a cornerstone of the EU agenda on circular economy, which has set minimum recycling and re-use standards for certain type of waste (Favoino & Giavini, 2024). Given the large quantities of bio-waste produced and the differences in its collection and treatment across countries, its effective management has become a central problem of European policy.

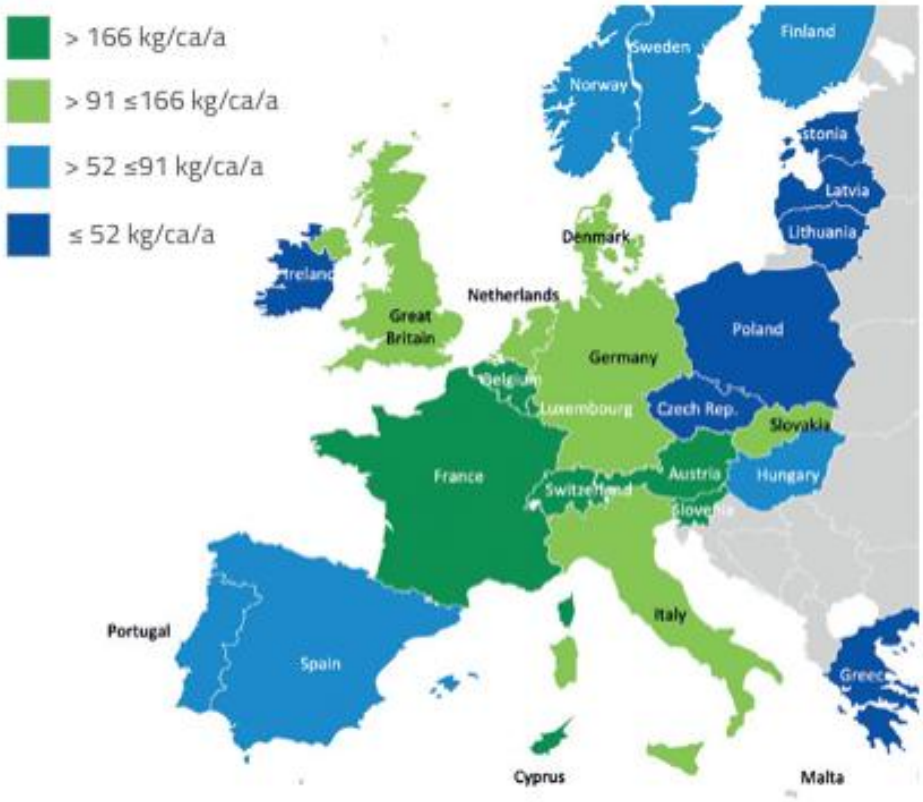

Figure 1.1.1 The amount of separately collected bio-waste per capita per year in European countries in 2022. Kg/ca/a = kilograms per capita per annum. Sources: ECN data report 2022, Compost and digestate for a circular bioeconomy

1.1.3. European policies for bio-waste management

For over 20 years, the EU has been discussing the topic of bio-waste management through evolving legislation (Jędrczak, 2018). The regulation of recycling and disposal of bio-waste began with the Directive on Packaging and Packaging Waste (1994/62/EC) (EUR-Lex, 1994), the Directive on the Landfill of Waste (1999/31/EC) (EUR-Lex, 1999b), and the Waste Framework Directive (2008/98/EC) (EUR-Lex, 2008), which first introduced the definition of bio-waste and encouraged its selective collection for composting or AD. In the same year (2008), a policy dialogue was initiated on the condition of sustainable bio-waste management with the Green Paper on bio-waste. In 2015, the European Council launched the Circular Economy Package and the three core waste Directives were revised (Kircher et al., 2023), setting new goals for bio-waste management:

- Recycling municipal waste up to 70% by 2030
- Phasing out landfilling for recyclables by 2025
- Reduce food waste generation by 30% by 2025
- Mandatory separate collection of bio-waste by 2023
- Promotion of recovery and recycling

Currently, the principal policy driver is the European Directive (EU) 2018/851, also known as New Waste Framework Directive, which introduced the "Principle of Proximity", according to which waste should be recycled close to the source of generation to avoid costs and pollution associated with collection and transport. This principle promotes the treatment of bio-waste at the local level, carried out at single home scale or at community level, for the joint treatment of neighborhood's bio-waste in a common location close to the homes (Alves et al., 2024). In the last years the European policy has also been enriched with other Regulations which enhance the integration of compost and

digestate into the circular economy such as the Regulation EU 2019/1009 on fertilizing products, that defines quality requirements for recovered organic materials.

1.1.4. The environmental impacts of bio-waste

Landfilling and incineration have been traditionally the most used techniques for disposing municipal solid waste thanks to their cheap operational investment and ease of operation (Kamaruddin et al., 2015). However, this approach has led to several environmental concerns such as leachate production, which can contaminate surface and groundwater, causing a threat to aquatic ecosystems (Salam et al., 2021). Areas nearby landfill sites have been found to be rich in carcinogenic molecules containing heavy metals (Parvin & Tareq, 2021). Approximately 8% of total greenhouse gas (GHG) emissions come from bio-waste, mainly in the form of methane produced by the decomposing organic matter under anaerobic conditions typically occurring in landfill (Scialabba, 2015). Methane is a GHG 85 times stronger than carbon dioxide and many countries in the world have committed to reduce methane emissions by 30% by 2030 (Myhre et al., 2014; Somers, 2025). Municipal waste is generated in massive amounts worldwide and, for 2050, it is estimated that the world will generate 3.4 billion tonnes of it (Anuar et al., 2025). Especially developing nations are facing problems in utilizing bio-waste due to its heterogeneity, however, efficient management of bio-waste presents a substantial opportunity for resource recovery because it can generate biofuels, organic fertilizers and amendments, electricity and chemical compounds (Md Nasir et al., 2025; Srivastava et al., 2020). The diversion of organic waste depends on households' participation and general community engagement, shaped by incentives, preferences and education programs. Despite the costs for the local jurisdictions, it can yield social and environmental benefits by reducing GHGs emissions (Somers, 2025).

1.2. Composting of bio-waste

Building on the environmental and regulatory pressures outlined above, circular economy has been widely recognized as a fundamental aspect for waste management and numerous technologies have been developed or made more efficient by researchers and industry to reduce the disposal of bio-waste to the landfills and recover valuable resources (Budiyono et al., 2021; LeClerc et al., 2022; Nawaz et al., 2022; L. Wang et al., 2023; Zakarya et al., 2023). These include composting, anaerobic digestion, hydrothermal liquefaction or carbonization and microbial fuel cells. Among them, composting is currently the most frequent method used for bio-waste treatment due to its simplicity and low cost. Although it can be less economically advantageous than other technologies on a large scale, it can still offer economic and environmental benefits including financial savings, increased soil quality, reduced pollution and a reduced use of chemical fertilizers (Md Nasir et al., 2025; Vaverková et al., 2020). Composting is a biologically mediated process which aerobically decomposes biodegradable materials, making it possible to return organic matter and nutrients to the soil though the production of organic fertilizers and contributing to the development of a more sustainable agriculture (Jędrczak Andrzej, 2018). Nonetheless, many concerns have emerged over the years regarding the limitations of this technology. Large-scale applications require extensive land, unproper management may release methane and unpleasant odors which may disturb the population living near to the composting plant, groundwater contamination may occur due to leachate production, long time is needed to obtain mature compost, usually around 2 months, and not always there's enough demand for organic fertilizers (Adhikari et al., 2008; Cerda et al., 2018; S. Wang & Zeng, 2018). For all these reasons, researchers are focusing especially on ways to reduce the process timing and to increase the value of the final product, by designing optimal feedstock mixtures, by planning more efficient composting systems and by using bioaugmentation (Guidoni & Vannini, 2024; X. Liu et al., 2025; Q. Wang et al., 2024; Zhu et al., 2024). Bio-waste has a rapid degradation and a high nutrient content, allowing biological treatments to be a suitable solution for their disposal.

However, to have a successful composting process, it is important to evaluate the physio-chemical properties of the feedstock (Alves et al., 2024; Zakarya et al., 2025).

1.2.1. Parameters affecting composting

Several factors can influence the composting process and determine the strength of the biological activity. The main factors are temperature, moisture, pH, C/N ratio, aeration, particle size, turning frequency and electrical conductivity.

1.2.1.1. Temperature

This factor is generally used as an indicator to assess the level of microbial activity and strongly depends on the biodegradability of the feedstock. The evolution of temperature during the composting process can be divided into four phases: mesophilic, thermophilic, cooling and maturation (Adhikari, 2007). The thermophilic phase is the one with the highest temperatures, which should range between 52°C and 60°C to facilitate microbial degradation and to favor pathogen, parasites and weed seeds removal (Noble & Roberts, 2004). If temperature is too high (>60°C), the efficiency of the process might be affected and microorganisms will be killed (Imbeah, 1998).

1.2.1.2. Moisture

It affects oxygen uptake, free air space, microbial activity and temperature (Petric et al., 2012). The initial moisture of the material should range between 60 and 65% and should never go below 40% throughout the process otherwise all the biological processes will be slowed down and eventually stop completely when moisture goes under 20% (Adhikari, 2007). This is because water is fundamental for the distribution of soluble nutrients to microorganisms (R. Guo et al., 2012). On the other hand, if moisture is too high (>70%) the process might become anaerobic and emit methane (R. Guo et al., 2012).

1.2.1.3. pH

The pH of bio-waste is usually acid or sub-acid and it further declines at the beginning of the composting because of organic acid release caused by microorganisms breaking down mainly sugars (Adhikari, 2007). Very low pH values might inhibit microbial activity and affect the transition from the mesophilic to the thermophilic phase (Paradelo et al., 2013). After, proteins start to degrade and ammonia is released, causing an increase of pH which, at the end of the process, should range between 6.5 to 8 (Hachicha et al., 2009). The increase of pH increases the ammonia/ammonium ratio, causing the volatilization of ammonia (Onwosi et al., 2017).

1.2.1.4. C/N ratio

Carbon is used as an energy source while nitrogen serves to build cells structure (Iqbal et al., 2015). A desirable starting C/N ratio ranges from 15 to 30 and ensures a correct nutrient availability for the microbial community (Adhikari, 2007). If too much nitrogen is present (low C/N ratio), unwanted byproducts are produced, foul odors are emitted, and ammonia volatilization increases. If the nitrogen content is limited (high C/N ratio) it causes nutrient deficiency and reduces the carbon consumption rate, slowing down the composting process (H. Zhang et al., 2016). A wide variety of bulking agents (BAs) like sawdust, woodchips, rice husks and straw are used to increase and optimize the C/N ratio and the porosity of bio-waste (Onwosi et al., 2017). C/N ratio tends to decrease overtime because carbon is used from microorganisms circa 30 to 35 times faster than nitrogen, mature compost should have a C/N ratio of 15-20 (Adhikari, 2007).

1.2.1.5. Aeration

Composting is an aerobic process, thus the presence of oxygen is critical (Cerda et al., 2018). Oxygen demand is very high during the thermophilic phase, as microbial activity is particularly strong. If low oxygen is supplied, the compost temperature rapidly decreases. Oxygen demand slowly decreases during cooling and maturation (Adhikari, 2007).

1.2.1.6. Particle size

It determines aeration and water holding capacity (L. Zhang & Sun, 2014). If particles are too coarse the organic matter (OM) will be degraded slowly due to the low surface-to-volume ratio. Conversely, if it is too small it can compact the composting material, reduce porosity and create anaerobic conditions (M. P. Bernal et al., 2009a).

1.2.1.7. Turning frequency

In case of a passive aeration system, turning is the operational procedure to aerate the compost. The optimization of the turning regime is fundamental because low frequency may not satisfy the oxygen demand of the microbial community while too frequent turning could result in heat dispersal and pollutant emissions (M. K. Awasthi et al., 2014). Several turning regimes have been reported by many authors, ranging from once a day to weekly turning, depending on the feedstock and on the composting phase (Li et al., 2015; Ros et al., 2006). The optimization of the turning regime yields nutrient retention and the sanitization of the material (Onwosi et al., 2017).

1.2.1.8. Electrical conductivity

Electrical conductivity (EC) tends to increase during composting due to the concentration of nutrients released from the decomposition of the OM (Chan et al., 2016). The maximum threshold in mature compost has not been defined yet, as some authors claim that it should be below 2 mS/cm while for others should be lower than 4 mS/cm (M. K. Awasthi et al., 2014; Oarga Mulec et al., 2016; Turan, 2008). Too high EC affects the germination rates and might inhibit plant growth, hence, compost should be used in reduced quantities and mixed well with the soil or potting mix before use (Gao et al., 2010; Onwosi et al., 2017).

1.2.2. Bulking agents

To start the composting process, four essential components are generally required: organic matter, moisture, oxygen, and microorganisms (Zhou et al., 2022). Bio-waste, especially the food-waste fraction, is usually characterized by excessive moisture, low C/N ratio, low porosity and acidic pH; conditions that are far from being optimal and make bio-waste unsuitable to be composted on its own (Cerda et al., 2018). Co-composting with BAs has become essential to correct these imbalances by improving the physical structure, stimulating microbial activity, regulating the moisture content, increasing the C/N ratio, buffering the pH, reducing GHGs and odor emission and increasing the temperature profile, enzyme activity and nutrient availability (Zahra et al., 2023; Zhou et al., 2022). Bulking agents are structural amendments often made of lignocellulosic material such as sawdust, woodchips, peanut shells, rice husks, wheat straw or bran. The choice of a BA and its quantity is among the most critical factors before commencing the composting process (Guidoni & Vannini, 2024). Its selection must account for the bio-waste composition range (Margaritis et al., 2023), which often is influenced by cultural differences, variations in locally available products and seasonal fluctuations (Adhikari et al., 2008). Typically, BAs proportions range from 20% to 60% of the pile fresh weight and a large variety of BAs have been identified during the years in literature, each with distinct strengths and weaknesses. Many lignocellulosic BAs (sawdust, paper) have relevant water absorption capacities that help maintain the moisture in the optimal range of 60-65% and reduce leachate production (Rich et al., 2018). Furthermore, their high carbon content increases C/N ratio, reduces ammonia volatilization and supports microbial activity (Huang et al., 2024). Woodchips also feature high C/N ratios, usually above 80, help conserving carbon by reducing CO_2 emission, increase

porosity and discretely enhance the aeration (Huang et al., 2024). In some cases, however, woody material can have a relatively low pH which does not make it ideal for acid feedstocks like food-waste (Adhikari et al., 2008; Zhou et al., 2022). Wheat straw tends to have a higher water-holding capacity which seems to limit nitrogen losses though it may, if over-moistened, augment ammonia and methane emissions most likely due to the creation of anaerobic niches (C. Wang et al., 2022). Also, inorganic BAs like perlite, vermiculite and zeolite, demonstrated to be beneficial for porosity and temperature evolution. In the case of zeolite, its ion-exchange property allowed to reduce nutrient losses (Margaritis et al., 2018). The choice of BAs depends on many factors, primarily on local availability and economic advantages. For example, in rice-producing regions, rice husks may be abundant and cost-effective, whereas mountainous areas can more easily supply woody materials like sawdust and woodchips. Also, seasonal fluctuations can influence the selection: wood-based BAs can become more expensive during colder months while agricultural residues may be dependent on harvest cycles. Seasonal variations may also affect bio-waste composition, especially when there's a large proportion of food waste, and adjustments in BAs proportions to maintain optimal composting conditions might be necessary. For example, moisture and nitrogen content of the bio-waste were found to be higher in summer months (Adhikari et al., 2008). Looking ahead, future research should aim to develop BA blends for specific types of waste, climatic conditions and geographical areas and eventually explore the possibility to have a synergistic effect with the integration of microbial inoculants, which could contribute significantly to the degradation of recalcitrant molecules and in the increase of the quality of the final product (Onwosi et al., 2017).

1.2.3. Microbiology of compost

1.2.3.1. Analytical tools

The microbial communities driving composting are studied using numerous techniques, categorized into two main groups: culture-based and culture-independent methods. Culture-based techniques, such as measuring adenosine triphosphate (ATP) for microbial activity (Horiuchi et al., 2003), assessing metabolic potential via BIOLOG sole-carbon utilization tests (Borrero et al., 2006), or quantifying microbial biomass and diversity (Ryckeboer et al., 2003), rely on isolating microorganisms in pure culture on AGAR plates. Their analytical simplicity and specificity made them attractive for large-scale monitoring processes, particularly before the advent of culture-independent techniques. Unfortunately, these methods can provide a very selective overview of compost microbiota, less than 1%, as most microorganisms can't grow on cultivation media (Chandna et al., 2013). This limitation obscures most of the diversity and functionality of unculturable species, which may dominate composting processes or perform key functions (Finore et al., 2023). Culture-independent molecular tools analyze microbial groups directly from the compost, without the need of cultivation (Handelsman, 2005). These include DNA and RNA extraction, PCR amplification of target regions and sequencing of the resulting fragments. These are the core components of metagenomics, a term introduced by Handelsman in 1998. Metagenomics studies the genetic material extracted directly from the environmental sample and provides an overview of the microbial community. This approach can be sequencing-based, which uses metabarcoding to identify taxa and predict functionality, or function-based, which aims to discover new genes encoding enzymes for biotechnological applications (Finore et al., 2023). These are among the most attractive technologies that have been used in the last years that have helped compost diagnostics by surpassing the limitations of cultivation. The wide use of these technologies in the last years was made possible by the gradual reduction of their costs and by the development of bioinformatics, which allowed the analysis of increasing amounts of data (Chandna et al., 2013; Sánchez et al., 2017). Despite advances, challenges persist. Results comparison from culturing approach and molecular approach have sometimes led to contrasting results. For example, Ishii and Takii (2003) reported discrepancies between 16S rDNA analyses and traditional culturing. While microbial isolation has its own limits, molecular approaches are affected by PCR biases and inefficient DNA extraction that can distort

results (Jurado et al., 2014). In light of all these considerations, both culture-based and culture-independent should be considered complementary and not exclusionary (Cerda et al., 2018). Culture-based methods remain fundamental for the isolation of strains for biotechnological use, such as bioaugmentation or enzyme production, whereas metagenomics can investigate the rich ecological interactions and diversity of the microbial community. Hybrid strategies combining both approaches provide a more detailed view of microbial diversity and functionality, and many researchers adopted this approach in their studies (Antunes et al., 2016; Guidoni & Vannini, 2024). Despite extensive research, compost ecosystems remain mostly unexplored, with different feedstocks and process conditions that influence microbial communities (Finore et al., 2023).

1.2.3.2. Role of microorganisms in composting

Composting is mediated by different types of aerobic microorganisms, mainly fungi and bacteria, that collectively transform heterogeneous OM into humus-like substances and releasing CO_2, heat and water (S. K. Awasthi et al., 2020; Finore et al., 2023). The microorganisms that play a role in composting are provided by the feedstock itself and their presence is influenced by the composition of the mixture, by changes in temperature, nutrient availability and by other parameters like moisture, oxygen and pH (S. K. Awasthi et al., 2020; Ryckeboer, Mergaert, Coosemans, et al., 2003). Bacteria from compost are mainly decomposers, belonging to the group of chemoorganotrophic microorganisms. Genera like *Pseudomonas, Burkholderia, Zymomonas* and *Xanthomonas* can be found. Also nitrifying bacteria which convert ammonium into nitrites and nitrates like *Nitrosomonas* and *Nitrospira* are present. Fungi can be found especially during the first and final phases of the composting process (Insam & de Bertoldi, 2007; Ryckeboer, Mergaert, Coosemans, et al., 2003; Ryckeboer, Mergaert, Vaes, et al., 2003). The most representative genera are *Aspergillus, Acremonium, Chrysosporium, Fusarium, Mortierella, Penicillium,* and *Trichoderma* (Anastasi et al., 2005; Sánchez et al., 2017). Characterizing and monitoring microbial communities provides important information for the optimization of the composting process, the sanitization of the material and production of a high-quality product (S. K. Awasthi et al., 2020). However, there's still a limited knowledge of the composition and functionality of microbial communities that occur during composting (L. Zhang et al., 2016). The microbial community controls the temperature evolution and the enzymatic activity, from which depends the degradation of lignocellulose and the transformation of nitrogen, thus, its study can provide important information on the maturity of the product and the presence of certain microorganisms may reflect the quality of it (S. K. Awasthi et al., 2020; Ryckeboer, Mergaert, Coosemans, et al., 2003). For example, several free-living diazotrophic organisms (i.e. *Stenotrophomonas, Alcaligenes, Pseudomonas, Klebsiella, Xanthomonas, Achromobacter* and *Caulobacter*) were isolated from compost and have shown to increase the nitrogen content of the end product (Pepe et al., 2013). Other microbial groups provide suppressive characteristics to compost, avoiding the development of certain plant diseases thanks to the production of antibiotics or by competition (Onwosi et al., 2017). With the development of new technologies such as HTS, the investigation of the microbial communities in various environments has deepened (Guidoni & Vannini, 2024; Sun et al., 2019; Zhong et al., 2020). Nonetheless, our current knowledge of the dynamics of microbial populations is still limited (L. Zhang et al., 2016). The first step of biodegradation is performed by mesophile organisms, that are growing at temperatures between 30 and 45°C and exploit easily degradable substrates. Bacterial belonging to the genera *Pseudomonas, Petrimonas* and *Proteiniphilum* alongside yeasts and other fungi like *Penicillium* rapidly metabolize easily degradable compounds, mainly sugars and starches (S. K. Awasthi et al., 2020; L. Zhang et al., 2016). The presence of yeasts during this phase is favored by simple sugars and low pH (Ryckeboer, Mergaert, Coosemans, et al., 2003). This first step can last from 1 to 3 days. As temperatures trespass 45°C, the total microbial population decreases and thermotolerant and thermophilic organisms settle and start to break down proteins, lipids, cellulose and hemicellulose (Onwosi et al., 2017). In this context, also known as active phase, nutrients are still abundant, and the decomposition proceeds at a high rate. Bacterial taxa like *Bacillus, Thermobifida*

and *Streptomyces* start to secrete cellulases, hemicellulases, peroxidases, laccases and xylanases, which decompose complex molecules like lignin and hemicellulose (S. K. Awasthi et al., 2020; Martins et al., 2013; L. Zhang et al., 2016). At the same time, fungi such the genera *Thermomyces* and *Aspergillus* contribute to the degradation of lignocellulosic material (M. K. Awasthi et al., 2015). Proteases transforms proteins into amino acids (ammonification), while the increasing microbial activity gives rise to carbon dioxide emission and temperature (Finore et al., 2023; Ryckeboer, Mergaert, Coosemans, et al., 2003; L. Zhang et al., 2016). When peak temperatures of 55-60°C are reached, the sanitization of the material takes place by the inactivation of pathogens (*E. coli, Salmonella, Listeria, Phytophthora*) and weed seeds (Onwosi et al., 2017). ~~Non~~ Non-thermophilic microorganisms persist in the composting pile only if capable of producing heat resistant spores or at the margins of the pile where temperatures are cooler (S. K. Awasthi et al., 2020; Cerda et al., 2018; Ryckeboer, Mergaert, Coosemans, et al., 2003). Ammonia-oxidizing bacteria and archaea (e.g. *Nitrosomonas*) convert ammonium (NH_4) into nitrite (NO_2) and nitrate (NO_3) while in some anoxic pockets might enable some denitrification to N_2O (Sánchez et al., 2017). High temperatures help promote enzymatic degradation of cellulose and a majority of the overall composition of microorganisms present are cellulolytic at the end of the thermophilic phase (Ryckeboer, Mergaert, Coosemans, et al., 2003). When most simple nutrients are transformed, temperature slowly drops to ambient, and mesophiles restart to colonize the composting material. During this phase, humic substances start to be produced (Antil et al., 2014; Biyada et al., 2021) and bacteria are taken over by fungi, that become dominant after the thermophilic phase like *Aspergillus* spp. and *Mucor* spp. (S. K. Awasthi et al., 2020; Ryckeboer, Mergaert, Coosemans, et al., 2003). In the final maturation stage, fungal diversity increases and through humification keeps converting the remaining OM into humic acids (Sánchez et al., 2017). Towards the end of compost maturation amyloses and proteins become fully degraded and cellulose is inaccessible as undergraded lignin prevents it degradation (Ryckeboer, Mergaert, Coosemans, et al., 2003). This causes a decline in the presence of cellulolytic, amylolytic and proteolytic organisms (Diaz-Raviña et al., 1989). At the end of the process the microbial activity lowers significantly due to lack of nutrients, resulting in the stability of compost (Ryckeboer, Mergaert, Coosemans, et al., 2003). The high microbiome richness and diversity found during the composting process is influenced by different environmental conditions. These facts have led some authors to select microorganisms of interest to further be used in biotechnological applications, like bioaugmentation (Cerda et al., 2018; Kinet et al., 2015). The use of bioaugmentation to inoculate composting material with target organisms can further enhance specific functional activities of the microbial community, offering the opportunity to optimize the composting process and yield a better final product.

1.2.3.3. Use of bioaugmentation

Bioaugmentation consists in the addition of specific microorganisms (Nakasaki & Hirai, 2017), microbial consortia (Ke et al., 2010) or even mature compost (Karnchanawong & Nissaikla, 2014) to enhance biodegradation, regulate pH, reduce foul odors, promote nutrient retention and, ultimately, increase the final product's agronomic value (Cerda et al., 2018). It is often described as an environmentally friendly and cost-effective solution for optimizing waste management. However, there are contrasting opinions regarding its efficacy. Several works support the effectiveness of bioaugmentation. For example, the addition of *Thermomyces vulgaris* has been shown to reduce composting times and improve compost quality (Ke et al., 2010). Addition of the yeast *Pichia kudriavzevii* eliminated the lag phase between the mesophilic and thermophilic stages by preventing excessive pH drops, which can inhibit the microbial activity especially in food-waste composting (Nakasaki & Hirai, 2017). For lignocellulosic waste, supplementation with cellulase-secreting bacteria like *Bacillus, Cellulomonas* and *Pseudomonas* spp. or fungi like *Trichoderma*, *Aspergillus* and *Sclerotium* spp. improves the degradation of structural carbohydrates, thereby raising humic acids levels (M. K. Awasthi et al., 2015; Rastogi et al., 2020). In some cases, microbial inoculation increases the activity of the indigenous microbial community (Kostov et al., 1991). Microbial blends,

comprising multiple microorganisms with similar functionality, have also proven their efficacy. For example, a consortium of anti-acidification microorganisms including *Pseudomonas, Bacillus* and *Lactobacillus* spp. has successfully stabilized pH at the beginning of the composting process and contributed to higher humic acid content (Ding et al., 2016). Some of these mixtures are commercialized as "Effective microorganisms" (EM) and have shown their potential in mineralizing the OM and reducing gaseous emissions and composting time (Manu et al., 2017). Nevertheless, in several cases the simple use of mature compost as an inoculant has yielded better results than commercial EM, possibly due to prior adaptation of microorganisms to the composting environment (Karnchanawong & Nissaikla, 2014).

The effectiveness of bioaugmentation depends on several factors:

- **Feedstock type**: A diverse indigenous population already exists in the feedstock; it competes with the inoculum and makes its establishment difficult (Hubbe et al., 2010). The incompatibility between the feedstock type and the inoculum is another issue (Vargas-García et al., 2007). High-fat substrates, for example, require lipolytic microorganisms, while a fiber-rich lignocellulosic waste requires lignocellulolytic microorganisms, because different substrates have different enzymatic requirements.
- **Inoculum concentration and timing**: single-stage applications may not sustain inoculant vitality throughout the process, especially if the application is done in the wrong composting phase. Multi-stage applications can sustain the survival of the inoculum throughout the composting process without being outcompeted by the indigenous community (B. Xi et al., 2015; B. D. Xi et al., 2012).
- **Composting system**: enclosed systems are more beneficial for bioaugmentation applications as they prevent the colonization of the material from the surrounding environment (Fan et al., 2018).

The outcomes of bioaugmentation reported in the literature are mixed, but generally more positive than negative and seem to be more effective on lignocellulosic substrates compared to MSW (Fan et al., 2018). The effectiveness of bioaugmentation seems to be limited to specific compost parameters. It has been associated with shorter composting time, higher humic acid production, enhanced degradation of OM, higher germination index and better nutrient retention (Che Jusoh et al., 2013; Ding et al., 2016; Ke et al., 2010; Manu et al., 2017). On the other hand, numerous studies have reported little effect of microbial inoculants. The study published by Pandey et al. (2016) did not report significant differences in composting parameters when using a commercial EM composed by thermophilic bacteria, and Nair and Okamitsu (2010) found no improvements when inoculating lignocellulolytic microorganisms on kitchen waste and green waste. Vargas-García et al. (2007) concluded that the efficacy of bioaugmentation depends on the combination of inoculum and feedstock type. A list of studies on bioaugmentation on different feedstocks is shown on Table 1.1. Some authors argue that simply controlling biochemical parameters (C/N ratio, moisture, pH, etc.) is sufficient to stimulate the indigenous microorganisms, making additional inoculation unnecessary (Fan et al., 2018). The inconsistencies observed across different studies and the difficulties in reproducing results are likely associated with the high complexity of the composting process and the variable nature of organic waste (Cerda et al., 2018). These challenges highlight the need for further research and the development of a decision-making framework that aligns the type of inoculant with the type of feedstock. This would help reduce unnecessary interventions during composting.

These challenges partly explain why the implementation of this approach in large-scale treatment plants is not adopted (Fan et al., 2018). To move forward, comprehensive feedstock characterization is required, along with intense research into the substrate-specific microbial community associated with different types of waste. After that, an appropriate inoculant can be chosen along with its dosage

and timing of application. Moreover, assessing the effectiveness of inoculants requires a highly monitored environment, to allow the rigorous measurement of indicator parameters such as temperature, pH, and gas emissions. Finally, a cost-benefit analysis should weigh the economic viability of bioaugmentation relative to its benefits. Currently no universal inoculant is compatible with all the composting scenarios, hence, more research is needed to understand this methodology and the conditions under which bioaugmentation can be most effective.

Table 1.1 Studies investigating the effect of bioaugmentation on different feedstocks

Reference	Feedstock	Inoculum	Findings
(Ke et al., 2010)	Food waste	*Thermomyces vulgaris* A31 (thermotolerant, lipolytic actinomycete)	Reduction fat content, decreasing maturation time
(Nakasaki & Hirai, 2017)	Food waste, sawdust	*Pichia kudriavzevii* RB1 (organic acid degrader)	Higher OM degradation; higher organic acid degradation; decrease maturation time
(Ding et al., 2016)	Food waste	Consortium of anti-acidification bacteria (*Pseudomonas, Bacillus, Lactobacillus*, etc.)	Early acidification prevented; higher humic-acid content
(H. Wang et al., 2011)	Wheat straw and cattle/chicken manure	*Penicillium expansum* (lignocellulolytic)	Higher germination index, higher humus content, higher lignocellulose degradation
(Zeng et al., n.d.)	Agricultural waste	*Phanerochaete chrysosporium* (lignocellulolytic)	Faster cellulose/lignin degradation, enhanced thermophilic phase
(Zhao et al., 2016)	Chicken manure and maize straw	*Streptomyces* sp. and *Micromonospora* sp (lignocellulolytic)	Accelerated degradation of OM, especially celluloses
(Zhao et al., 2017)	Corn staw and dairy manure	*Streptomyces* and *Actinobacteria* (lignocellulolytic)	Increase of humic acids, increased degradation of cellulose
(Manu et al., 2017)	Food waste, grass and leaves	Commercial EM phototrophic bacteria	Active decomposition time reduced, fester degradation of lignocellulosic compounds
(Karnchanawong & Nissaikla 2014)	Food scraps and dry leaves	Mature compost and two commercial EM	Only mature compost slightly stabilizied C/N ratio earlier and reduced VOCs emission
(B. Xi et al., 2005)	Municipal solid waste	One blend of *Bacillus* spp. and one blend of cellulolytic fungi	Enhanced thermophilic phase, odor reduction, increased respiration
(Rashad et al., 2010)	Rice-straw and okara	*Trichoderma reesei* and *Phanerochaete chrysosporium* (cellulose degrading fungi)	Enhanced thermophilic phase, decrease of C/N, increase in N and P, increased respiration
(Che Jusoh et al., 2013)	Rice straw, goat manure, green waste	Commercial EM (lactic acid bacteris, yeasts and phototrophic bacteria)	Enhanced thermophilic phase, decrease of TOC/OM, decrease of C/N, increase of NPK
(C. Ma et al., 2019)	Sewage sludge and sawdust	Mature compost	Increased enzymatic content and bacterial diversity. Reduced peak of thermophilic phase.

Table 1.1 (continues)

Reference	Feedstock	Inoculum	Findings
(Varma et al., 2015)	Vegetable waste, cow dung, sawdust and dried leaves	*Phanerochaete chrysosporium*	VOS reduction, prolonged thermophilic phase, higher OM degradation, higher N and P
(Fersi et al., 2019)	Green waste, olive mill wastewater	*Trametes trogii*	Improved degradation of OM and lignin; higher stalk lenght and biomass
(B. D. Xi et al., 2012)	Municipal solid waste and dry grass	*Nitrobacter* and *Thiobacillus*	Improved degradation of aliphatics, proteins and polysaccharides; increase of humic acids
(Vargas-García et al., 2007)	Agricultural waste	*B. shackletonni, S. thermovulgaris* and *U. thermosphaericus*	Improved degradation of cellulose and lignin for *Ureibacillus thermosphaericus*
(Pandey et al., 2016)	Food waste, horse manure, and green waste	Commercial EM (thermophilic microorganisms)	No significant differences vs. control
(Nair & Okamitsu, 2010)	Kitchen waste, paper, grass, sawdust	Commercial EM and *Trichoderma*	No significant differences vs. control
(Kostov et al., 1991)	Sawdust and bark	*Bacillus, Cephalosporium* and *Streptomyces*	Increase of microbial population and respirometric index
(Maboeta & Van Rensburg, 2003)	Sewage sludge and woodchips	*Pseudomonas, Lactobacillus, and Saccharomyces spp.*	Bioaccumulation of heavy metals

1.2.4. Compost maturity and stability assessment

Evaluating compost maturity and stability is crucial for its application in agriculture, horticulture and for environmental management, as these parameters directly reflect compost quality (Kong et al., 2024). Maturity indicates the degree to which the composting process has been completed, while stability is more related to the microbial activity and is typically assessed through respiration rates (Mahapatra et al., 2022). Immature compost can compete for oxygen and nitrogen with soil microbiota and plants and can release phytotoxic compounds like organic acids and ammonia. These factors can inhibit the growth of plants, deplete soil fertility and reduce productivity (M. P. Bernal et al., 2009b; Jamroz et al., 2020; Luo et al., 2023; Mazumder et al., 2020). A wide range of indicators are available to assess compost maturity and stability, each with advantages and limitations (Kong et al., 2024). Due to the diversity in composting materials and processes, maturity and stability cannot be assessed by just considering a single parameter, but rather a multiple set (Mahapatra et al., 2022). In Italy, regulatory guidelines such as Legislative Decree 75/2010 define maturity and stability requirements for organic amendments. Traditionally physical, chemical and biological indicators have served as a basis for the compost maturity assessment, however, also metagenomic techniques have been recently applied (Estrella-González et al., 2020).

Physical indicators include temperature, color, odor and moisture content and rely on the observable properties of compost. These provide a quick, easy and cost-effective qualitative assessment; however, they are inherently subjective and can lack precision. A mature compost is typically cool, with a dark brown color and earthy smell (Kong et al., 2024).

Chemical indicators provide quantitative data and include pH, electrical conductivity, C/N ratio, dissolved organic carbon and humification. These need to account for the feedstock properties and on the scope of application, which can complicate the interpretation. For example, while EC values

above 4 mS/cm are often considered inhibitory to seed germination (Chung et al., 2021), composts made from sheep manure or sludge may exceed this threshold while still achieving a high germination index (GI) (Wu et al., 2019; J. Yang et al., 2022). C/N ratio is usually one of the most used indicators and its values should ideally fall between 15 and 10 at the end of maturation (Hsu & Lo, 1999). This metric is not applicable for feedstocks like chicken manure or sewage sludge where the C/N ratio is already very low at the beginning of the composting process (Ma et al., 2022; Yuan et al., 2016). Humification, the microbial transformation of organic matter into humus-like substances, is another index for the evaluation of maturity because it occurs especially during the late phase of composting (M. Bernal et al., 2017).

Biological indicators, like germination and growth tests are also widely used and assess phytotoxicity (Kong et al., 2022; Luo et al., 2018). Germination tests or germination index (GI) are often performed with Cress (*Lepidium sativum*) (M. P. Bernal et al., 2009a; Mahapatra et al., 2022). A GI higher than 60% is usually indicative of mature compost, although national standards may vary. Despite its wide use, GI results are very variable due to different seeds used, compost extract preparation, cultivation and germination protocols, which lead to problems in replication (Jalili et al., 2019; Lerma-Moliz et al., 2023; Soré & Ouoba, 2023). Microbial respiration indicators, like CO_2 production and O_2 uptake, are primarily used for stability. Immature compost exhibits an intense microbial respiration due to the presence of bio available OM in the material, as soon as the OM is transformed into humus, the oxygen consumption and CO_2 production decrease (M. Bernal et al., 2017; Samal et al., 2022). Unlike chemical parameters, biological tests are universally applicable across different feedstocks, however, methodological standardization is required for results comparison (Kong et al., 2024).

Many studies involving metagenomics and metabarcoding have investigated compost biodiversity (Antunes et al., 2016; Guidoni & Vannini, 2024; Langarica-Fuentes et al., 2014; K. Wang et al., 2018) but its correlation with maturity parameters have been poorly studied. For this reason, microbial diversity indicators associated with compost maturity have not been identified yet. Correlation between diversity indexes like Chao1 and Shannon with traditional maturity indexes like GI, humification rate and oxygen demand has been found by Estrella-González et al., (2020). Moreover, negative correlations were found between bacterial diversity and both the C/N ratio and nitrification index, whereas fungal biodiversity correlated positively with compost moisture. GI was also positively linked to microbial diversity.

Despite abundant research, maturity and stability assessment still lack standardization. The wide array of parameters available should be tailored to feedstock and operational variability as each maturity evaluation index is applicable to specific raw materials and conditions. A universally applicable framework seems unrealistic, instead, different composting conditions should be treated with specific approaches.

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Aims and objectives

Bio-waste constitutes the largest fraction of MSW and its sustainable managements is fundamental to mitigate greenhouse gases emissions, prevent water pollution and reduce health risks for the population. Composting is currently the primary technology for treating bio-waste, however, it faces many challenges such as lack of efficiency, long processing times and an end product of generally low quality. Moreover, the high heterogeneity of organic waste complicates standardization, hence, process conditions need to be designed for each feedstock composition. Scientific research on composting enhancement has demonstrated that an improved control over process parameters can sensitively enhance the quality of the final product and the velocity of the composting process. Furthermore, the increasing studies on microbial communities inhabiting compost environment are showing their complex dynamics whose understanding is critical for developing new biological parameters for compost quality assessment and using microorganisms for biotechnological purposes.

This thesis has the general aim of contributing to the enhancement of composting processes by investigating the microbiology of compost and the critical factors that often hinder efficiency or slow down compost production. Specifically, this project focuses on designing an optimal feedstock composition for bio-waste composting that provides favorable physiochemical conditions for organic matter transformation, advancing our knowledge of the microbial communities involved in composting, contributing to the production of pathogen-free compost, and exploring the potential of biotechnologically relevant species to optimize the process and enhance the agronomic value of the final product.

For these reasons, this thesis had the following objectives:

- Defining the optimal balance between organic waste and bulking agents, in order to set the best physiochemical conditions for composting. This includes testing and comparing different feedstock compositions and evaluating their impact on major composting parameters.

- To study microbial community dynamics during composting, with a focus on analyzing its changes during the main composting phases and between feedstock composition. This includes investigating the abundance and distribution of taxa associated with specific environmental conditions and evaluating their role in assessing compost quality and maturity.

- To encourage green waste producers, like plant nurseries, to establish their own circular waste management system through the development of simple operational procedures for producing pathogen-free compost. This involves evaluating and comparing different diagnostic techniques for monitoring pathogen presence both during the composting process and in the final product.

- To assess the effect of bioaugmentation on the composting process, with the goal to increase microbial activity, improve the degradation of complex organic molecules and increase the quality of the final product.

CHAPTER II - Food Waste Composting with Wood Chips: Preliminary Analysis of the Bacterial and Fungal Communities

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ABSTRACT

Wood chips represent an important component in improving the composting of food waste by contributing to the optimization of the carbon/nitrogen ratio and providing porosity and aeration of the mass, which are determinant factors for proper aerobic fermentation and maturation processes carried out by microbial communities. This work evaluated the taxonomic complexity and functionality of the microbial communities associated with the composting process of three different w/w (wet weight) ratios of food waste and wood chips using traditional isolation methods and high throughput sequencing techniques (HTS). Results highlighted differences in the presence of specific taxa associated with certain ratios. The obtained results make a substantial contribution to understanding microbial dynamics during food waste composting.

2.1. Introduction

Disposal and treatment of food waste pose challenges, potentially leading to soil consumption, greenhouse gas emissions, foul odors, and leakage (Palaniveloo *et al*., 2020). Composting offers a solution to convert food waste into a valuable product; however, it is not straightforward to produce safe and quality compost since the starting material has physiochemical characteristics that hinder the aerobic degradation of the organic matter (Cerda *et al*., 2018). To overcome these technical problems, bulking agents were added to tune the balance between carbon and nitrogen and to allow proper aeration of the material. The most used bulking agents were woody residues (Li *et al.*, 2013) and biochar (Waqas *et al*., 2018). The choice of a suitable bulking agent and its quantity were among the most critical factors to consider before starting the process because high moisture, poor aeration, and inadequate pH levels can promote undesirable fermentation. Conversely, specific aerobic microbial taxa capable of degrading recalcitrant molecules, regulating pH levels, and promoting the production of humic substances are crucial. Since the presence of certain taxa is related to specific physiochemical parameters (Palaniveloo *et al*., 2020), detecting these taxa could give important information about compost quality or warn about the presence of unfavorable conditions. This study evaluated the microbial communities of three different weight-to-weight (w/w) ratios of food waste and wood chips (2:1; 3:1 and 5:1 w/w) using traditional isolation methods and high throughput sequencing (HTS) techniques. The aim was to determine the effects of different amounts of wood chips on food waste composting performance and on the microbial communities, leading to the development of a methodology capable of detecting specific taxa associated positively or negatively with the composting process and its maturity.

2.2. Materials and methods

Composting material included seasonal fruits and vegetables. Before composting, the material was shredded manually to reach a particle dimension of about 5-8 cm. The bulking agent was composed

of woodchips. Composting was carried out using rotating composting bins of 180 L. Three different ratios of food waste to wood chips were studied based on a wet weight ratio (w/w): 2:1, 3:1, and 5:1. Composting process lasted 60 days (from 1/06/2023 to 31/07/2023) during which samples of 10 grams used for microbial isolation were taken from each composting bin thermophilic phase (day 4), cooling phase (day 20) and at the end of the maturation phase (day 60). Samples for metagenomic analysis were collected following temperature changes on ranges of 10 °C each during the active phase (until day 30), while every 15 days during the maturation stage (from day 31 to 60).

2.2.1. Chemical and Physical Analysis

Temperature was measured using a long-stem thermometer (Humboldt Mfg. Co., Range of –50 to 150°C, Stem length 20 cm). The moisture content was determined by drying at 105 °C for 24 h. The pH was measured in a 1:10 (w/v) water extract. Total organic carbon (TOC) and nitrogen (N) were determined following the standards UNI EN 15936:2012 (DIN, 2012) and UNI EN 16168:2012 (En, 2012) respectively. Organic matter content was assessed by determination of weight loss on ignition at 550 °C.

2.2.2. Microbial isolation

Fungi and bacteria were isolated from a suspension obtained by adding 10 g of fresh material to 90 mL of sterile saline solution (0.9% NaCl in distilled water). The suspension was shaken (150 rpm) at room temperature for 30 min. After that, a 10-fold serial dilution method was performed and 100 µL aliquots of each dilution were spread out in five replicates of Rose Bengal Agar and five of PCA Agar plates. Fungi and bacteria were allowed to grow for 72 and 24 h respectively at 30 °C (mesophilic and mature stage) or 45 °C (thermophilic stage). Results were expressed as colony-forming units (CFU) per milliliter of suspension. All different morphotypes identified were isolated and maintained in the same conditions as above and regularly transferred to fresh agar.

2.2.3. Molecular identification of the isolates

The sequence of ITS and 16S regions was used for fungi and bacteria identification respectively. For fungi, genomic DNA was extracted from the pure microbial cultures, using the kit “NucleoSpin Plant II” (Macherey– Nagel Gmbh & Co. Düren, Germany), according to manufacturer instructions. For bacteria, amplification was done directly by scrapping single colonies. The amplification reaction was carried out using primers ITS1 (5’- TCCGTAGGTGAAC CTGCGG-3’)/ITS4 (5’ - TCCTCCGCTTATTGATATGC-3’) for fungi and the 16s rRNA gene primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) for bacteria. Negative controls were used at each stage. The PCR reaction mix comprised 12.5 µL of GoTaq® Hot Start Master Mix (Promega, Madison, WI, USA), 1 µL for each primer, 1 µL of Bovine Serum Albumine (ThermoFisher Scientific, Waltham, MA, USA), and 1 µL of template for a total volume of 25 µL. For bacterial isolates, the microliter of template was not added since DNA extraction was not performed. The presence of the amplicons was determined through electrophoresis on a gel with 1% of agarose and visualized by staining with Gel Red (Biotium). Amplicons were cleaned using the NucleoSpin Gel and PCR Clean-up Reagent kit (Macherey–Nagel; Düren, Germany), following the manufacturer's specifications, and quantified via “Quant-it Assays” (Invitrogen, Eugene, Oregon, USA). The suitably prepared samples were sent to Eurofins genomics (Luxemburg; http://www.eurofins.com) for Sanger sequencing. The sequences obtained were analyzed using the Bioedit Sequence Alignment Editor software and were compared with those present in the NCBI (National Center for Biotechnology Information) database using the NCBI Blast function (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.2.4. High Throuhput Sequencing

For the HTS analysis, two replicates of DNA extraction (0.5 g of material each) were performed for each sample (for both fungi and bacteria). Total DNA was extracted using the kit “Nucleospin Soil”

(Macherey–Nagel Gmbh & Co. Düren, Germany) according to the manufacturer's instructions and then pooled together. The PCR reaction mix comprised 12.5 µL of GoTaq® Hot Start Master Mix (Promega, Madison, WI, USA), 1 µL for each primer, 1 µL of Bovine Serum Albumine (ThermoFisher Scientific, Waltham, MA, USA), and 1 µL of template for a total volume of 25 µL. Multiplexing PCR was carried out using a set of barcoded primers with a dual indexing primer targeting the ITS2-5.8S-ITS2 and 16S regions for fungi and bacteria respectively. Two replicates of PCR were conducted, and the products were pooled. Amplicons were purified using the MagJET NGS Cleanup (ThermoFisher Scientific, Waltham, MA, USA), quantified with the Qubit dsDNA HS assay kit, and pooled at equal concentrations for sequencing. Paired-end sequencing (2 × 300 bp) was carried out on an Illumina MiSeq sequencer by Fasteris (Genesupport SA, Plan-les-Ouates, Switzerland).

2.2.5. Bioinformatic analysis

The bioinformatics pipeline of (Morales-Rodríguez *et al*., 2021) was followed to decrease false assignments and cross-contamination, to perform quality filtration, trimming, merging, OTU clustering, and OTU identification. Qiime2 (Bolyen *et al*., 2019) was used for metacommunity analysis of composition "ANCOM" (Mandal *et al*., 2015).

2.3. Results and discussion

2.3.1. Temperature and physiochemical characteristics of compost

After 24h from the beginning of the trial, the temperature of the material started to increase by reaching the peak after 3-4 days. After that, the material started cooling and reached the ambient temperature around the 20th day. Ratio 3:1 showed to have a stronger thermophilic phase compared to the others, with temperatures above 50 °C and maintaining the material above 40°C for a week (Figure 2.1). The amount of humidity, was particularly high in the ratio 5:1 (76%) due to smaller amount of bulking agent while was considered acceptable in the ratios 2:1 and 3:1. Nonetheless, at the end of the 60th day, humidity was below the limit of the Italian national regulation in all three cases (Dlgs. 75/2010, published in G.U. n.126 26/05/2010). The C/N ratio was low for all three trials, due to the high amount of nitrogen in food waste (Wang & Zeng, 2018). This condition caused the loss of precious nitrogen probably through leachate in the 5:1 ratio as its C/N ratio increased sensitively into mature compost and its (total nitrogen) N tot decreased by 28%. Regarding trials 2:1 and 3:1 C/N ratio remained constant, and a lower amount of nitrogen was lost thanks to better starting conditions. The results of the physiochemical analysis of the starting material and the mature compost are displayed in Table 2.1.

2.3.2. Microbial isolates

The colony-forming units (CFU) count revealed a notable prevalence of bacteria over fungal populations during the composting period (Figure 2.2). The thermophilic phase was characterized by a generalized low microbial load. This occurred because high temperatures created a hostile environment for most of the microorganisms, favoring the proliferation of specific bacteria, and decreasing the other taxa (Ryckeboer *et al*., 2003). After the thermophilic phase, where the degradation of recalcitrant molecules such as lignin and hemicellulose took place (Zhu *et al*., 2021), the decrease in temperatures created a favorable environment for the growth of fungi and other bacterial species which resulted in a higher CFU/ml count. The higher presence of lignocellulosic bulking agents in the ratios of 2:1 and 3:1 appeared to promote the growth of fungi into mature compost, while in the ratio of 5:1 a lesser presence of fungi was evident. Notably, the 3:1 ratio exhibits a consistent increase in both fungal and bacterial presence over time. These trends may be attributed to the unique capacity of fungi to degrade lignocellulosic molecules, to the superior physiochemical characteristics of the compost mix 3:1, and to its more intense thermophilic phase, which helps the effective degradation of complex molecules (Turan & Ergun, 2008). The overall increase of the

microbial biomass in mature compost matches with the findings of Li *et al.*, (2013) and Pot *et al.*, (2021) which demonstrated the positive correlation between maturity and microbial diversity. The taxa isolated from single colonies (Table 2.2) belonged to nine different bacterial genera including members of the *Bacillus amyloliquefaciens* group, classified as Plant Growth Promoting Bacteria (PGPB) (Ngalimat *et al*., 2021). Among members of the genus *Pseudomonas*. *Pseudomonas mucidolens* which is capable of degrading low-density polyethylene (Krishnaswamy *et al*., 2022), and *Pseudomonas aeruginosa* which can potentially pose a risk to human health (Bassetti *et al*., 2018) together with the bacteria *Klebsiella pneumoniae* (Li *et al*., 2014). Regarding fungal diversity twelve genera were isolated, many of them described as capable of degrading cellulose, lignin, and hemicellulose such as *Acaulium* spp. (He *et al*., 2022) and *Bjerkhandera* spp. (RobledoMahón *et al*., 2020) or to regulate the level of organic acids in compost like *Pichia kudriavzevii* (Nakasaki & Hirai, 2017). There are several explanations for the existence of pathogens in compost, including secondary colonization from the surrounding environment (Palaniveloo *et al*., 2020); yet, it's also likely that they were present in the starting material and survived the entire composting process.. Interestingly, the ratio of 3:1 did not show the presence of bacterial pathogens; possibly, its stronger thermophilic phase contributed to the removal of the other hazardous organisms.

2.3.3. Microbial community characteristics

ANCOM analysis (Mandal *et al*., 2015) allowed the identification of specific taxa in compost mixes. For fungi, *Barnettozyma californica* was found particularly abundant in the ratio of 5:1 where N amount was higher, and its loss was greater during composting. This yeast can degrade edible oil thanks to lipase production (Ciafardini *et al*., 2006) and has an interesting ammonium reduction (Falih & Wainwright, 1995) and aerobic denitrification capability (Fang *et al*., 2021) which could explain its more abundant presence in the ratio of 5:1. Among bacteria, the ratio of 5:1 was particularly enriched by anaerobic bacterial genera such as *Bacteroides* (Wexler, 2007) and *Phascolarctobacterium* (Wu *et al*., 2017) which are commonly found inhabiting the human body but also by *Advenella* sp. which included some species associated with N-rich environments such as slurry wastewaters (Matsuoka *et al*., 2012) and others that can harbor plant growth promoting activities like phytase production (Singh *et al*., 2014). *Bordetella* and *Delftia* instead, were more present in the ratio of 2:1. Genus *Bordetella* includes several species, some of them well studied because are causing diseases in humans such as *B. pertussis* and others isolated from environmental samples which are less known (Kamanova, 2020). *Delftia* spp. is known to be particularly useful for bioremediation thanks to its capacity to store heavy metals (Benndorf & Babel, 2002).

2.4. Conclusions

The findings indicated that the use of wood chips at a 3:1 ratio demonstrated superior chemical and physical properties, resulting in a more pronounced thermophilic leading to the production of a safer product. Conversely, ratios of 2:1 and 5:1 exhibited suboptimal performance. Microbiological analysis carried out in this study demonstrated the predominance of the bacterial population over the fungal population and the importance of compost as a source of potentially useful microorganisms such as *Pseudomonas mucidolens* and *Pichia kudriavzevii* which could serve as valuable microbial starters in future applications to enhance composting. Metabarconding allowed the detection of specific microorganisms associated with suboptimal composting parameters such as *Bacteroides* spp., thriving in anaerobic conditions, and *Barnettozyma californica*, prevalent in nitrogen-rich environments. The results of this study contributed to a more efficient use of wood chips in food waste composting and deepen the knowledge regarding microbial communities involved in the composting process.

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Table 3.1 Results of the physiochemical analysis of the three compost mixes at the beginning (day 0) and at the end of composting cycle (day 60). The expressed ratios are between agri-food waste and wood chips.

Index	U.M	2:1 (0)	3:1 (0)	5:1 (0)	2:1 (60)	3:1 (60)	5:1 (60)
pH		4.5	3.9	3.9	6.2	6.8	7.6
Humidity	%	53	70	76	41	45	46
TOC	%ss	35	32	29	31	27	35
N tot	%ss	1.9	2.1	2.1	1.5	1.6	1.5
C/N		18	16	13	20	16	23

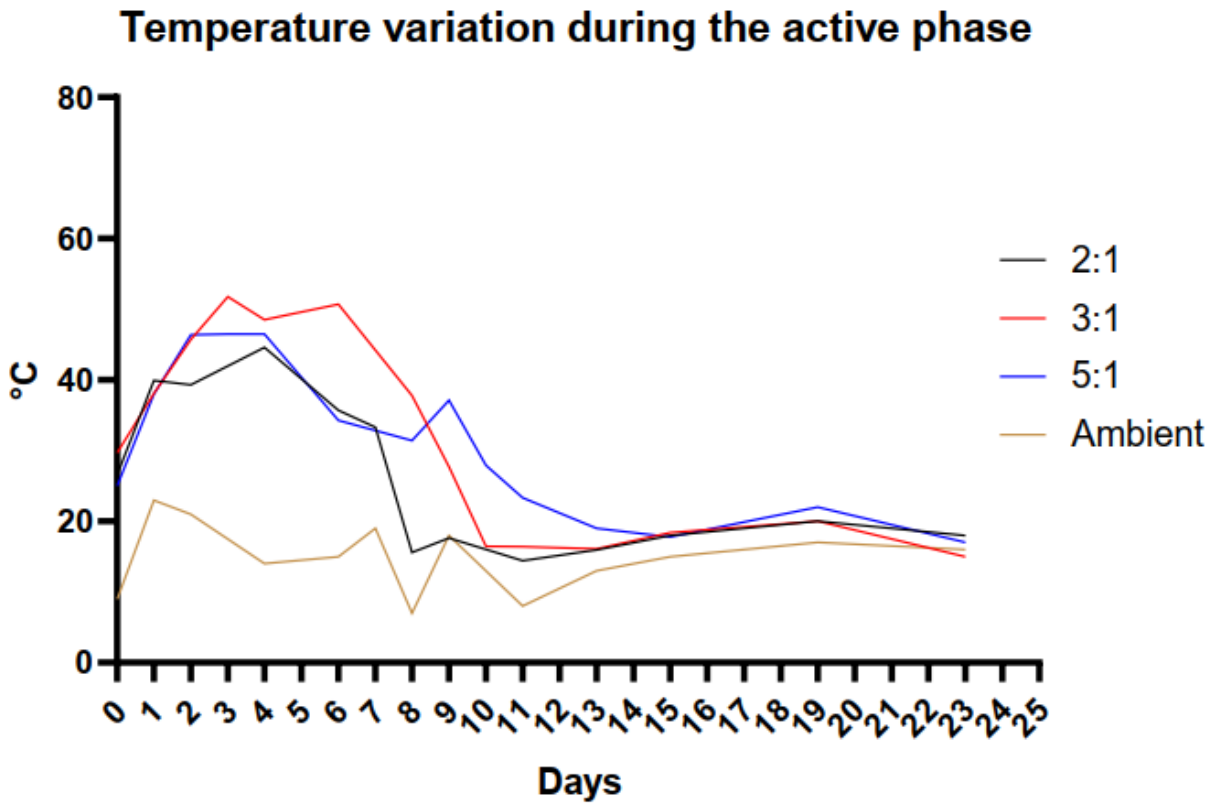

Figure 2.1 Temperature trend in the first 25 days of the three compost mixes and of the ambient temperature.

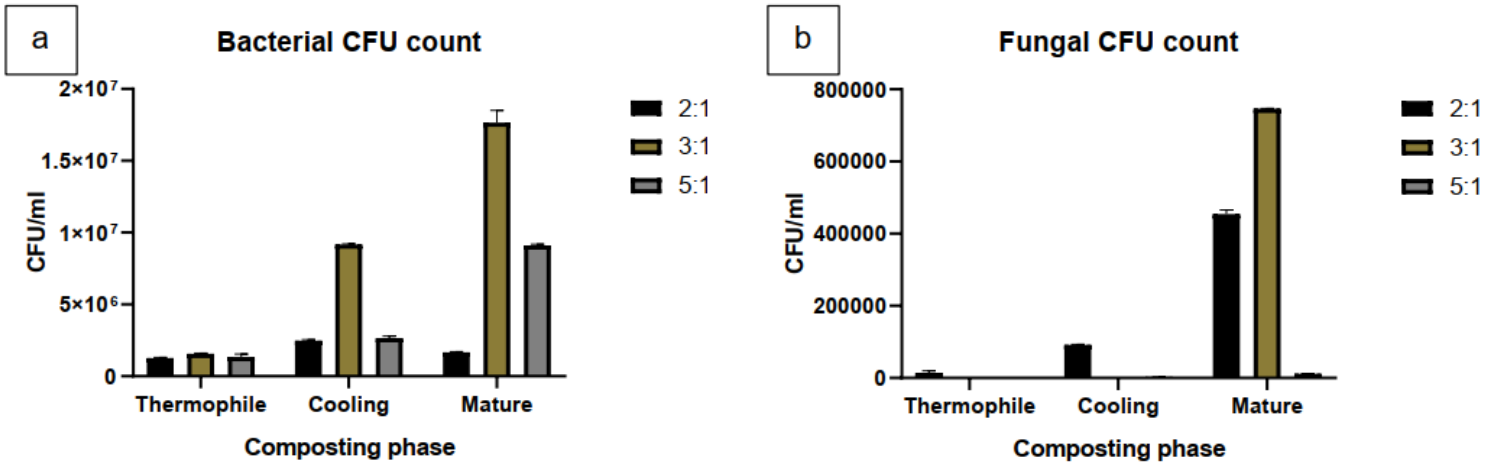

Figure 2.2 Bacterial (a) and fungal (b) CFU count for each composting ratio measured during the three main phases of the process.

Table 2.2 Fungal and bacterial taxa isolated from the three different compost mixtures, where "x" means presence. The letter into the brackets indicate the phase of isolation: T=thermophile, C=cooling and M=mature.

Fungal taxon (Phase)	2:1	3:1	5:1	Bacterial taxon (Phase)	2:1	3:1	5:1
Aspergillus flavus (T,C,M)	x	x	x	*Microbacterium* spp. *(T,C)*	x	x	
Trichurus spiralis (T,C,M)	x			*Klebsiella pneumoniae (T,C)*	x		
Lichteimia corymbifera (T,C)	x	x	x	*Pseudomonas aeruginosa (C,M)*	x		
Geotrichum candidum (T,C,M)	x	x	x	*Brucella gallinifaecis (C,M)*	x		
Parascedosporium putredinis (C,M)	x		x	*Kestersia gyiorum (C)*	x	x	x
Aspergillus fumigatus (C,M)	x		x	*Bacillus subtilis (T,C,M)*	x	x	x
Cladosporium spp. *(C,M)*	x			*Lysinibacillus pakistanensis (C)*	x	x	
Pichia kudriavzevii (T,C)	x		x	*Bacillus amyloliquefaciens* group *(T,C,M)*	x	x	x
Bjerkandera adusta (C)			x	*Kurthia gibsoni (T,C)*	x	x	
Acaulium album (M)			x	*Pseudomonas mucidolens (M)*	x		
Penicillium polonicum (M)	x			*Pseudomonas composti (M)*		x	
Lomentospora prolificans (T,C,M)	x	x	x	*Cellulosimicrobium composti (M)*	x		
Mucor fragilis (M)		x					

Chapter III - Fungal communities in bio-waste composting: a comparative study of multiple ratios of woodchips vs. a perlite-cardboard blend as bulking agents

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ABSTRACT

The large production of biowaste can cause environmental risks if not managed properly. Composting is considered a sustainable solution for the disposal of this material, but generating high-quality compost requires proper design of feedstock composition and operational procedures. Microorganisms mediate the degradation of organic matter into a nutrient-rich substrate yet their response to different compost recipes is still poorly understood. In this study, fruit and vegetable biowaste was co composted with woodchips or a perlite + cardboard mix in four different recipes (two representing optimal composting conditions and the other two were unideal composting conditions). Then, using Illumina high throughput sequencing, was defined the core fungal community shared by all treatments, mapped fungal succession through all composting phases and treatments, and identified indicator taxa associated to compost's specific recipes or phases. Our results confirm the presence of a core microbiome common to all compost piles dominated by Aspergillus but also reveal that every recipe has a degree of uniqueness in its fungal community and that the type of bulking agent used can significantly affect the composition of the fungal community during the latest stages of composting. At least one biomarker was associated with every recipe, with some reflecting the environmental conditions occurring such as the yeast P. kudriavzevii when more biowaste was present or T. lanuginosus when higher temperatures were reached during the thermophilic phase. These findings will contribute to increasing the knowledge of microbial dynamics during composting and provide useful information for the future use of biological parameters to assess compost quality.

Keywords: Metabarcoding; Microbiome; Biomarkers; Mycobiota; Compost; Fungi

3.1.Introduction

Grocery stores and food markets generate significant amounts of biowaste, with one third of food produced worldwide being wasted every day. In addition, organic material represents the largest fraction of municipal solid waste generated by households (Nasir et al., 2025; Nenciu et al., 2022). In

response, in 2020 the European Union's Circular Economy Action Plan has set a target to reduce food waste production by 30% and to recycle 60% of municipal waste by 2030, with composting recognized as a sustainable method for treating organic waste (Circular Economy Action Plan, 2020).

Composting transforms organic waste into a nutrient-rich organic amendment for agriculture. However, biowaste is characterized by high humidity, low porosity, and a low carbon-to-nitrogen (C/N) ratio which, when decomposing in uncontrolled conditions, can lead to methane (CH4) and ammonia (NH3) emissions, pathogens proliferation, nutrient loss, and unpleasant odors (Bernstad and La Cour Jansen, 2012; Nenciu et al., 2022; Singh et al., 2011). High humidity can lead to the formation of leachate, a byproduct found in various waste streams (Bogdan et al., 2021). Leachate often contains high concentrations of nutrient elements like nitrogen and phosphorus; therefore, reducing its production can prevent nutrient losses from the compost (Wang et al., 2021), Moreover, if not managed properly, leachate can cause significant environmental damage, particularly in aquatic environments where it can lead to oxygen depletion (Hashemi and Khodabakhshi, 2016).

Effective biowaste composting relies on the addition of bulking agents (BAs) to regulate the physicochemical properties of the feedstock (Adhikari et al., 2009). Common BAs include straw, woodchips, zeolite, perlite, paper and sawdust (Adhikari et al., 2009; Francou et al., 2008; Margaritis et al., 2018). The choice and quantity of BAs is crucial to ensure a successful process, as any alteration in the initial organic matter composition influences microbial communities that drive composting (Franke-Whittle et al., 2014).

Fungal and bacterial communities are central to the biotransformation of organic matter and their structure and composition are influenced by both the feedstock type and the physicochemical conditions that develop throughout composting (Leow et al., 2018). Therefore, studying compost microbial communities is essential for understanding the underlying mechanisms of composting and the response of microbial communities to changing composting environments. In recent years, the combination of traditional isolation methods with high-throughput sequencing (HTS) has significantly improved our understanding of microbial succession during composting, consisting of the change in microbial taxa overtime according to the environmental conditions (Guidoni and Vannini, 2024; Ryckeboer et al., 2003). These advancements have helped identify key taxa involved in the process and isolate microbes with potential biotechnological applications. The presence of specific taxonomic groups can influence the process either positively or negatively (Palaniveloo et al., 2020). While some genera, such as Bacillus and Aspergillus spp., are ubiquitous in compost environments, other taxa are more specific to feedstocks or composting phases. For instance, yeasts are typically found in the early stages of composting (López-González et al., 2015).

The increased knowledge of the microbial communities has led researchers to propose that some bioindicators might be considered, along with traditional parameters, for the challenging task of assessing compost quality, stability, and maturity (Estrella-González et al., 2020; Pot et al., 2021). Still, a data gap remains in identifying taxa which could be a biological indicator of a particular composting condition. Recent advances in molecular diagnostic techniques, such as highly specific and sensitive Recombinase Polymerase Amplification (RPA) and CRISPR-Cas12-based detection now enable the rapid identification and quantification of target organisms (e.g. viruses, human and plant pathogens, etc.) (Guo et al., 2023; Qiu et al., 2022). These technologies have the potential to revolutionize compost monitoring by providing real-time insights into microbial community composition and function during the main phases of the process: in raw materials (Start phase), when temperatures are above 40-45°C (Thermophilic phase), when the pile starts to cool down (Cooling phase) and when the material stabilizes and humic acids are forming (Maturation phase). Moreover, the identification of substrate-specific taxa will allow a more targeted choice of species used for compost bioaugmentation, favoring those adapted to the feedstock of interest. Much of the research

has focused on microbial succession during the main phases of composting under optimal composting conditions, whereas less attention has been given to microbial communities in unsuccessful composting processes. A deeper understanding of microbial populations under varying conditions could lead to the identification of substrate-specific biomarkers that represent biological indicators of compost quality alongside physicochemical parameters. The ability to associate microbial species with environmental conditions offers a powerful tool for compost quality assessment.

The current knowledge on microbial communities in biowaste composting is limited (Gonçalves et al., 2024) and most studies focused on determining only bacterial dynamics because they were considered dominant during the transformation process, and because most fungi can't survive at high temperatures (i.e. >65°C) which may occur during composting (Bonito et al., 2010; López-González et al., 2015). However, the role of fungi in composting has recently been reconsidered due to the role of some taxa in degrading recalcitrant molecules during composting's maturation phase and since several thermophilic and thermotolerant species have been isolated and identified (Bonito et al., 2010; Wang et al., 2018).

This study investigates the fungal communities associated with fruit and vegetable composting, identifying both common fungal taxaacross 4 different feedstock recipes and those unique to specific conditions. This approach allowed us to: (1) identify fungal taxa that are ubiquitous to biowaste composting across every treatment, (2) assess the effects of different BAs on fungal biodiversity and abundance, (3) characterize fungal successions associated with the four different composting phases and (4) identify fungal taxa (i.e. indicators) associated with specific feedstock recipes and/or composting stages. This is among the first studies to link fungal community dynamics with specific biowaste composting conditions and phases. The findings contribute to optimizing the use of BAs and to enhance the understanding of fungal succession and distribution, supporting the development of novel indicators for compost maturity and quality. We hypothesize that the fungal community is significantly affected by the type and quantity of bulking agent used and that certain taxa can be used as indicators of certain physiochemical conditions.

3.2.Materials and methods

3.2.1. Composting protocol, temperature and leachate measurement

Biowaste was co-composted with two different BAs: woodchips and a mixture of perlite with shredded cardboard. Four feedstock recipes were designed: two with unideal BA quantities (both woodchips-based, one with an excessive amount of BA and another with an insufficient amount of BA), and two with optimal composting conditions (i.e. one woodchips-based and one with perlite and cardboard). Each feedstock mixture had two replicates. In total, eight composting bins were used in parallel, two for each mixture. Woodchips were made from beech (Fagus sylvatica L.) and European hophornbeam (Ostrya carpinifolia Scop.), with particle diameters ranging from 1-5 cm. The perlite had a granulometry of 2-6 mm and cardboard strips were obtained from shredded cardboard boxes. Cardboard strips, with a mean dimension of 5 cm in length and 1 cm in width, were chosen for their potential to reduce leachate formation due to the adsorption properties of cardboard. Biowaste was provided by two local shops in the University area and consisted of fruits, vegetables and green tissues of horticulture plants no longer salable because they had gone bad, such as apples, melons, cucumbers, lettuce, eggplants, tomatoes, potatoes, artichokes and cauliflowers. Before the start of the composting process, this biowaste material was crushed into pieces ranging from 1-15 cm. The feedstock (wet weight) biowaste-to-woodchips mix ratios were 2-to-1, 3-to-1 and 5-to-1 (will be referred to as ratios 2:1, 3:1 and 5:1 respectively). The biowaste-to-perlite-to-cardboard ratio was 8-1-1 (referred to as ratio p+c). The choice of the amount of woodchips used to design the optimal and unideal feedstock mixtures was based on previous studies (Guidoni and Vannini, 2024), which demonstrated that ratio 3:1 yielded better results compared to the other two ratios in terms of

temperature regime during the thermophilic phase and chemical properties of the final product. The same principle was used for p+c, where the quantity of BA was adjusted based on the proportions of cardboard (Francou et al., 2008) and perlite (Margaritis et al., 2018) used for biowaste composting in similar studies. The composting processes were carried out using 90L rotating domestic composters (AOSOM Italy Ltd, Assago, Italy) and the total initial weight of all mixtures was 30 kg (S1). The composting process started on April 1st, 2024, and lasted 60 days. During the first ten days the temperature was assessed thrice for each bin on Days 1, 2, 3, 4, 5, 7, 9 and 10 using a 20cm long-stem thermometer (Humboldt Mfg. Co., Range of –50 to 150°C), then mean and standard error mean (SEM) were calculated. Then, when the temperature started to decrease, it was assessed every four or five days just to evaluate the stability of the material. After each temperature assessment, the composter was rotated 360° to toss and aerate the mix. To assess changes in temperature trends across different ratios, a mixed-effects model was performed using GraphPad Prism v. 8.0.1. Post hoc multiple comparisons were done using Tukey's test correction. The reported p-values reflect this correction. Leachate production was collected by placing a plastic tray under each composting bin allowing liquids to pour from the aeration holes of the composters. Every day, leachate production was measured with a graduate cylinder. The liquid was kept at -18°C for chemical analysis.

3.2.2. Chemical analysis

The chemical analysis of the leachate on Day 0-16 was conducted using a 450 mL sample, obtained by pooling liquid collected from each treatment. For the compost (Days 0 and 60) the analysis was performed after pooling together the two representative samples coming from the replicates of each feedstock mixture. Samples were processed by Agribioeco s.r.l., Pomezia (Italy), a laboratory accredited by the Italian accreditation body (ACCREDIA).

3.2.2.1. Bio-waste compost

Humidity content was determined by the gravimetric method by drying compost at 105 °C for 24h. The pH, total organic carbon (TOC), salinity and humic and fulvic acids were measured following the procedures indicated in the Italian Ministerial Decree for the analysis of fertilizers (Decreto Ministeriale [DM] 17/06/2002 Gazzetta Ufficiale [GU] n° 220 of 19/09/2002). Total nitrogen (Ntot), organic nitrogen (Norg) and germination index (GI) were determined according to the standards Ente Nazionale Italiano di Unificazione (UNI) European Norm (EN) 16168:2012, UNI 10780:1998 App. J2.2 + J3.2 + UNI EN 16168:2012, and UNI 10780:1998 App. K, respectively. The Norg/Ntot and C/N ratios were calculated.

3.2.2.2. Leachate

The leachate analysis was done on all woodchip-based treatments but could not be performed on the p+c one due to its too low volume produced. The salinity, pH and Ntot were determined according to the same standards as for compost. Chemical oxygen demand (COD) was measured following the standardized methodology indicated by the Italian "Agency for Environmental Protection and Technical Services (APAT), the National Research Council (CNR) and the Water Research Institute (IRSA) method #5130 for COD and the Manual 29 of standardized analytical procedures (2003)". Heavy metals, phosphorous and potassium were measured with the standard "Environmental Protection Agency (EPA) #3052 (microwave-assisted acid digestion; 1996) + EPA #6010D (Inductively Coupled Plasma - Optical Emission Spectrometry; 2018)", except for mercury, which followed "EPA #7473 (Mercury in Solids and Solutions by Thermal Decomposition, Amalgamation, and Atomic Absorption Spectrophotometry; 2007)". Ammonium was measured using the standard "UNI 10780:1998 App. J3.2". All the leachate liquid produced by the ratio treatments was stored until the last day of leachate production (Day 16), then 150mL of leachate from each treatment were pooled together as a representative sample.

3.2.3. Sampling and library preparation for HTS

During the composting process, samples were collected on Days 1, 4, 8, 12, 20, 30 and 60, resulting in a total of seven different sampling times. Samples were divided into groups according to the sampling time: Start (Day 1), Thermophilic (Day 4), Cooling (Days 8 and 12) and Maturation (Days 20, 30 and 60). For each composter bin, at every sampling time (n=7), 25g of material were collected from three different locations (two distal and one central) and combined. Then samples were ground with a kitchen blender prior to taking 1g subsamples for downstream analyses, resulting in a total of 56 working samples. The material was kept in sterile 2mL Eppendorf microtubes at -18°C. Total DNA was extracted for each sample using the kit "Nucleospin Soil" (Macherey–Nagel Gmbh & Co. Düren, Germany) according to the manufacturer's protocol. The Internal Transcribed Spacer 1 (ITS1) region was amplified via a multiplex PCR using the ITS1F/ITS2 primer pair (White et al., 1990) tagged with dual indexing barcodes. The PCR reaction mix comprised 12.5 µL of GoTaq® Hot Start Master Mix (Promega, Madison, WI, USA), 1 µL of each primer, 1 µL of Bovine Serum Albumin (ThermoFisher Scientific, Waltham, MA, USA), and 1 µL of template DNA for a total volume of 25 µL. The thermal cycle was an initial 5-min. step at 95 °C, followed by 35 cycles of 30s at 95 °C (denaturation), 30s at 55 °C (annealing), and 1 min. at 72 °C (extension), with a final extension step of 5 min. at 72 °C. For each sample, three replicates of PCR were carried out and the PCR products were pooled. Amplicons were purified using the MagJET NGS Cleanup (ThermoFisher Scientific, Waltham, MA, USA), quantified with the Qubit dsDNA HS assay kit, and pooled at an equimolar concentration for sequencing. Paired-end sequencing (2 × 300 bp) was carried out on an Illumina MiSeq sequencer by Fasteris (Genesupport SA, Plan-les-Ouates, Switzerland).

3.2.4. Bioinformatic analysis

The raw sequence and quality score data (FASTQ files) provided by the sequencing center were processed using the Nextflow (Version 24.10.3.5933; Di Tommaso et al., 2017) workflow management system to run the nfcore/ampliseq pipeline (Version 2.12.0) (Ewels et al., 2020a, Ewels et al., 2020b). Singularity (version 3.5.3) (Kurtzer et al., 2017) was used to input the configuration profile, allowing customization of the computational resources of the machine used. In brief, the pipeline allowed to specify the data type input, did quality control on the raw sequence files (FastQC version 0.12.1), trimmed reads and removed primers (Cutadapt version 4.6) (Martin et al., 2011), used DADA2 (Version 1.30.0) (Callahan et al., 2016) for inferring Amplicon Sequence Variants (ASVs), for filtering ASVs and assigning fungal taxonomical classification (n.b. for further details, see section 2.4.1) with the UNITE database (Version 2024.04) (Nilsson et al., 2019) and then used MultiQC (Version 1.25.1) (Ewels et al., 2016) to populate a pipeline results summary. Details on the ampliseq pipeline parameters are available in S4.

3.2.4.1. Training the classifier

The ITS1 region is highly variable, contains repeats, and can lead to inconsistent results for taxonomic assignment when using NCBI BLAST, especially when processing high-throughput datasets (Raja et al. 2017). Therefore, to improve precision of taxonomic assignments and augment reproducibility, a Naïve-Bayes classifier was trained with QIIME2 (Version 2023.7) (Bolyen et al., 2019) for DADA2 data. The workflow provided by Quensen (n.d.) was used on the UNITE fungi developer database version 2024.04.

3.2.4.2. Diversity and differential analysis

The resulting ASV and taxonomy tables artifacts (.qza), along with the metadata file, were uploaded in Rstudio (Posit software, PBC, v. 4.3.1; R Core Team (2023)) and converted into a phyloseq object (Version 1.46.0) (McMurdie and Holmes, 2013) with the "qza_to_phyloseq" command for further analysis. Amplicon sequence variants with a relative abundance < 0.1% were removed and additional filtering steps were processed with the phyloseq functions "transform_sample_counts", "filter_taxa", "tax_glom" and "prune_taxa". Data was normalized with the "median" function (R package Stats,

Version 4.3.1; R Core Team (2023)). Using the phyloseq function "estimate_richness", Shannon and Simpson diversity indexes were calculated. Pairwise comparisons (function pairwise.wilcox.test; Stats) and rank sum test (kruskal.test; Stats) of the calculated diversity indexes were calculated to assess the data's non-parametric distributions. For beta diversity data, Permutational Multivariate Analysis of Variance Using Distance Matrices (PERMANOVA) was measured with a Bray-Curtis matrix using vegan v. 2.6-10 (Oksanen et al., 2019). Boxplots for alpha and beta diversities were populated using ggplot2 (Version 3.4.4) (Wickham, 2016). Linear Discriminant Analysis Effect Size (LEfSe) analysis was performed to search for potential species (indicators) unique to single composting phases using the "diff_analysis" function and from the R package MicrobiotaProcess v. 1.14.1 (Xu et al., 2023). Only species with Linear discriminant analysis effect size (LDA) >3 were considered as statistically significant. LEfSe's line segment plots were generated with ggplot2. For all statistical tests, $p < 0.05$ with Benjamini-Hochberg correction was set as the minimum requirement for statistical significance. An Upset plot was made with the R package UpSetR v. 1.4.0 (Conway et al., 2017) to visualize the abundance of fungal ASVs in each treatment, how many were commonly found in >1 mix and how many were unique to a specific ratio. Line Graphs of the daily mean temperature (°C) and the SEM for each composting treatment were plotted using GraphPad Prism v. 8.0.1. The same software was used to create line graphs of the leachate yielded (mL/kg) by the four composting treatments and their SEM. Heatmap at the genus level was generated using the R package ampvis2 v. 2.8.9.

3.3. Results and discussion

3.3.1. Temperature peaks and leachate production

At the start of the composting process, all mixes had a temperature of 26°C (Fig. 1). Ratios 2:1 (Fig. 1a) and 5:1 (Fig. 1c) were intentionally formulated with unbalanced feedstock; consequently, their peak temperatures were lower and reached later: 44.0±0.0°C for ratio 5:1 after four days and 46.0±2.0°C for ratio 2:1 after five days. Ratio 3:1 (Fig. 1b) reached a peak of 59.8±1.8°C within 24h, making it the only treatment reaching mean temperatures around 55°C for three days, a necessary threshold for pathogen inactivation (Gurtler et al., 2018). Although high temperatures like those of ratio 3:1 were expected for ratio p+c (Fig. 1d), its peak temperature was only 45.5±0.5°C after three days. This lower peak may be attributed to an excessive amount of perlite, which increases aeration and porosity (Margaritis et al., 2018) and likely prevented this mix from reaching higher temperatures. Nonetheless, it achieved a longer thermophilic phase (i.e. temperature > 40°C) compared to ratios 2:1 and 5:1. The thermophilic phase lasted seven days for ratios 3:1 and p+c, four days for ratio 2:1, and three days for ratio 5:1. The composting material cooled down to reach ambient temperature across all treatments by the ~15th day. Mixed-effects analysis showed a significant influence of the treatment ratio on temperature evolution during composting ($p<0.05$). Multiple comparison revealed differences between ratio 3:1 and 2:1 on day 2 ($p<0.05$) and on day 7 between ratios 3:1 and 5:1, 3:1 and p+c as well as between 5:1 and p+c ($p<0.05$). These differences highlight the effect of bulking agent proportions on the dynamics of the thermophilic phase during composting. The most productive leachate was found in feedstocks containing woodchips as BAs versus perlite and cardboard (S2). Ratio 5:1, which contained the most biowaste and hence was more humid, by Day 16 had a mean cumulative production of leachate of 148±2 mL/kg of compost. Ratios 2:1 and 3:1 had smaller production yields, 113±2 mL/kg and 116±4 mL/kg, respectively. After the 16th day, leachate production ceased in every treatment. As expected, given the absorbing properties of the cardboard contained in ratio p+c combined with the lack of fruit and vegetable (biowaste) to start, this treatment produced the least amount of leachate, only 5±1 mL/kg of leachate.

3.3.2. Chemical analysis

On Day 0, humidity was higher in the ratios with more biowaste (i.e. 5:1 and p+c; 76% and 72%, respectively) but, on Day 60 all ratios were below the minimum requirement threshold (i.e. 50%) for

mature compost from the Italian Legislative Decree n° 75/2010 (Table 1). All four treatments' parameters were within the limits of the Decree, except for the C/N values of ratios 2:1 and 5:1. This would make the final product non-marketable. While woodchips-based treatments had particularly low pH values on Day 0, the higher pH observed in ratio p+c (6.1) was likely due to the use of cardboard, which increases compost's pH (Francou et al., 2008). Ratio 5:1 exhibited a more marked increase in pH over time (from 3.9 to 7.6) which is usually attributed to a significant loss of ammonia (Gao et al., 2010). The strong foul odor emitted by this treatment supports this hypothesis. Woodchip-based treatments had nitrogen losses (Ntot), which led to an increase of C/N ratio at maturation. Carbon-to-nitrogen ratio typically decreases during composting because organic carbon is mineralized more rapidly than nitrogen but, persistent nitrogen losses can reverse this trend (Cesaro et al., 2019). Nitrogen can be lost in different ways, but mainly through leachate or gas emission (Bernal et al., 2009). Woodchips don't absorb many liquids, and the leachate which originated from biowaste was the primary driver of nitrogen loss, as shown by the high concentration of nitrogen in the leachate (Table 1). Also, factors like temperature, aeration and humidity regulate the amount of nitrogen that is lost (Bernal et al., 2009). In addition to leachate production, each treatment was subject to unique conditions that could enhance the loss of nitrogen: low aeration in ratio 5:1, high temperatures in ratio 3:1, and a higher fraction of low degradable organic carbon in ratio 2:1. Ratio p+c did not suffer from significant nitrogen loss, thanks to the presence of cardboard preventing leachate formation, which would have taken nitrogen away from the composting pile. For this reason, p+c was the only treatment showing a slight increase in C/N ratio. Leachate production not only reduces compost quality by removing precious nutrients, but also poses environmental risks, as it can contain heavy metals and exhibit high COD. While heavy metals contamination is more commonly associated with municipal solid waste (Roy et al., 2018), in our case, only low levels of metals, such as chromium, nickel, mercury, lead, and copper were detected. However, the COD level was particularly high (3273 mg/L), confirming the risk for aquatic animals and plants if leachate percolates into the water table. Despite these concerns, the high concentration of nutrients in the leachate suggests that it should no longer be considered merely a waste byproduct, but rather a valuable resource for the sustainable production of fertilizers. While the bigger loss of humidity at maturation in ratios 5:1 and p+c (-30% and -24%, respectively) increased the TOC probably due to a concentrating effect, high temperatures in ratio 3:1 helped achieve a stronger mineralization of the TOC (-10%). Woodchip-based treatments exhibited higher levels of inorganic nitrogen at maturation, affecting both salinity and GI. On the other hand, the absence of inorganic nitrogen and the low salinity of ratio p+c helped in achieving higher GI.

3.3.3. Fungal reads, taxa and ASVs distribution

While the usage of sequence alignment for the ITS1 region to identify fungal species is not unanimously variable enough for robust taxonomy of all fungi at the species level (e.g. Fusarium, Aspergillus), it is still a recognized barcoding region that can provide accurate identification in many instances or, at least yield meaningful insights and potential identifications (Raja et al., 2017), O'Donnell et al., 2022). The curated UNITE database and the trained classifier were combined for more accurate identifications and, given that the analysis of the different factors (temperature, acidity, bulking agent, etc.) was consistent with the potential species identified in this paper (in particular for the LeFse), the primary identifications are considered acceptable for the purpose of this article. Readers are still advised to take species tentative identifications cautiously since there are taxa within Risk Group 2 (RG2) (A. fumigatus, A nidulans, P. kudriavzevii and S. chartarum) due to their potential as human pathogens and the toxins they can produce. Limited resources could not support testing additional loci (e.g. beta-tubulin, elongation factor, calmodulin, etc.) that could have provided more robust taxonomic identification needed in a more regulated context than for composting, such as trading market of plant materials. After quality filtering a total of 3,136,230 reads, 717 ASVs and 217 different fungal species were found. All treatments shared 189 ASVs (S3), ~26% of the total taxa, which represents the core fungal community that can be found in various composting

environments, regardless of feedstock. The group sharing the second most ASVs included woodchip treatments, owning 118 common ASVs, suggesting that using the same BA, even if in different quantities, enriches the fungal community with specific taxa. On the other hand, p+c had the highest number of unique ASVs (n=116; n.b. taxonomic identifications further discussed in section 3.5). The presence of perlite and cardboard instead of woodchips introduced different microhabitats in the compost pile, affecting more radically the composition of the fungal community. Ratios 3:1, 5:1 and 2:1 had respectively 50, 19 and 18 unique ASVs.

3.3.4. Alpha and beta diversity analysis

With diversity analysis, single phases for each ratio were analyzed and compared. In the plot of the Shannon diversity index, for all ratios, raw (i.e. Start Phase) feedstock was characterized by the highest alpha diversity values (Figure 2a), which reflects a large species diversity that is favored by the abundance of easily degradable organic molecules in the biowaste (Bernal et al., 2009). After that (Fig. 2b), rising temperatures caused selective pressure on the native community, decreasing microbiota diversity in all the samples and allowing the colonization of a few thermophilic and thermotolerant species. Alpha diversity values also kept decreasing during the cooling phase (Fig. 2c). This trend of decreasing fungal diversity up until the cooling phase has already been described and confirms that temperature is not the only driver for fungal succession, and that time and other factors like the decrease in the nutrients availability inside the composting material also have an impact (López-González et al., 2015). Differences among ratios were concentrated during the maturation (Figure 2d), where Kruskal-Wallis statistical test resulted significant for Shannon index ($p < 0.05$). For the other phases (Start, Thermophilic and Cooling), differences were not statistically significant. No significant results either were obtained for Simpson diversity index (Fig. 2 e-h). Simpson diversity index shows values near the maximum value (i.e. 1), which means that the microbial community was dominated by a few taxa. This domination decreases only during the cooling phase (Fig. 2g), probably due to the recolonization of the compost pile by other fungi after the thermophilic phase. At maturation (Fig. 2h), the fewer dominant species take over again. Pairwise Wilcoxon tests were not significantly different for any index and could not segregate any specific ratio at a specific composting phase. This probably occurred because of some overlaps in the community structure caused by more abundant species and by the great variability in the diversity values within certain replicates, like in ratio 5:1 which is characterized by a large Standard Deviation. Pairwise PERMANOVA of Bray-Curtis distance matrix displayed significant ($p < 0.05$) differences among ratios during maturation between p+c vs. 2:1 (R2=0.28), p+c vs. 5:1 (R2=0.26) and 2:1 vs 5:1 (R2=0.26). In the early stages of composting, similar fungal taxa are found among different feedstocks, and then differences arise during the last two stages of composting, eventually affecting the microbiological properties of the final product. Similar results were shown by Neher et al. (2013), who observed that after a similar starting composition, differences become more marked after the thermophilic phase.

3.3.5. Changes in fungal community structure throughout the composting process

The heatmap displayed in Figure 3 shows the 28 most abundant genera, representing 83% of the total reads. No more genera could be added without affecting the readability of the plot. The graphical display of only the genera was selected because unlike the family level, these taxa can provide detailed information regarding the functionality of each taxon without being too pressing for the ITS region (i.e. identification at the species level can be difficult or even impossible with only the ITS1 locus). Their representativeness was lower at the starting phase, consisting of 73% of the total reads. This percentage, however, increases up to 83% during cooling and maturation, because of the reduction of fungal diversity in the later stages. At the beginning of the process (Day 1), compost's diversity is composed of multiple different taxa that will slowly disappear overtime. In every

treatment, there is a great abundance of Aspergillus, Penicillium and Fusarium together with many yeasts like Hanseniaspora, Meyerozyma, Wickerhamomyces, Pichia and Clavispora. Aspergillus is commonly shared in all trials, and it remains the most abundant genus throughout all phases. Aspergillus is a saprophytic fungus well-known to be dominant during composting of various feedstocks (manure, greenwaste, etc.) thanks to its heat tolerance and capacity to degrade various substrates, including lignocellulosic material (Franceschini et al., 2016). Penicillium and Fusarium are also cellulose degraders and are commonly found on spoiled food waste (Tian et al., 2017). Yeasts are favored by the acidic environment caused by the release of organic acids from food waste and are commonly associated with the initial stages of composting (Nakasaki and Hirai, 2017). At the thermophilic phase (Day 4), the distribution of these genera was not affected because mesophilic taxa can survive to high temperatures in the outer edges of compost or as spores and can eventually recolonize during cooling and maturation (Ryckeboer et al., 2003). The higher temperatures reached in ratio 3:1 (i.e. 59.8°C), however, were a significant driver in favoring some population over others, allowing the growth of Thermomyces, Thermoascus and Thermochaeotides. These are all thermophilic fungi capable of producing thermostable laccase, cellulase and peroxidase, which are highly effective in degrading lignocellulosic material (Neher et al., 2013; Peixoto-Nogueira et al., 2008). After the thermophilic phase, most of the yeasts found in the early stages of composting across all ratios tend to disappear and then fewer genera take over the communities. Cellulose degradation has been favored during the thermophilic phase as cellulolytic fungi (incl. Trichoderma and Aspergillus) become more abundant. Kernia, a fungus capable of degrading refractory organic matter (Zheng et al., 2024), can be found across all phases and treatments and its abundance increases as composting occurs. Certain taxa became specific to certain treatments, in particular, at maturation for ratio p+c, during which it got enriched with many genera nearly absent in the other treatments: Stachybotrys, Scopulariopsis, Cephaliophora, Iodophanus and Microascus. These are lignocellulolytic taxa that are commonly found in the latest stages of composting, where they take part in the degradation of complex molecules and the formation of humic substances, which are higher in p+c compared to other ratios (Table 1) (Hubbe et al., 2010; López-González et al., 2015; Ryckeboer et al., 2003; Wang et al., 2018; Zhao et al., 2023). The presence of cardboard in ratio p+c might have favored the higher diversity of lignocellulolytic fungi given that it is a more easily accessible source of carbon compared to woodchips. In the woodchips-based treatments, instead, limited availability of nutrients caused the proliferation of fewer taxa.

3.3.6. LeFse analysis for indicator fungal species

A total of nine potential indicator species were found to be associated with a single treatment at a specific composting phase. While it was possible to find significant differences during the cooling (Fig. 4a) and maturation (Fig. 4b) phases, no indicators were found in the start and thermophilic phases. Ratios 2:1 (cooling), 3:1 and 5:1 (maturation) each had a single indicator, with ratio 3:1 having Thermomyces lanuginosus as indicator during both cooling and maturation phases. This fungus is found mainly during the thermophilic phase of different feedstocks (Hubbe et al., 2010; Neher et al., 2013) but, in this study it remained significantly more abundant during cooling and maturation. It is probable that the higher temperatures reached in ratio 3:1 helped deactivating part of the fungal community, reducing competition and leaving enough resources for T. lanuginosus to develop and remain in the substrate. Similar dynamics have also been observed for the fungus Mycothermus thermophilus during cow manure composting (Wang et al., 2018). Many common fungi active during the thermophilic phase are thermotolerant rather than true thermophilics, like Aspergillus fumigatus, which has an upper temperature limit of 52 °C (Lott Fischer et al., 1998). Temperatures above this threshold restrict its development and instead favor the growth of other fungi. Thermomyces lanuginosus appeared to thrive in the hotter (65–70°C) and more aerated parts of the composting feedstocks (3:1), correlating with previous reports of being more susceptible to low temperatures and low oxygen levels (Zhang et al., 2016). Both fungal indicators detected for ratios 2:1 (Cephalotrichum cylindricum) and 5:1 (Pichia kudriavzevii) are known to play important

roles in the degradation of the organic substrate during composting and have been previously applied as inoculants for the enhancement of composting. Their presence, moreover, seems to be directly linked with the environmental conditions which characterize each ratio. Pichia kudriavzevii is a mesophilic yeast capable of increasing compost's pH by degrading organic acids. Moreover, it has been successfully utilized for the bioaugmentation of different types of composts (Nakasaki and Hirai, 2017). While yeasts are commonly found at the beginning of composting, favored by the lower pH which inhibits the growth of other fungi and bacteria, they tend to disappear in the later stages (Ryckeboer et al., 2003). The abundant presence of P. kudriavzevii in ratio 5:1 during the cooling phase was probably favored by mild temperatures during the thermophilic phase (~35°C) and by the higher proportion of food waste increasing humidity and organic acids. Cephalotrichum cylindricum is effective in rapidly producing high yield enzymes degrading cellulose and lignin and has been tested on different lignocellulosic material such as wheat bran and wheat straw composting, proving to increase compost quality and being suggested for the solubilization of wheat bran (Hart et al., 2003). The high lignocellulosic content of ratio 2:1 combined with the decrease of easily degradable molecules, typical of the later stages of composting, might have favored organisms more efficient at processing lignin, like C. cylindricum. Ratio p+c was particularly differentiated from the other trials, with a total of six indicator taxa distributed among cooling and maturation. Aspergillus nidulans, Fusarium rubicola, Plectosphaerella nieneijeranum and Stachybotrys chartarum were the indicator taxa in the cooling phase. Stachybotrys chartarum and F. rubicola were indicator taxa also in the maturation phase together with Scopulariopsis brevicaulis and Cephaliophora tropica. All these fungi play a well-known role in the degradation of organic matter and are often associated with composting environments (Ibrahim et al., 2022; Jurado et al., 2014; Liu et al., 2025; Robledo-Mahón et al., 2020). Some of them, such as S. chartarum and A. nidulans, are linked with the latest stages of composting and are appreciated for their capacity to produce enzymes with very high potential for the degradation of lignocellulosic material (Hubbe et al., 2010; Wang et al., 2018). Aspergillus nidulans is a thermotolerant species which has also been proposed as an inoculant to enhance the composting process (Sánchez et al., 2017). Part of the indicators found in p+c, however, are also considered RG2 human pathogens so, their abundance is not desirable because it jeopardizes the biosafety of the final product. The genus Fusarium also includes human pathogens such as F. oxysporum (Liu et al., 2025) and many other plant pathogens including F. rubicola, which is the causal agent of potato stem rot disease (Riaz et al., 2022). Stachybotrys chartarum is known to produce mycotoxins responsible for skin allergy reactions in workers handling inoculated potting substrates (Beffa et al., 1998). Most of the species detected in the LeFse analysis seem to have their abundance associated with composting conditions that are complementary to their ecological niche. These data would likely provide valuable insights if applied to compost maturity assessments and bioaugmentation, because it would allow the detection and exploitation of target taxa which are naturally adapted to specific feedstocks conditions.

3.4. Conclusions

Biowaste composting can be underperforming or even unsuccessful if BAs are not properly selected. In this study, most of the fungal communities were not affected by the different physiochemical properties among treatments, and the distribution of dominant taxa along with their temporal turnover was very similar early on. Most of the differences in the fungal communities emerged during the later stages of composting. These differences were accentuated in the treatment where woodchips were substituted with perlite and cardboard, whereas variations in the amount of woodchips had a lesser impact. All biomarker fungal species were identified during the cooling and maturation phases; in some cases, their presence was closely-associated with the environmental conditions of the respective treatment like the fungus T. lanuginosus in ratio 3:1 and P. kudriavzevii in ratio 5:1. Overall, ratio p+c gave better results in terms of compost physiochemical quality and leachate retention capacity, however, a slight reduction of aeration by decreasing the amount of perlite should be tested for reaching higher temperatures during the thermophilic phase and ensuring the sanitization of the end

product. This study is one of the few to look into fungal communities rather than bacteria during composting, and the results demonstrate the effects of changing environmental conditions driven by BAs on fungi. These findings will contribute to a better understanding of the microbiological dynamics in biowaste composting and aid in the identification of specific taxa associated with both positive and negative composting conditions.

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Table 3.1. Results of the chemical analysis for leachate (first 16 days) and for compost at the beginning and end of the composting process. The column "Min. req." displays the minimum requirements for mature compost according to the Italian Legislative Decree n° 75/2010. U.M.= unit of measure; dS/m = decisiemens per metre; dm=dry matter; TOC= total organic carbon; Ntot= total nitrogen; Norg= organic nitrogen; GI= germination index; COD= chemical oxygen demand, N/A = Not applicable.

		Leachate (Total yield between Day 0-16)								Min. req.	U.M
Parameter analysed	ph	6.9								N/A	N/A
	Salinity	3.4									dS/m
	COD	3273								N/A	mg/L O_2
	Phosphorous	63									mg/kg
	Potassium	730									
	Heavy metals – Chromium	<0.25									
	Heavy metals – Nickel	<0.3									
	Heavy metals – Mercury	<0.01									
	Heavy metals – Lead	<0.25									
	Heavy metals – Copper	<1									
	Ammonium NH_4	87.2									mg/L
	N_{tot}	243									mg/L N
		Compost feedstock									
		Day 0				Day 60					
	Ratios	p+c	2:1	3:1	5:1	p+c	2:1	3:1	5:1		
Parameter analysed	ph	6.1	4.5	3.9	3.9	8.3	6.2	6.8	7.6	6-8.5	N/A
	Salinity	0.1	1.1	0.7	1.7	1.6	4.1	6.7	5.7	N/A	dS/m
	Humidity	72	53	70	76	48	41	45	46	≤50%	%
	TOC	27	51	50	41	32	47	40	47	≥20%	%dm
	Humic and fulvic acids	7	10	11	11	12	7	8	8	≥7%	%dm
	N_{tot}	2.2	1.9	2.1	2.1	3	1.5	1.6	1.5	N/A	%dm
	N_{org}	2.2	1.8	1.9	2	3	1.3	1.3	1.2	N/A	%dm
	C/N	13	27	23	19	10	31	25	31	≤ 25	N/A
	N_{org}/N_{tot}	99	94	92	95	100	91	86	83	≥80%	%dm
	GI	80	71	70	70	118	74	73	79	≥60%	%

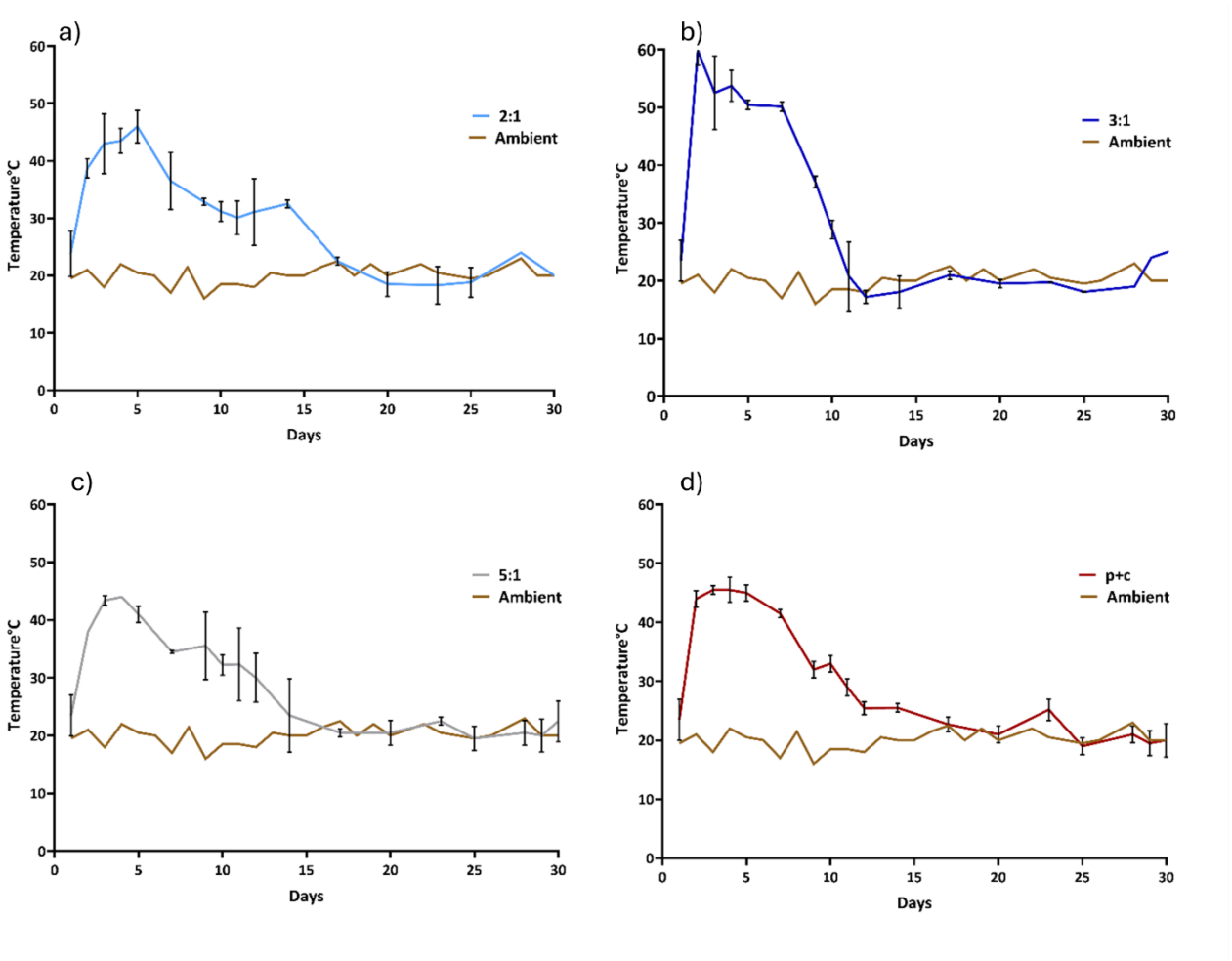

Figure 3.1. Mean ± SEM temperature (°C) for composting treatments (a) ratio 2:1, b) ratio 3:1, c) ratio 5:1 and d) p + c during the first 30 days of composting. Together with every treatment, ambient temperature is displayed.

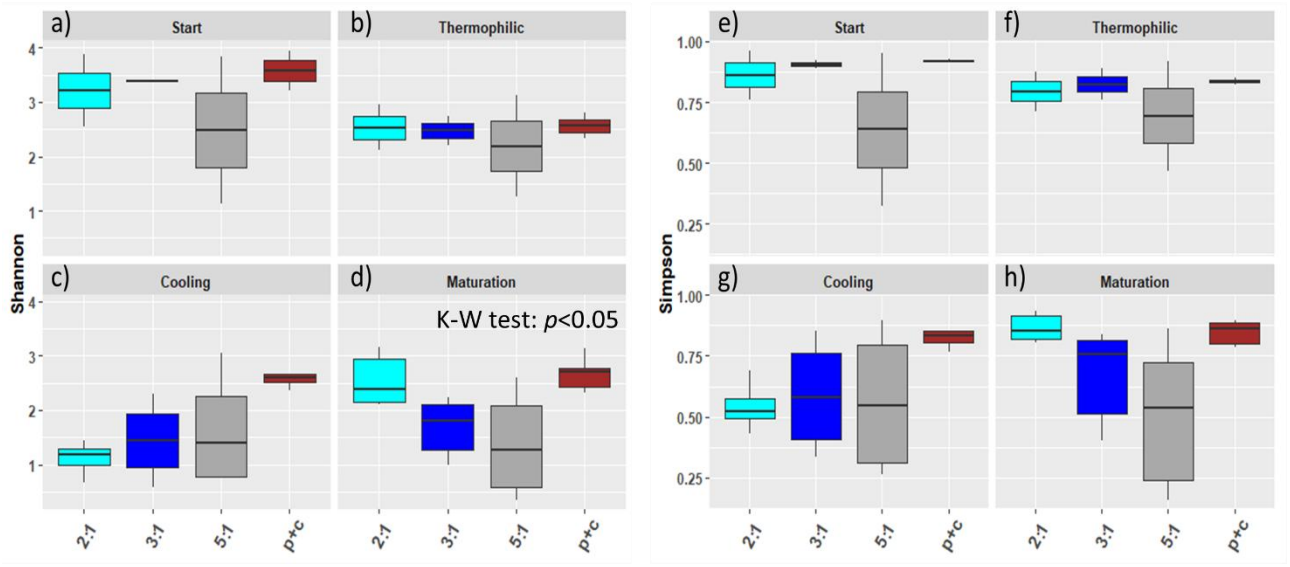

Figure 3.2. Mean ± SD of Alpha diversity values for Shannon (a,b,c,d) and Simpson (e,f,g,h) indexes for each composting treatments during different stages. Stages showing significant differences after Kruskal-Wallis statistical test are noted.

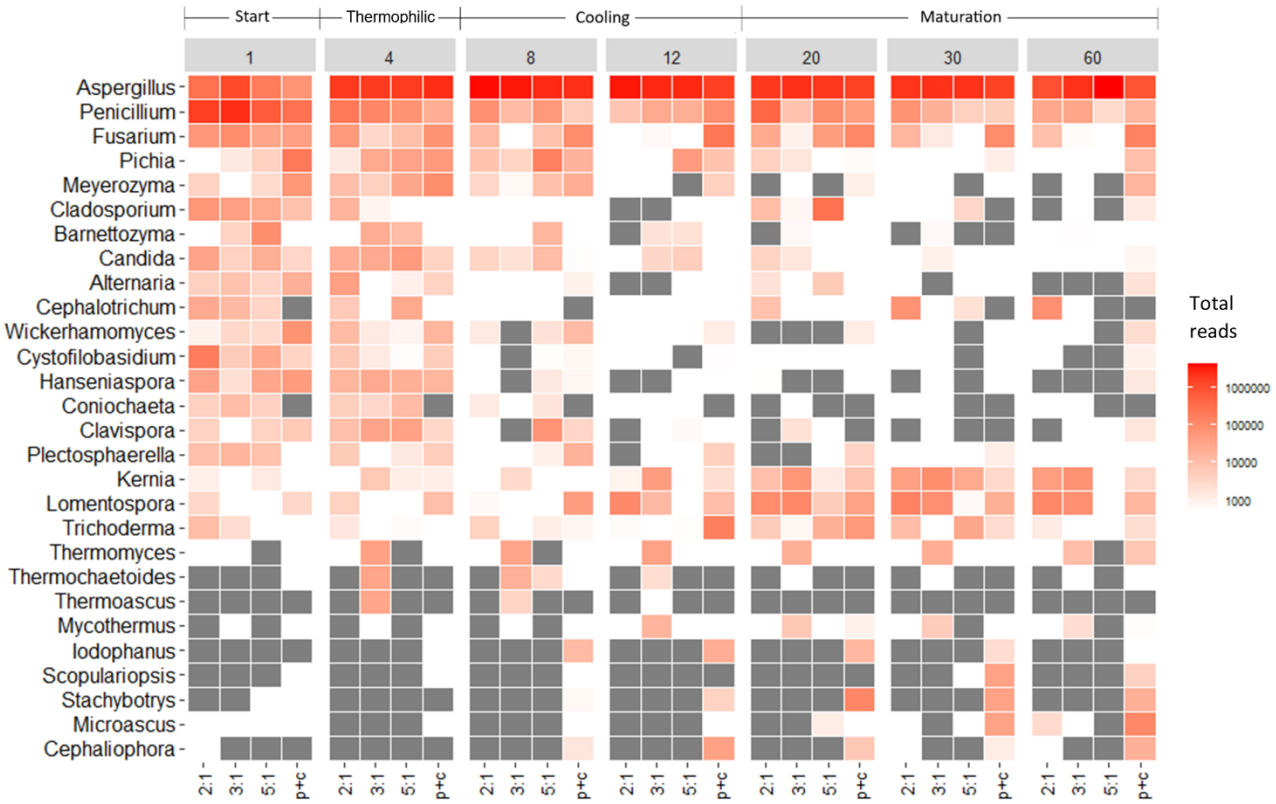

Figure 3.3. Heatmap of the 28 dominant genera in the fungal community. The color intensity is associated with the total number of reads of the genus in each sample. Grey boxes represent the absence of the genus.

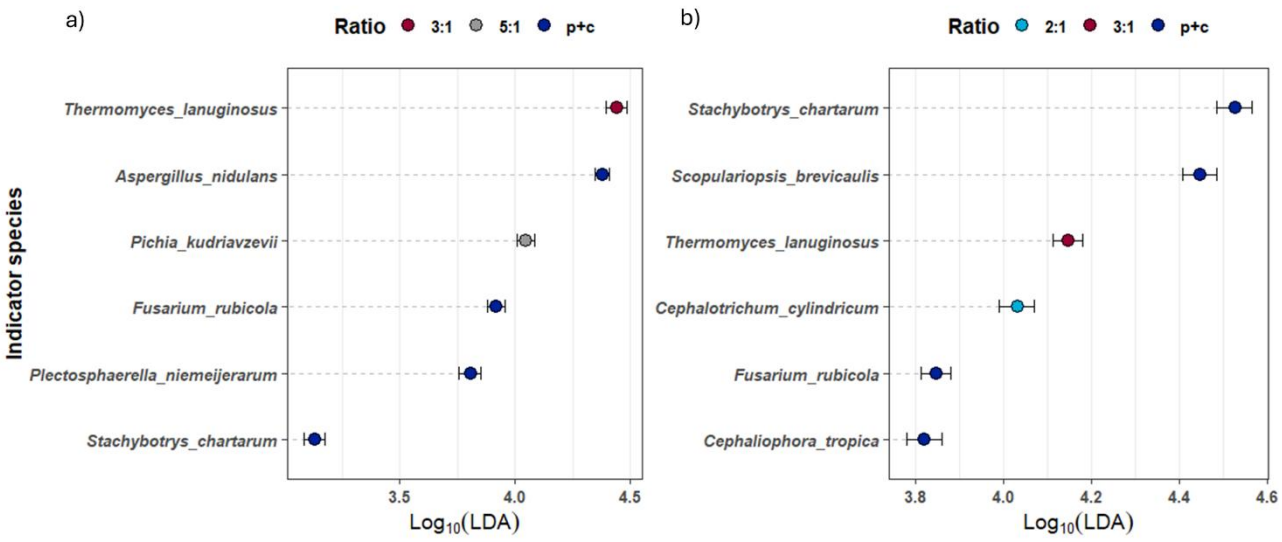

Figure 3.4 Output of LEfSe analysis displaying fungal markers associated with specific treatments during cooling (a) and maturation (b).

Chapter IV - Survival of plant pathogens during composting of bio-waste: a case study for oomycetes and fungi

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ABSTRACT

European countries have implemented national strategies to reduce the use of peat in horticulture due to its environmental impact. Studies demonstrated the possibility of reducing peat consumption by using compost as a substitute without affecting the growth and development of potted horticulture plants. However, any substitute must be produced considering quality standards and requirements that are adopted for peat. Among others, the risk of contamination of compost with plant pathogens is particularly high. Furthermore, the assessment of the survival of specific plant pathogens during the composting process requires proper detection methods. This study evaluated the survival/presence of the oomycete *Phytophthora cinnamomi*, as a model plant pathogen, in woody chips of artificially inoculated *Castanea sativa* saplings used as a bulking agent in composting processes. Detection techniques for assessment included isolation in pure culture, quantitative PCR (qPCR) and High Throughput Sequencing (HTS). The pathogen was easily detected in woody chips at the beginning of the composting process with baiting and molecular barcoding. By the end of the maturation phase, no *P. cinnamomi* was detectable by any diagnostic method including baiting, confirming the efficacy of a proper composting process in eradicating the pathogen. HTS approach was also able to detect throughout the process the DNA of plant pathogenic fungal genera naturally present in the green residues and bulk agents. These findings are crucial for developing diagnostic pipelines to be included in protocols for compost biosafety certification. Finally, the present study demonstrated the possibility of processing recycled horticulture bio-waste to obtain high-quality and safe compost without the need for complex and expensive composting plants.

Keywords: composting; bio-waste; *Phytophthora cinnamomi*; metabarcoding; qPCR; compost biosafety; fungi

4.1.Introduction

Peat is a fossil material that has been the primary component of growing media for horticulture in Europe for decades. Due to its impact on climate, some European countries have implemented national strategies to reduce the use. However, a unified global approach would ensure more equitable and effective results compared to individual national efforts. Peat can be replaced with alternative biomass-growing media (Hirschler et al., 2022). Research efforts have been made to find alternatives

for the use of peat as growing media in ornamental horticulture and contribute to reducing the anthropic pressure on peatland ecosystems, and the costs related to the purchase and shipping of this expensive material. Studies demonstrated the possibility of reducing peat consumption by using compost as a substitute without affecting the growth and development of potted horticulture plants (Aleandri et al., 2015; Kir, 2025; Núñez et al., 2008; Farrel and Jones, 2010). However, any substitute must be produced considering quality standards and requirements that are adopted for peat. Composting is a sustainable method to recycle organic waste, mitigating climate change by diverting waste from landfills, and significantly reducing greenhouse gas emissions (Bernstad Saraiva Schott et al., 2016). Moreover, composts are considered an alternative to chemical fertilizers since they are rich in organic matter and nutrients, improve water retention, and contain plant growth regulators and helper microorganisms (Atiyeh et al., 2002; Awasthi et al., 2020; Castaldi et al., 2023; Cerda et al., 2018; Tilston et al., 2002). Horticulture, including ornamental plants, commonly produces large amounts of green and woody plant residues generated from cultural practices useful to be used as compost feedstock. However, these residues are often burned or piled somewhere for long periods and left unutilized (Schiffer-Forsyth et al., 2023). The use of horticulture bio-waste for on-site production of compost is at the basis of a circular value chain system and it is an efficient alternative to manage the plant residues and produce valuable substrates for cultivation, resulting in environmental and economic advantages. Satisfying results can be obtained using simple methodologies such as composting piles (Schiffer-Forsyth et al., 2023), but particular care must be taken during the process since the heterogeneous composition of bio-waste causes challenges in composting (Adhikari et al., 2008), leading to unwanted by-products such as leachate, ammonia and methane (Yang et al., 2013). Feedstock composition and its physiochemical condition are also affecting the fungal and bacterial communities which are central to the biotransformation of organic matter by producing enzymes during composting (Chen et al., 2024; Leow et al., 2018). The increased knowledge of the microbial communities has led some microbiological and biodiversity parameters to be proposed as indicators for the challenging task of assessing compost quality, stability, and maturity (Estrella-González et al., 2020; Pot et al., 2021; Villar et al., 2016).

Improper composting conditions affect the thermophilic phase which may not reach adequate temperatures for the pasteurization of the mass. It has been shown that temperatures above 55°C kept for several days are essential to reduce the risk of plant pathogens' survival in the mass (Noble & Roberts, 2004). Specifically for plant nurseries, the risk of contamination with plant pathogens is particularly high (Rani et al., 2019). For instance, species of the oomycete genus *Phytophthora*, notorious for causing root rot and other diseases in a wide range of plants in open fields as well as in nurseries, are massively present in traded ornamental and horticulture plants (Jung et al., 2016) and associated commodities (soil, potting mix). Their eradication from horticulture commodities, such as compost, was investigated in a few papers. Swain *et al.* (2006) demonstrated the efficacy of heat and composting treatment on the eradication of high levels of inoculum of *Phytophthora ramorum* using *Umbellularia californica* leaves as vector. Hoitink (1976) hypothesized the complete eradication of *P. cinnamomi* and additional plant pathogens in static piles after 12 weeks at 40-60°C, excluding the inhibitory effect of microorganisms and feedstock by performing laboratory experiments. In a similar study carried out on green waste static composting, plant pathogens (fungi and oomycetes) were not detectable in piles with temperatures peaking at 70°C after 2 (the Basidiomycota *Armillaria mellea*, and the nematode *Tylenchulus semipenetrans*) and 21 (*P. cinnamomi*) days (Downer et al., 2008). However, these studies all applied a classical detection method based on isolation in pure culture of fungal pathogens, which is known for its low sensitivity (Burgess et al., 2021; Vettraino et al., 2010). Pure culture isolation can lack sensitivity due to its dependence on media, timing, bait material, volumes/ratio, temperature, or container shape used (Burgess et al., 2021); moreover, it cannot be applied to unculturable organisms (Rani et al., 2019). Hence the probability of false negatives is high.

Survival of plant pathogens during composting can be favored by cool zones and dry pockets at the pile edges, suggesting that frequent turning is necessary to expose pathogens to lethal temperatures

(Noble & Roberts, 2004; Swain et al., 2006). Accordingly, temperature cannot be the only factor that certifies the absence of pathogens in compost. Hence, sensitive diagnostic methods must be employed to ensure the biosafety of the product. Because contaminated commodities including potting-mix components (e.g. compost) are among the most important pathways for plant pathogen's introduction and spread in new areas, stringent protocols must be elaborated and released to effectively reduce the risk of the presence of viable plant pathogens.

Currently, diagnostic methods for pathogen detection include highly sensitive molecular methods such as quantitative PCR (qPCR), and, more recently, high-throughput sequencing (HTS) technologies (Chandelier et al., 2021; Downer et al., 2008; Rani et al., 2019). Studies have compared HTS and qPCR trying to understand which technology is more effective for sensitive and specific detection of target microorganisms. They revealed a wide range of outcomes, underlining the context-dependent nature of this comparison (Blackman et al., 2020; Dritsoulas et al., 2020; Harper et al., 2018). In some cases, qPCR demonstrates to be more sensitive (Chandelier et al., 2021) while in other cases sequence proportion in HTS was positively associated with qPCR score, suggesting that HTS might be also indicative of DNA concentration (Harper et al., 2018). qPCR has been always considered a predominant method for the detection and quantification of pathogens and other microbes (Le Dréan et al., 2010; Malorny et al., 2008). However, at least four studies demonstrated the ability of the metabarcoding approach to be quantitative within a single Operational Taxonomic Units (OTUs) (Bastianelli et al., 2024; Turco et al., 2021; Ercolini, 2013; Segata et al., 2011).

This study aims to evaluate the survival of *P. cinnamomi* as a model plant pathogen, during the on-site composting process using simple yet effective composting techniques and reliable detection protocols. qPCR and HTS protocols will be compared to test their effectiveness in detecting the target pathogen and to be candidates for inclusion in a compost certification protocol for biosafety in terms of the absence of threatening plant pathogens. *Phytophthora cinnamomi*, was chosen as the model pathogen because it is considered one of the most polyphagous and aggressive plant pathogens, with a large spectrum of hosts, around 5000 including relevant species in forestry, agriculture and horticulture (Hardham & Blackman, 2018), and its widespread presence in productive nurseries worldwide (Jung et al., 2013). The HTS approach will be also tested in this study to monitor the DNA presence of additional putative plant pathogenic genera during the process.

4.2. Materials and methods

4.2.1. Preparation of infected wood chips

Infection of Castanea sativa Mill. was carried out on 1-year-old living seedlings. The inoculum of *P. cinnamomi* was prepared according to Vettraino et al. (2001) with some modifications. Inoculum was grown on a substrate prepared by adding 100 g millet seeds and 100 mL carrot juice in a 500-mL Erlenmeyer flask. The flasks were autoclaved at 121 °C for 20 min for two consecutive days and inoculated on the 3rd day after the substrate had cooled down. The inoculum per flask consisted of agar plugs (5-mm diameter) cut from a 14-day-old culture of *P. cinnamomi* (isolate 3B) grown on V8 agar. Flasks were shaken and sealed with parafilm, covered with aluminum foil, and incubated at 25 °C for 5 weeks. The inoculum was thoroughly mixed with vermiculite and sand (1:1 v/v) before transplanting the seedlings. Fifty seedlings were transplanted in a 14-cm-diameter pot (one per pot) containing the infested substrate. Potted seedlings grew at 24 °C and 65% RH. A photoperiod of 15 h of light was maintained throughout the trial. Pots were flooded once for 48 hours to stimulate the production of sporangia and then maintained at water-holding capacity. The potted seedlings were assessed for visual symptoms every week for two months. At the end of this period, dead plants were removed from the pot, the root system was gently rinsed with tap water and small pieces of necrotic root lesions and segments of fine roots were plated on Malt Extract Agar (MEA) to reisolate the

pathogen. Seedlings that resulted positive for the presence of *P. cinnamomi* were chipped using a shredder (Einhell Italia S.r.l., Binago, Italy) to produce the bulking agent to mix with bio-waste.

4.2.2. Composting process and temperature and moisture measurements

Green residues comprised fruits and green tissues of horticulture plants such as apples, lettuce, tomatoes, eggplants, peppers, potatoes, pears and melons. Before processing, this material was crushed into pieces ranging from 1cm to 15cm. Two composting processes were carried out in parallel using rotating domestic composters of 90l (AOSOM Italy Ltd, Assago, Italy) (Figure 4.1)

The bulking agent was prepared with 50% woody infected chips and 50% commercial wood chips of Fagus sylvatica L. The mixing ratio of green residues/bulking fractions (expressed in wet weight) was 3:1. A total of 26 kg of the mix was loaded in each composter. The composting process started on July 18, 2023, and lasted 60 days. During the thermophilic phase (10 days) the temperature was assessed on days 1,2,3,4,6,7,9, and 10. After, the temperature was assessed at a variable interval of 3-5 days (Figure 4.2). After each temperature assessment, the composter was rotated 360 degrees to turn and aerate the mix. Turning was additionally performed on days 20 and 30. Moisture content in both composters was assessed on days 1, 4, 10, 19, 26, 39, and 60 by sampling 50 g of composting material and applying the gravimetric method, which involved oven drying at 105 °C.

4.2.3. Effect of temperature on *P. cinnamomi* inoculum

Three Erlenmeyer flasks containing *P. cinnamomi* inoculum (isolate 3B), were prepared as described in paragraph 2.1. The experiment started with incubation at 28°C per 24h. (1st day). On the second day, the millet seeds were incubated at 60°C for 24h, which is the maximum average temperature recorded during the composting tests. Subsequently, at 24h intervals, the temperature was decreased by 4°C until down to 44°C, 1°C below the lethal temperature described for *P. cinnamomi*, which is 45°C (Erwin and Ribeiro, 1996). Specifically, the following temperature regime was set in the incubator containing *P. cinnamomi* inoculum: 1st day, 28°C; 2nd day, 60°C; 3rd day, 56°C; 4th day, 52°C; 5th day, 48°C, 6th day, 44°C. Five millet seeds were collected from the flask and plated on 5 different 9 cm Petri dishes with PARPNH media amended with 10 µg/mL pimaricin, 200 µg/mL ampicillin, 10 µg/mL rifampicin, 25 µg/mL PCNB, 50 µg/mL nystatin, and 50 µg/mL hymexazol (Erwin and Ribeiro 1996) and kept at 25°C to assess *P. cinnamomi* mycelial growth.

4.2.4. Assessment of *P. cinnamomi* survival during the composting process

4.2.4.1. Baiting

Baiting was carried out at the beginning and the end of the composting process (1st and 60th day) by sampling 50g of material from each composter. Once in the laboratory, soil samples were baited as described by Jung et al. (1996) within 24 hours of the collection. Compost was placed in a plastic tray (15 × 25 × 5 cm) flooded with distilled water and baited with 20 leaf disks (1cm in diameter) of rhododendron. The baiting was performed in a growth chamber at 20 °C and 12/12 h of photoperiod. After 7 days, baits were removed, surface sterilized in 70% ethanol for 30 s, washed twice with sterile distilled water, dried on filter paper, and plated on PARPNH. The plates were incubated at 25 °C in darkness and examined daily under the dissecting microscope for Phytophthora-like hyphae.

4.2.4.2. Sampling and DNA extraction

During the active phase of both composters, samples were collected at different temperatures of the thermophilic phase, at 28°C, 56°C, 44°C, 39°C, and 27°C (average between the two composters) on days 1, 3, 13 and 22. Additionally, samples were collected at the beginning and the end of the maturation phase on day 30th and 60th respectively resulting in a total of 6 different sampling times. For each composter, at every sampling time, 25g of material was collected, minced, and two replicates of 1g were taken, resulting in a total of 24 samples analyzed in this study. The material was kept in

sterile 2ml Eppendorf at -20°C. Total DNA was extracted for each sample using the kit "Nucleospin Soil" (Macherey–Nagel Gmbh & Co. Düren, Germany) according to the manufacturer's protocol.

4.2.4.3. HTS

The internal transcribed spacer 1 (ITS1) was amplified in a multiplexing PCR using a set of barcoded primers with a dual indexing primer using the tagged primer pair ITS6/ITS7 (Cooke et al., 2000) for oomycetes and the ITS1F/ITS2 for fungi (White et al., 1990). The PCR reaction mix comprised 12.5 µL of GoTaq® Hot Start Master Mix (Promega, Madison, WI, USA), 1 µL for each primer, 1 µL of Bovine Serum Albumin (ThermoFisher Scientific, Waltham, MA, USA), and 1 µL of template for a total volume of 25 µL. The thermal cycle for oomycetes was an initial 5-min step at 95 °C, followed by 30 cycles of 40 s of denaturation at 95 °C, 40 s of annealing at 58 °C, and 40 s of extension at 72 °C, with a final extension step of 10 min at 72 °C (Ruiz Gómez et al., 2019). The thermal cycle for fungi was an initial 5-min step at 95 °C, followed by 35 cycles of 30 s of denaturation at 95 °C, 30 s of annealing at 55 °C, and 1 min of extension at 72 °C, with a final extension step of 5 min at 72 °C. For each sample, three replicates of PCR were carried out and the products were pooled. Amplicons were purified using the MagJET NGS Cleanup (ThermoFisher Scientific, Waltham, MA, USA), quantified with the Qubit dsDNA HS assay kit, and pooled at equal concentrations for sequencing. Paired-end sequencing (2 × 300 bp) was carried out on an Illumina MiSeq sequencer by Fasteris (Genesupport SA, Plan-les-Ouates, Switzerland).

4.2.4.4. qPCR

qPCR was conducted according to Drais et al. (2025) in 10 µL reaction volumes, each containing 1 µL of template DNA extracts from compost material, 300 nM of both forward and reverse primers, 100 nM of TaqMan probe, and 5 µL of 2X GoTaq® Probe qPCR Master Mix (Promega, Madison (WI), EEUU). The primers used in this study were developed by Drais et al. (2025), targeting the internal transcribed spacer ITS region of *P. cinnamomi*. Each primer set and the probe was designed based on ITS gene fragments from various isolates of *P. cinnamomi*.

Amplification was performed on Rotor-Gene Q (Qiagen, Hilden, Germany) with an initial denaturation at 95°C for 2 minutes, followed by 40 cycles of 95°C for 10 seconds, 64°C for 15 seconds, and 72°C for 20 seconds. Fluorescence was measured once per cycle at the end of the extension step, and Cq values were automatically determined by the instrument. Each sample was run duplicate.

Absolute quantification of *P. cinnamomi* DNA was achieved by generating a standard curve, from which target DNA concentrations in the analyzed samples were extrapolated based on the Cq values. The detection limit of the assay was set at 10 fg of total genomic DNA (Drais et al., 2025).

4.2.4.5. HTS data analysis

Only readings with the pairing of the expected 3′barcode and reverse primer and the 5′barcode and forward primer were coupled and taken into consideration for subsequent studies to reduce the likelihood of incorrect assignments and cross-contamination. The raw sequence data were processed using the DADA2 pipeline (version 1.18.0) in R (version 4.0.5). The forward and reverse reads were quality-filtered and reads containing ambiguous bases (Ns) were removed. Primers were identified and removed using Cutadapt (version 3.4). The primer sequences were trimmed from both the forward and reverse reads. Dereplication of sequences was performed using a 100% clustering threshold, and sequence variants were inferred. The forward and reverse reads were merged to obtain the full denoised sequences. Sequences identified as chimeric were removed before downstream analysis. For Oomycetes, the plugin of Qiime2 (v. 2024.10) feature-classify classify-sklearn was utilized for taxonomic classification, using a classifier based on NCBI trained with the Qiime2 plugin feature-classify fit-classifier-naïve-bayes (Bokulich et al., 2018). For fungi, the plugin of Qiime2 (v. 2024.10) feature-classify classify-sklearn was utilized for taxonomic classification, using a UNITE

(v. 2024.04) classifier trained with the Qiime2 plugin feature-classify fit-classifier-naïve-bayes (Bokulich et al., 2018). GraphPad Prism version 8.0.1 for Windows, (GraphPad Software, Boston, Massachusetts USA) was used for non-linear regression analysis and descriptive statistics. Linear discriminant analysis Effect Size (LEfSe) analysis was performed using the R package Microbiota (v.1.14.1), results were considered significant when p-value <0.05. Ecological guild of fungal taxonomic units was annotated with the support of the open source FUNGuild database (Nguyen et al., 2016). The detection limit for both oomycetes and fungi was set at a relative abundance lower than 0,001% (Parks et al., 2013; Morales-Rodríguez et al., 2024).

4.3. Results

4.3.1. Composting process

The mix temperature at the beginning of the composting process was 28°C for both composters. A peak of 59,8 °C was reached after 24 hours. During the thermophilic phase, temperatures were above 40°C for one week, with peak values above 50°C lasting five consecutive days. After that, the material started cooling and reached an ambient temperature around the 20th day (Figure 4.2). The moisture content during the composting process is shown in Figure 4.2. It gradually decreased from 68.8±2,9 (SE)% at day 1 to 39,9±9.3% at day 60.

4.3.2. Effect of temperature on *P. cinnamomi* inoculum

Millet seeds plated and incubated at 28°C were all positive (n=5) for *P. cinnamomi* mycelial growth. From the second day, after 24h at 60°C, *P. cinnamomi* mycelial growth was no longer observed from any millet seed plated on PARNPH media.

4.3.3. Baiting

In the baiting carried out on day 1 of the composting process, necroses were visible on 7 out of 20 leaf baits (35%). *P. cinnamomi* was re-isolated from 3 of them (15%). In the baiting carried out on day 60 of the composting process, no necroses were noticed on any of the leaf baits; no *P. cinnamomi* growth was observed after plating on PARNPH.

4.3.4. HTS analysis

4.3.4.1. Oomycetes

After filtration and trimming, 489 ASVs from 599618 reads were obtained (Table S1). Of these 214 were assigned as class, 101 as family, 174 at genus or species. Overall, 20 out of 24 samples were analyzed. Indeed, four samples, one for days 1, 3, 13 and 30, were not included in the analysis due to the poor quality of the libraries. The relative abundance of the most represented genera at each sampling day is shown in Figure 4.3. *Phytophthora cinnamomi* was the most abundant taxa of the genus. It was detected in 9 samples, with a total of 22976 reads, representing 80 and 6 % of the total reads for Phytophthora and oomycetes, respectively. *Phytophthora cinnamomi* was detected on days 1 (3 positives), 3, 13 and 30 (two positive samples each) (Table 4.1). Relative abundance values were always above the detection limit (0,001%).

The highest relative abundance was recorded on day 1 (28°C), 2,6%, 16,3% and 17,8% respectively. Data on *P. cinnamomi* relative abundance during the composting process were fitted by least square regression with an exponential decay curve (R square=0,61) (Figure 4.4). Replicates test for lack of fit rejected the inadequacy of the model (F=0,25; P=0,85). Residuals passed the normality test (D'Agostino-Pearson, p>0,05). The presence of the pathogen declined gradually until day 30 when its relative abundance was 0,25±0,12%. At the end of the maturation phase (day 60), *P. cinnamomi* was not detected in any of the 4 replicates analyzed (Table 4.1 and Figure 4.4).

Three more pathogenic oomycetes genera were detected. *Peronospora* was detected on day 1 with a relative abundance of 6,6% and 1 sample at day 3 with a relative abundance of 0.4%. *Plasmopara* was detected on day 1 with a relative abundance of 0,3% and 22,3% on day 3. Both the genera were not detectable after day 3. *Phytium* s.l. was the most abundant taxa of pathogenic oomycetes, recorded at all the sampling dates including day 60 (Figure 4.3). No significant differences were found between sampling dates at ordinary one-way ANOVA (F=0,6; p=0,7).

4.3.4.2. Fungi

After filtration and trimming a total of 559 ASVs from 3.474.161 reads were obtained (S2). Overall, 22 out of 24 samples were analyzed. Indeed, two samples, one for each day 1 and 3, were not included in the analysis due to the poor quality of the libraries. The relative abundance of the most represented taxa on each sampling day is shown in Figure 4.5. Heath-tolerant genera such as *Mycothermus*, *Thermomyces, Thermochaetoides* and *Aspergillus* were detected as the most abundant from day 3 (Figure 4.5). LEfSe analysis confirmed the negative impact of the thermophilic phase on the plant pathogenic genera *Penicillium* and *Cladosporium*, and the positive effect on thermotolerant taxa such as *Thermoascus* and the less represented thermotolerant yeasts *Issatchenkia, Rasamsonia* and *Kluyveromyces* (Figure 4.6). The presence of DNA of plant pathogenic fungi during the composting process is shown in Table 4.2. The genera *Aspergillus* and *Penicillium* resulted in the most abundant. *Aspergillus* spp. declined during the composting process but was still very abundant on day 60 (16,1%). Significant differences between dates were found with ordinary one-way ANOVA (F=16,55; p<0,0001); Tukey's multiple comparisons test results are summarized in S3. *Penicillium* spp. decreased during the thermophilic phase down to day 22 and then increased its abundance to day 60. Significant differences between dates were found with ordinary one-way ANOVA (F= 178; p<0,0001); Tukey's multiple comparisons test results are summarized in S4. The DNA of the plant pathogenic genera, *Acremonium, Alternaria, Cladosporium* and *Fusarium*, and the species *Botrytis cinerea* and *Gnomoniopsis castaneae* were all undetectable at day 60 (Table 4.2).

4.3.5. qPCR

Six samples resulted positive for *P. cinnamomi*, but only the 3 samples associated with day 1 were above the minimum threshold of 10 fg/µl, with Ct values ranging from 32.18 to 34.86 corresponding to 80 fg/µl to a minimum of 14 fg/µl, respectively. Three additional samples yielded a Ct value on days 2 and 3; however, they were considered negative for the presence of DNA associated with *P. cinnamomi*, as the values were below the detection limit (Figure 4.7). Data on *P. cinnamomi* inoculum level expressed in fg/µl during the composting process were fitted by least square regression with an exponential decay curve (R square=0,75) (Figure 4.7). Replicates test for lack of fit rejected the inadequacy of the model (F=0,0014; P>0,99). Residuals passed the normality test (D'Agostino-Pearson, p>0,05).

4.4. Discussion

One of the objectives of the present study was to assess the survival of *P. cinnamomi*, as a model pathogen, during a compost-turned process of green waste. *Phytophthora cinnamomi* was included as a living inoculum with artificially contaminated wood chips to monitor its presence and viability. The choice of this pathogen is not only due to its relevance as a plant pathogen but also because its survival during composting was investigated in previous studies although using different matrices, processes and detection methods (Hoitink et al., 1976; Downer et al., 2008).

Hoitink et al. (1976) studied the survival of *P. cinnamomi* buried in a hardwood bark compost stack (static), where *P. cinnamomi* was not detected using isolation on selective media or baiting of infected material retrieved from the pile, after 10-12 weeks. Downer et al. (2008) studied the survival of *P. cinnamomi* during composting of fresh green waste, as was done in the present study, but using static piles. Their study revealed that *P. cinnamomi* persisted alive for over 21 days during composting

while modelling curves based on pile temperatures indicated 30 days to inactivate *P. cinnamomi* propagules. In the present study, employing turning of waste mass, the temperature increased to 60°C in 24 h, after which, no *P. cinnamomi* propagules resulted alive at any of the temperatures tested in 'in vitro' experiment. Temperature is confirmed as one of the most relevant factors for inoculum inactivation during composting. Interestingly, Downer et al. (2008) suggested turning the pile to optimize its pasteurization through the movement of the pathogen's propagules, or infected tissues, in locations in the mass where they were more likely to be killed by heat, microbial attack or degradation. Indeed, in the present study, we adopted a compost-turned process that can explain the rapid inactivation of *P. cinnamomi* even into the infected tissues.

In the present study three different diagnostic methods were considered, i) baiting and isolation in pure culture; ii) qPCR (TaqMan technology); iii) HTS (Illumina MiSeq). Baiting followed by isolation in pure culture is a specific method for organisms such as oomycetes with motile spores (Erwin and Ribeiro, 1996). However, besides being very specific, this assay lacks sensitivity compared to molecular detection, especially for hemibiotroph organisms like those of the genus *Phytophthora* (Vettraino et al., 2010). Low sensitivity is likely associated with pathogen life cycle, and specifically on sporangial production, which can be inhibited by various factors such as nutrients, bacteria, aeration, temperature, and season (Butler et al., 1984). Therefore, a negative baiting assay does not definitively exclude the presence of alive *P. cinnamomi*. Indeed, *P. cinnamomi* can survive as resting chlamydospores in the host tissues (Fernandes et al., 2021). This is the reason why it is necessary and recommendable to couple molecular detection to classical isolation/baiting assays, to provide a better sensitivity. Molecular methods are independent of the pathogen's life cycle and rely on the detection of target DNA, even at low concentrations, offering high sensitivity. TaqMan assay used in the present work (Drais et al., 2025) fully confirmed the results of baiting, being able to detect *P. cinnamomi* over the detection limit, at day 1 only. Differently, Illumina MiSeq detection was able to produce a sensible number of *P. cinnamomi* reads, above the detection limit of the method (Parks et al., 2013; Morales-Rodríguez et al., 2024) during the composting process for up to 30 days. After this sampling date, the DNA of *P. cinnamomi* was no longer detectable. The decrease of detectable *P. cinnamomi* DNA during the composting process is likely the result of microbial attack or degradation, as previously suggested by Downer et al. (2008). Combining the results of baiting and independent molecular assays (qPCR and HTS), it can be stated that after day 30 or, more conservatively, at the end of the composting process (day 60), the compost is free of *P. cinnamomi*. The present study utilized *P. cinnamomi* as a model pathogen, but we cannot exclude the presence in the starting matrices of additional plant pathogens. This aspect was investigated in previous studies (Hoitink et al., 1976; Downer et al., 2008), showing that different pathogens are differently inactivated during composting. This aspect was also addressed in the present study using HTS analysis, investigating taxa naturally present in the green residues and bulking material entering the composting process. Of particular interest is the persistence in the mass of the DNA of *Pythium* s.l. after the thermophilic phase. *Pythium* s.l. includes many genera and species causing root and collar rot in a wide range of herbaceous and woody plant species (Boie et al., 2024). In the present study, the relative abundance of *Pythium* was not statistically different between sampling dates. Whether DNA presence corresponds to viable inoculum cannot be stated. However, Shavnam and Ray (2024), evidenced a sensible reduction of *Pythium* population viability with soil solarization with temperatures reaching 41,8°C in 40 days experiment, but not its complete eradication. The genera *Aspergillus* and *Penicillium* were among the most represented in terms of relative abundance, accounting for more than 78% of the total fungal relative abundance in green residues and bulk agent mixes on day 1. These genera include plant pathogenic and spoilage species responsible for the infection and decay of various fruits and vegetables (Alegbeleye et al., 2022; Zakaria, 2024). They also include many mycotoxins-producing species (Bastianelli et al., 2024). The relative abundance of *Aspergillus* spp. declined during the thermophilic and maturation phases although it was still abundant after 60 days. It cannot be stated if these organisms were alive after 60 days, because of the

gradual decrease of their abundance. However, their high relative abundance after 60 days likely suggests vitality even considering that the genus *Aspergillus* includes thermotolerant species such as *A. fumigatus* for which survival in compost heaps has been largely demonstrated (Shelton et al., 2022). More interestingly, *Penicillium* decreased close to the detection limit at day 22 and then increased in abundance until day 60, suggesting a natural re-colonization of the compost heap by inoculum naturally present in the environment. Indeed, the genus *Penicillium* is ubiquitous and among the most common and abundant fungi occurring in various environments, in soil and air (Yadav et al., 2018). As evident in Table 4.2, the genera *Alternaria, Acremonium, Cladosporium* and *Fusarium*, and the species *Botrytis cinerea,* and *Gnomoniopsis castaneae* were undetectable in the compost heap after day 60. *Botrytis cinerea* is one of the most relevant pathogens of fruits and vegetables including ornamentals, causing the so-called grey mold (Williamson et al 2007; Bika et al., 2021). Its presence in green residues from horticulture is not surprising at all due to its wide host range. Differently, *G. castaneae* is a specific pathogen of chestnuts causing brown rot of fruits and stem cankers (Vannini et al., 2017). It commonly lives endophytically in all chestnut tissues including bark and woody tissues. Thus, its presence is likely associated with the chestnut chips used in the present study. Sensitivity to high temperature of the genus *Fusarium* and the causal agent of chestnut brown rot, *G. castaneae*, was already evidenced in previous studies of post-harvest technologies by Morales-Rodriguez et al. (2022) and Bastianelli et al. (2024). In conclusion, frequent turning helped rapidly increase the mass's temperature and accelerate the thermophilic phase, exposing it more homogeneously to high temperatures. *Phytophthora cinnamomi* and some low-represented oomycetes and fungal taxa are presumably inactivated after 24 h exposure at 60°C. Detectable DNA immediately after the thermophilic phase is probably represented by inactivated vegetative or reproductive structures (sporangia) of these microorganisms that are readily degraded during the maturation phase. All taxa (oomycetes and fungi), represented in the starting material with a relative abundance above 20%, seem to be less affected or readily re-established in the compost heap thanks to their high colonization rate of organic matter and high presence in the environment. Although the detection of DNA cannot be considered proof of pathogen viability, on the contrary, and more conservatively, the disappearance of the pathogen DNA from the mass at the end of the process must be considered conclusive proof of sanitization. The adoption of these criteria could provide a stringent protocol for certification of the compost free of target-threatening microorganisms.

Mass sequencing technologies such as Illumina MiSeq, can provide an easy and solid method to determine in a single reaction the presence of multiple plant pathogens in the compost during and at the end of the process. Although the presence of DNA is not proof of vitality, its disappearance along the process is solid proof of sanitization of the final product. The application of this technology might extend to many different contexts including food safety, biodiversity conservation and human health. Furthermore, in the broader context of the circular economy, the bulking fraction—certified as sanitized, such as the wood chips used in this study—can be sieved from the compost at the end of the maturation phase and reused in subsequent composting cycles, thereby enhancing economic sustainability.

However, further investigations are needed on comparisons between molecular detection technologies such as HTS and qPCR techniques (Chandelier et al., 2021; Wood et al., 2019). The HTS pipeline, from DNA extraction to bioinformatic analysis, can be biased by errors and artefacts (e.g. primer design, quality of reference database for taxonomic classification) (Dritsoulas et al., 2020; Harper et al., 2018) which may affect efficiency also depending on the target organism. Overall, solving the discrepancies between HTS bioinformatic pipelines represent a paramount need.

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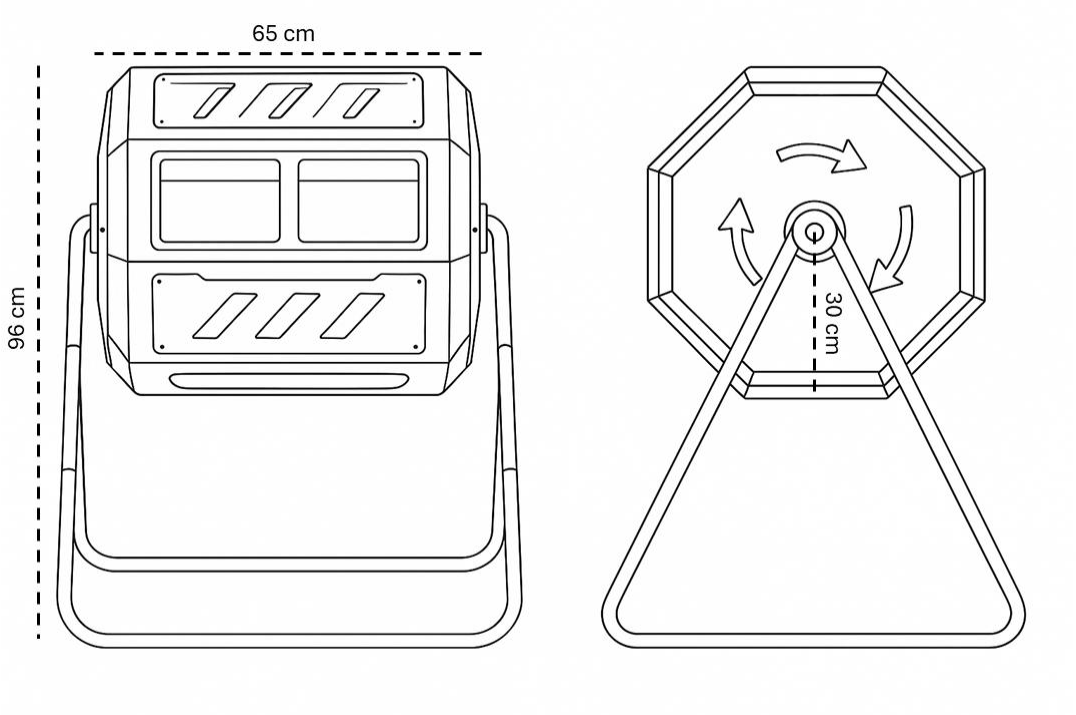

Figure 4.1 Schematic diagram of the composting bin with measures of height, width and radius.

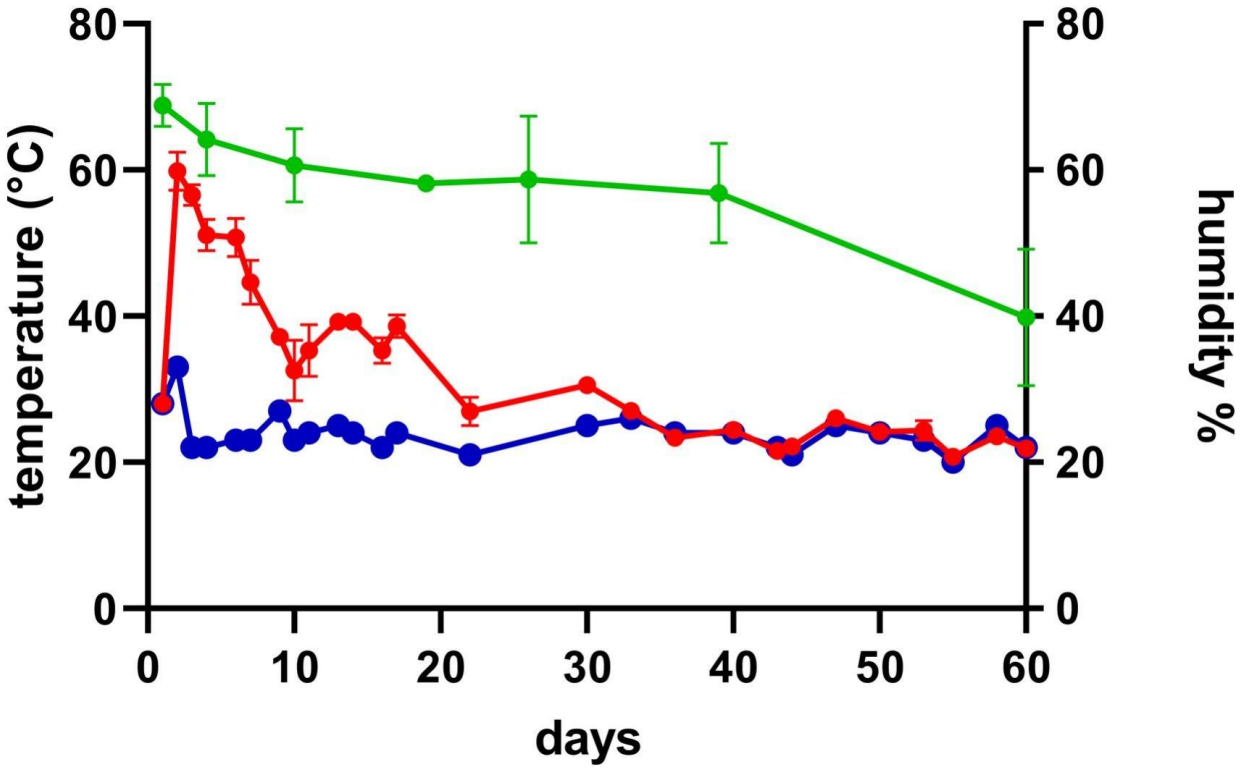

Figure 4.2 Temperature pattern of the mass during the composting process (60 days) (red line). Blue line represents the ambient temperature. Moisture content of the mass during composting process is also shown (green line). Bars represent SEM.

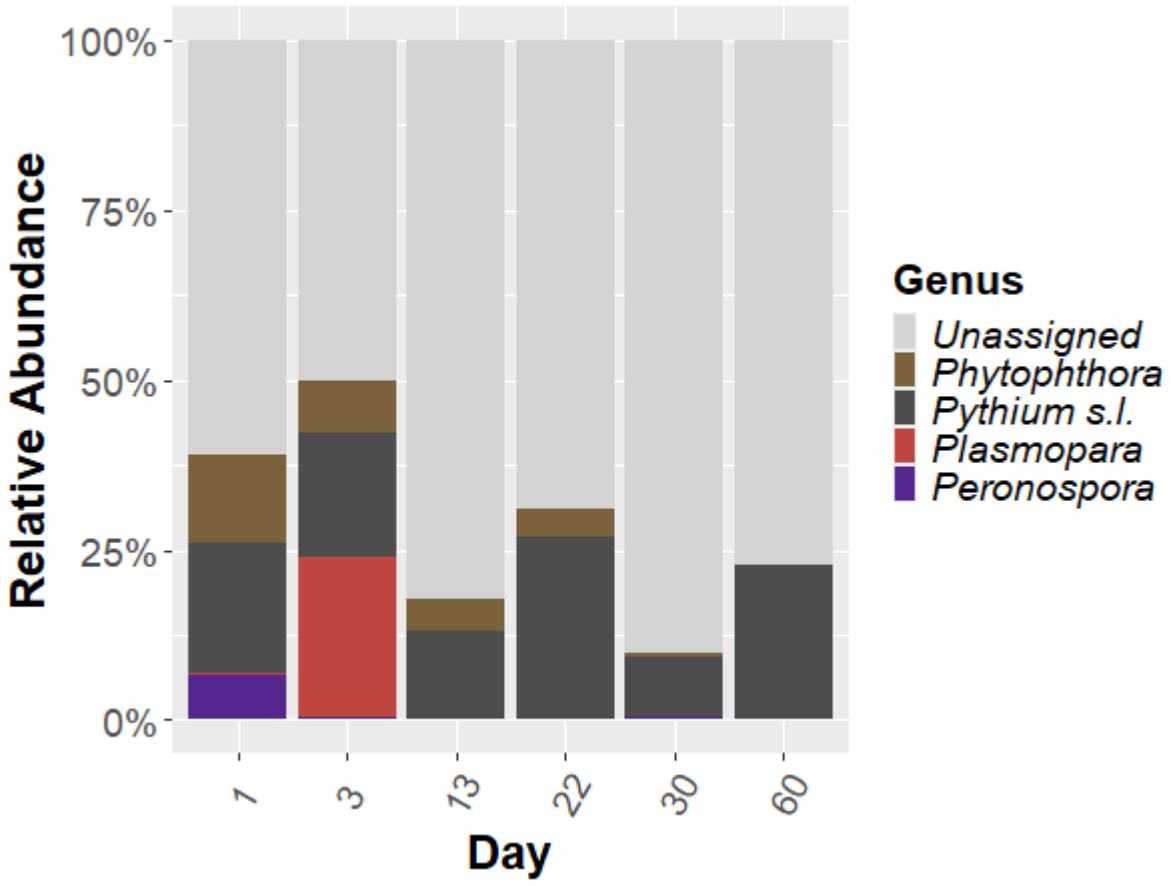

Figure 44.5.3 Relative abundance of the most represented oomycetes genera during the composting process

Table 4.1 Sampling dates, mass temperature and detection of P. cinnamomi by HTS expressed as the absolute number of reads and relative abundance (SEM=standard error).

Day	Temperature (°C±SEM)	N° of positives/total	N° of reads	Relative abundance (%±SEM)
1	28±0,1	3/3	678 8623 11240	13,2±5,2
3	59,8±1,8	2/3	469 344	8,2±4,1
13	39,2±0,2	2/4	363 66	2,8±2,7
22	26,5±	0/4	nd	nd
30	30,6±0,6	2/4	812 96	0,26±0,13
60	21,5±	0/4	nd	nd

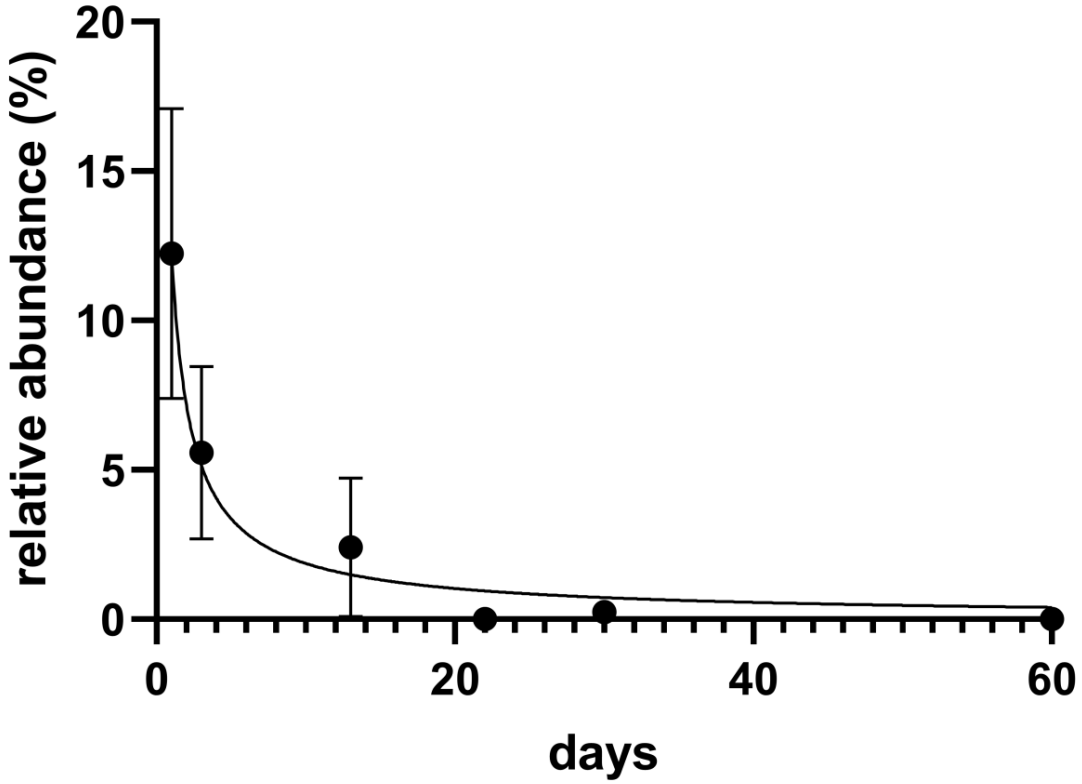

Figure 4.4 Relative abundance of HTS reads of P. cinnamomi throughout the composting process expressed as mean±SEM. Data were fitted with an exponential curve with a R-square = 0,61.

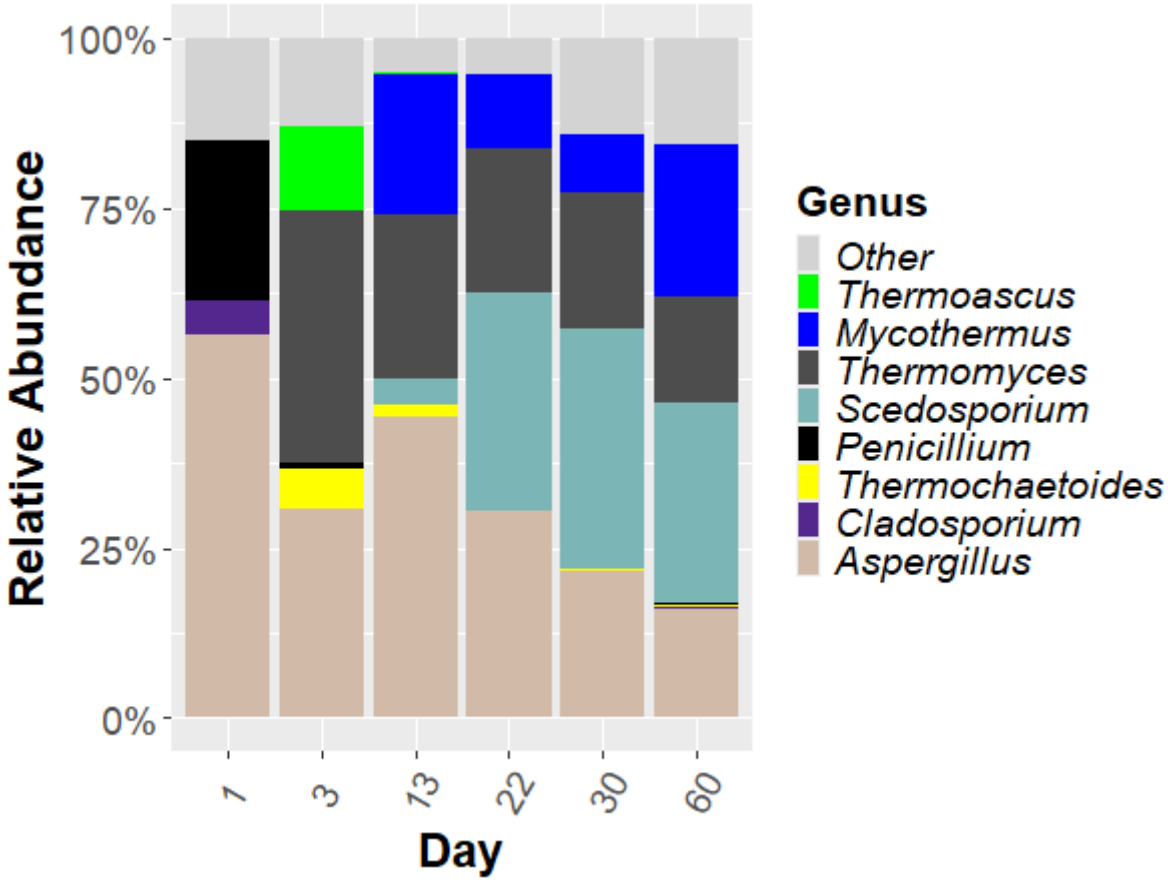

Figure 4.5 Relative abundance of the most represented fungal genera during the composting process

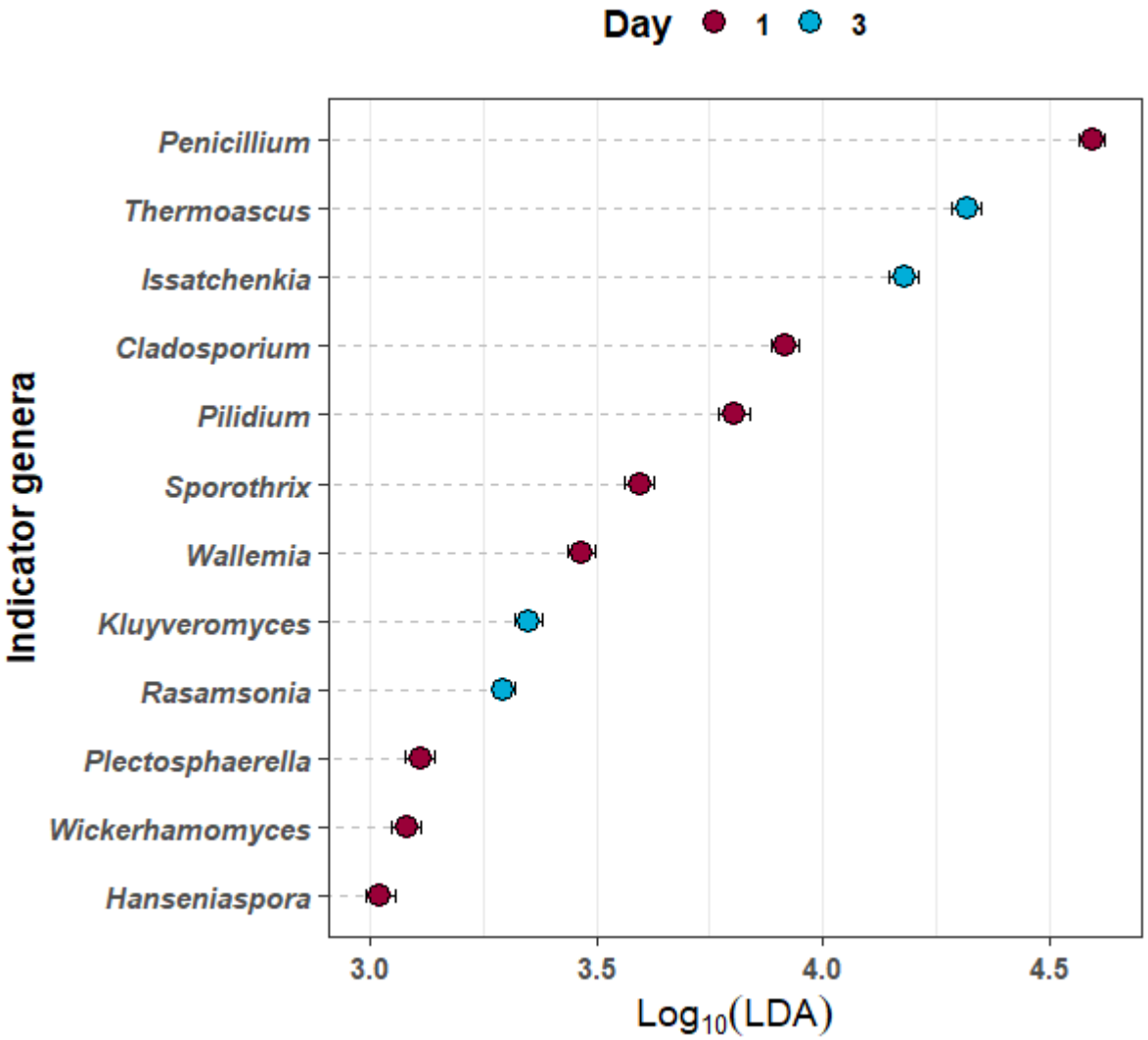

Figure 4.6 Results of LEfSe analysis of the relative abundance of fungal taxa during the composting process. Significant differences were found on days 1 and 3 ($p<0,05$)

Table 4.2 Relative abundance of plant pathogenic fungal taxa during the composting process. Different letters indicate significative differences in Tukey's multiple comparisons test (Aspergillus and Penicillium), and Unpaired t test (Cladosporium).

	Relative abundance/ Day (%)					
Taxa	**1 (n=3)**	**3 (n=3)**	**13 (n=4)**	**22 (n=4)**	**30 (n=4)**	**60 (n=4)**
Fungi						
Aspergillus spp.	55,2±2,1a	30,7±4,5bc	44,4±2,5ac	30,3±3,8bc	21,6±4,1b	16,1±3,1b
Penicillium spp.	23,2±0,4a	0,791±0,49b	0.146±0,04b	0.033±0,007b	0,089±0,019b	0,322±0,225b
Cladosporium spp.	4,9±1,1a	0,049±0,035b	0	0	0	0
Botrytis cinerea	0,105±0,074	0	0	0	0	0
Alternaria spp.	0,09±0,03a	0	0	0	0,04±0,04a	0
Fusarium spp.	0,084±0,04a	0,008±0,004a	0	0	0	0
Acremonium spp.	0,06±0,03	0	0	0	0	0
Gnomoniopsis castaneae	0,037±0,016	0	0	0	0	0

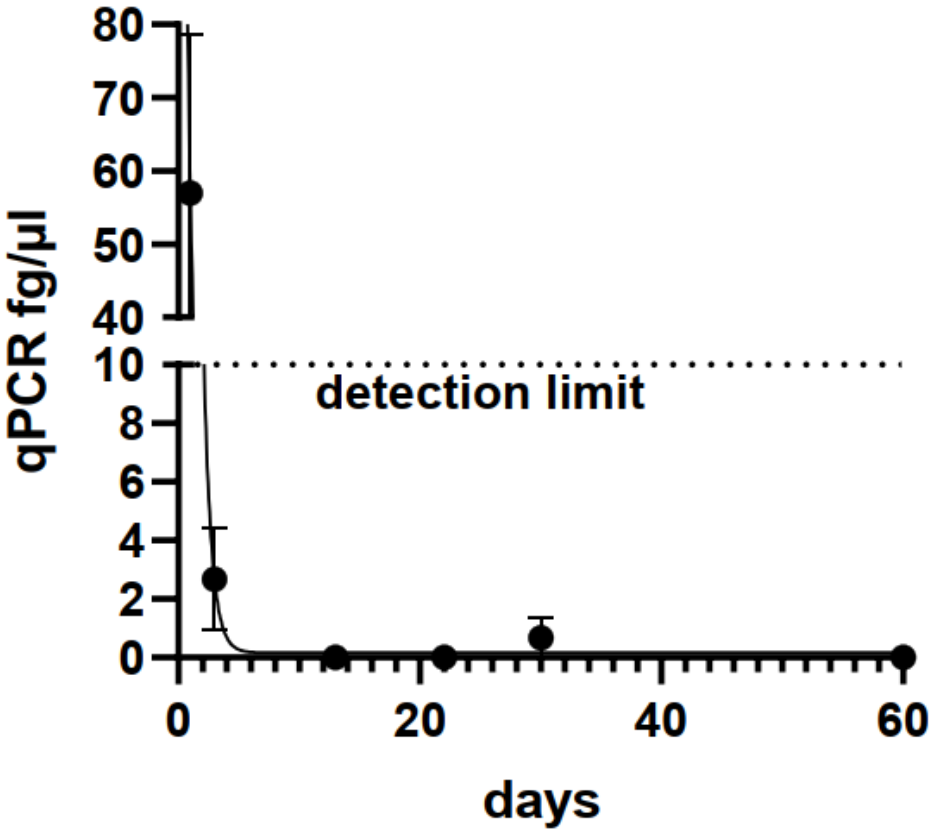

Figure 4.7 Detection of P. cinnamomi inoculum using qPCR. Values are expressed as mean±SEM. Data were fitted with an exponential decay curve with a R-square = 0,75

Chapter V – Compost inoculation with *Bacillus velezensis*: study on process efficiency and agronomic value of the product

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Abstract

Italy generates circa 30 million tons of municipal solid waste (MSW) every year, with bio-waste representing the largest portion of separately collected waste. Composting is the most spread method for the sustainable management of bio-waste, but its efficiency is limited by slow degradation rates and variable process conditions. This study evaluates compost bioaugmentation as a strategy to enhance decomposition and increase compost quality, using *Bacillus velezensis* (*Bv*), a bacterium known for its lignocellulolytic activity and plant growth-promoting potential but still poorly considered for these applications. Composting of bio-waste and woodchips was performed using steel reactors equipped with real-time sensors to monitor temperature, CO_2, and NH_3 emissions. Two treatments (*Bv*-inoculated and non-inoculated) were tested and compared. After 30 days of active composting and 30 days of maturation, composts were analyzed chemically to assess maturity and quality of the final product. Bioaugmented composts reached higher temperatures and maintained a longer thermophilic phase, indicating an increase in microbial activity and a faster degradation of organic matter (OM). CO_2 emissions were also higher in bioaugmented treatments, displaying higher carbon loss during early composting. However, chemical properties of the final compost showed little or no differences between treatments. The low *Bv*-inoculum concentration and competition with the indigenous microbial community may have limited the effectiveness of bioaugmentation. In conclusion, *Bv* showed interesting results for enhancing the active phase of composting, but further research is needed to explore different inoculation protocols and to evaluate the contribution of this bacterium in bio-waste composting.

5.1. Introduction

In the past decade, Italians have generated around 30 million tonnes of MSW each year, an average of 496 kg/capita. In 2023, 66.6% of this waste was separately collected, an increase of 573 000 tonnes (+3%) compared with 2022. The largest fraction of separately collected MSW is bio-waste, which is around 7 million tons (approximately 40% of all separately collected waste). Bio-waste is mainly composed of food waste from restaurants, canteens, households and markets, with a smaller contribution from the maintenance of gardens and parks (Rapporto Rifiuti Urbani, 2024). The valorization of bio-waste is fundamental to reaching the EU's recycling goals (recycling MSW up to 70% by 2030). Currently, composting is the most widespread recovery method for bio-waste: Italy in 2022 produced 1.9 million tons of finished compost (Consorzio Italiano Compostatori, CIC).

Aerobic composting transforms organic matter into a biofertilizer thanks to the metabolic activity of microorganisms that participate in the degradation of lignocellulosic, proteic and lipidic substrates (X. Liu et al., 2025). However, the efficiency of this process strongly depends on feedstock physiochemical conditions. Any imbalance might slow down and eventually stop completely decomposition, causing environmental problems including leachate production and methane emissions (Salam et al., 2021; Scialabba, 2015). Although composting is much simpler than other methods, like anaerobic digestion, it is characterized by a slower degradation rate and a longer processing time, which makes it economically less efficient than other techniques on a large scale. For this reason, research has focused on accelerating composting and increasing the quality of the end product. Various strategies have been studied to enhance composting efficiency, including the addition of chemical supplements such as magnesium oxide and di-potassium hydrogen phosphate (X. Wang et al., 2013), the use of different bulking agents (Huang et al., 2024) and the inoculation with bacterial and/or fungal microorganisms (Oviedo-Ocaña et al., 2023; Voběrková et al., 2017). Microbial inoculation, or bioaugmentation, has always been considered an eco-friendly approach: many studies reported increasing temperatures during the thermophilic phase, more efficient degradation rates and higher humic acids content (Hemati et al., 2021; Onwosi et al., 2017; Rastogi et al., 2020). Yet there's no full consensus on the efficacy of this approach as in some cases microbial inoculation has not led to significant changes (Karnchanawong & Nissaikla, 2014; Pandey et al., 2016). These unsuccessful outcomes are probably due to heterogeneity of the feedstock, composting methodology, inoculation timing and the characteristics of the indigenous microbial population (Fan et al., 2018; B. Xi et al., 2015). Accordingly, some authors have suggested the necessity of a decision framework for the application of microbial inoculation according to different composting scenarios (Fan et al., 2018). Among potential inoculants, *Bacillus* spp., has gained attention (Abdullah et al., 2013; L. Wang et al., 2023). The genus *Bacillus* is one of the dominant taxa during all composting phases and has been particularly considered because of its ease of manipulation, rapid reproduction and its adaptability (Li et al., 2022). Within this genus, members of the *B. amyloliquefaciens* group are often detected in compost microbiomes, however, few studies have investigated their use as inoculants. *Bacillus velezensis* (*Bv*), a member of this group, has attracted attention thanks to its capacity to produce a wide range of metabolic intermediates including antibiotics, enzymes and growth promoters, suggesting its potential use for biotechnological applications (Adeniji et al., 2019). Its lignocellulolytic enzymes such as xylanase, laccase and peroxidase are of particular interest for the composting industry, often struggling with the degradation of woody material in the feedstock. Moreover, *Bv* exhibits plant growth promoting effects that could represent added value for the final product, positively influencing the productivity of the fields where it is applied. To our knowledge, only one study investigated the potential use of *Bv* as microbial inoculant for cattle manure and wheat straw, showing improvements on compost maturity, lignocellulose degradation and compost fertility (L. Wang et al., 2023).

A key challenge in assessing bioaugmentation is the monitoring of compost parameters (e.g. temperature, gaseous emissions) and the assessment of compost quality and maturity, which often complicates results comparison and reproducibility. This study aims to evaluate the ability of *Bv* to enhance organic matter degradation during composting, and to increase the quality and maturity of the final product. To do this, composting trials were performed in highly monitored reactors, where automatic sensors measured basic composting parameters continuously for 30 days. After that, compost quality was assessed with chemical analyses.

5.2. Materials and methods

5.2.1. Inoculum preparation

Bv was isolated from the thermophilic phase of a previous bio-waste composting experiment. To assess heat tolerance, *Bv* colonies were incubated on tryptic soy agar (TSA) at four different

temperatures (45, 50, 53, 55 °C) for 48 hours. After the incubation, each colony was re-streaked onto fresh TSA to check viability.

Part of the viable colonies were transferred into 100 mL of Luria Bertani (LB) broth (10g/L tryptone, 5g/L yeast extract, 10g/L NaCl) and incubated for 24h at 35 °C. After that, colony forming unit (CFU) count was determined using serial dilutions. Ten milliliters of culture were added to 90 mL of sterile distilled water and shaken at 150 rpm for 30 mins. Serial dilutions ranged from 10^{-1} to 10^{-8}. For each dilution, 100 µL were spread onto TSA Petri dish using a sterile L-shaped glass rod. Each dilution had three replicates. Petri dishes were incubated at 35 °C for 24 hours. CFUs were counted only on plates with a range of 30 to 300 colonies while plates outside this range were discarded. Final CFU count is expressed as mean of valid replicates within this range.

To prepare the substrate for compost bioaugmentation, after 24 h of incubation 10 mL of *Bv* LB broth were added to 100 g of sterile organic substrate (S1) composed of vegetable scraps at 50% of moisture content. The mixture was kept at 35 °C for five days. Then, 25 g of the inoculated S1 were transferred to another container with 250g of sterile substrate of the same composition and moisture (S2) for another five-day incubation. This two-step transfer was done to ensure the gradual acclimatation of bacterial colonies from the nutrient-rich broth to the harsher and nutrient limited compost environment. After five days of incubation, on both S1 and S2, CFU count was performed to check bacterial concentration and viability, using the same protocol as above. But instead of using 10 mL of broth, 10 g of inoculated substrate were used.

5.2.2. Composting protocol

Two different feedstock mixtures were tested for the study, one bioaugmented with *Bv* and another without. Each treatment had two replicates.

Bio-waste was taken from local shops near ENEA Casaccia, Via Anguillarese 301, Rome, Italy, and included discarded fruits, vegetables and green plant tissues that were no longer salable. Examples include apples, melons, cucumbers, lettuce, eggplants, tomatoes, potatoes, artichokes and cauliflowers. Before composting, this material was crushed into pieces ranging from 1 to 15 cm.

Each feedstock mixture consisted of seven kilograms of bio-waste and three kilograms of woodchips with particle size ranging from one to five cm in diameter. In the bioaugmented mixtures, 200 g of *Bv*-inoculated substrate (S2) were added. The control mixtures received 200 g of the same substrate, but sterilized. The first set of replicates started on October 10th, 2024 (bioaugmented and non-bioaugmented), while the second replicates started on November 21st, 2024 (bioaugmented and non-bioaugmented). Each composting cycle lasted 60 days: 30 days inside the reactor and 30 days outside the reactor for maturation.

5.2.3. Measurement of composting parameters

Composting was carried out using 30 L cylindrical steel reactors insulated with glass wool. Temperature, CO_2 and NH_3 emissions were continuously monitored over the initial 30-day composting. Measurements were performed every 15 minutes, 24 hours a day. Results are reported as a daily average ± standard deviation. Each reactor had an inlet for forced aeration on the bottom and an outlet for gas exhaust and analysis on the top. Airflow was automatically regulated via mass flow controller (Sensirion SFC6000D-20slm; Sensirion, Stäfa, Switzerland). The system was regulated by an algorithm that increased airflow when CO_2 concentration exceeded 4% or when temperatures rise above 55°C. The control of these two parameters was performed every 20 minutes, and according to the measured value, the airflow was increased or decreased. These values were chosen to optimize the degradation of OM per unit of time, ensuring enough supply of oxygen and avoiding too high temperatures. Part of the exhaust gas was diverted to a sampling chamber for emissions analysis. CO_2 concentrations were analyzed using a 20mm NDIR sensor (IRNET-P 20mm; N.E.T. srl, Cornaredo, Italy; range: 0-20% vol), while NH_3 levels were monitored with two 3-

electrode electrochemical gas sensors, one with a range of 0-300 ppm with a resolution of 2 ppm and another with a range of 0-5000 ppm with a resolution of 20 ppm (NT-NH3-PL5000; N.E.T. srl, Cornaredo, Italy). Temperature was measured using digital thermometer located in the central part of the reactor (1-Wire® Digital Thermometer, Dallas Semiconductor, Mansfield, TX). To perform the carbon mass balance, starting from CO_2 emissions a series of calculations were done. The percentage of CO_2 recorded by the sensor was converted into parts per million (ppm) and then into mg/m^3 using a standard conversion tool (https://teesing.com/en/tools/ppm-mg3-converter). The airflow which was measured into L/min was converted to m^3/min to allow the calculation of the mass of CO_2 emitted from the reactor overtime. The obtained mass flow rate of CO_2 was then used to estimate the total CO_2 released during the time interval of one hour and then was cumulatively summed to obtain the total CO_2 emitted over the period of monitoring. These calculations assume that all the CO_2 originated from microbial respiration and that the conditions in the reactors were always aerobic.

5.2.4. Chemical analysis

Chemical analysis was performed for each replicate after maturation (60 days). For humidity and pH, measurements were carried out also on days 0 and 30. Samples were processed by Agribioeco s.r.l., Pomezia (Italy), a laboratory accredited by the Italian accreditation body (ACCREDIA).

Humidity content was determined by the gravimetric method by drying compost at 105 °C for 24h. The pH, total organic carbon (TOC), salinity and humic and fulvic acids were measured following the procedures indicated in the Italian Ministerial Decree for the analysis of fertilizers (*Decreto Ministeriale* [DM] 17/06/2002 *Gazzetta Ufficiale* [GU] n° 220 of 19/09/2002). Total nitrogen (N_{tot}), organic nitrogen (N_{org}) and germination index (GI) were determined according to the standards Ente *Nazionale Italiano di Unificazione* (UNI) European Norm (EN) 16168:2012, UNI 10780:1998 App. J2.2 + J3.2 + UNI EN 16168:2012, and UNI 10780:1998 App. K, respectively. The N_{org}/N_{tot} and C/N ratios were calculated.

5.2.4. Statistical tests

Comparison of the overall carbon consumption was performed calculating the area under the curve (AUC) using the trapezoidal rule and applying Friedman's test, since the data did not meet the assumption of normal distribution and homoscedasticity. Dunn's multiple comparison test was used as a post-hoc analysis to assess pairwise differences. Results were deemed significant when $p < 0.05$. All the analyses were performed using GraphPad Prism 8.0.1 (GraphPad Software, San Diego, CA, USA).

5.2.5. Plant growth test

Part of this study also included growth tests to assess the agronomic quality of the different composts, however, due to an unfortunate fire that involved our department, the results of this trial were lost.

5.3. Results

5.3.1. Inoculum preparation and concentration and heat tolerance

After 48 hours of incubation at 45, 50, 53 and 55 °C, all the colonies transferred to fresh TSA were vital, demonstrating the resistance of *Bv* to temperatures reached in most of the composting processes during the thermophilic phase. After 24 h in LB at 35°C, there was an average of 7.6×10^7 CFU/mL. The concentration in S1, after 5 days of incubation was 3000 CFU/g and in S2 after 5 days it was 6600 CFU/g.

5.3.2. Monitoring of compost parameters

Process performance was influenced by seasonal variation between the first replicates in October and the second replicates in late November due to a sensible drop in external temperatures. The forced aeration system introduced air coming from outdoor pipes, hence, the cooler air of November (20.3 ±1.0 °C) compared to the air of October (26.5±0.8 °C) negatively affected the microbial activity in the second replicates, limiting temperature rise and slowing degradation of OM.

At the start of composting, initial temperatures were ~23 °C for the first set of replicates and ~14 °C for the second set of replicates. Across all trials, bioaugmented compost reached higher temperatures and had a longer thermophilic phase (i.e. temperature >40°C) compared to non-bioaugmented treatments (Figure 5.1a).

- Bioaugmented replicates:
  - In October peaked at 51.3 ± 2.6 °C on day 5 and maintained a thermophilic phase for 10 days.
  - In November peaked at 50.8 ± 0.5 °C on day 5, with a 6-day thermophilic phase.
- Non-bioaugmented replicates:
  - In October peaked at 48.5 ± 0.4 °C on day 6, with a 7-day thermophilic phase.
  - In November peaked at 47.3 ± 0.8 °C on day 4, maintaining thermophilic conditions for 5 days.

The temperatures cooled down to ambient ~15th day (October replicates) and ~10th day (November replicates).
CO_2 emission was directly related to temperature profiles; all trials had their peak in CO_2 emission during the thermophilic phase (Figure 5.2b):

- Bioaugmented replicates:
  - In October peaked at 11.7 ± 6.3% on day 3
  - In November peaked at 4.7 ± 1.9% on day 2

- Non-bioaugmented replicates:
  - In October peaked at 10.3 ± 4.9% on day 2.
  - In November peaked at 4.8 ± 1.5% on day 2.

With the decrease in temperature, CO_2 production decreased, eventually dropping below 1% when compost reached ambient temperature. Cumulative carbon consumption (Figure 5.2) was evaluated by calculating the area under the curve (AUC) for each treatment group. Most of carbon consumption occurred during the first ten days, when temperatures were high. The highest AUC was observed in the bioaugmented treatment of October (947.1), followed by the non-bioaugmented treatment of the same month (870.5), bioaugmented treatment of November (783.4), and the non-bioaugmented treatment of November (706.1). Statistical comparison using the Friedman test followed by Dunn's multiple comparisons revealed significant differences among treatments. In October, no significant difference was found between bioaugmented and non-bioaugmented conditions. In contrast, in November, bioaugmentation significantly increased carbon consumption compared to the non-bioaugmented treatment ($p = 0.0416$). A seasonal effect was also found: both bioaugmented and non-bioaugmented treatments in October showed significantly higher AUCs than the corresponding November treatments (bioaug. Oct vs bioaug. Nov, $p = 0.0137$; non-bioaug. Oct vs non-bioaug. Nov, $p < 0.0001$). NH_3 emissions were negligible during the thermophilic phase but increased during the cooling phase: the bioaugmented trial of October peaked at 93.5 ± 7.6 ppm on day 27 (Figure 5.2c), the non-bioaugmented peaked at 71.0 ± 8.4 ppm on day 26. Both replicates of November had steady emissions of circa 8 ppm post-thermophilic phase, without marked peaks.

5.3.3. Chemical analysis

On the 60th day of composting, all compost treatments were respecting the minimum requirement threshold for mature compost from the Italian Legislative Decree n° 75/2010, except for humidity that was slightly above 50%. Minor differences were found among treatments, with most parameters falling within a narrow range. Levels of pH started acidic at the beginning of the process, then stabilized around an alkaline range by day 60. Moisture content did not decrease much until day 30, but it did during maturation. Heavy metals content remained negligible. The most marked difference was observed in TOC, where bioaugmented treatments could retain more carbon compared to the other treatments. Total nitrogen was uniform across all treatments, and C/N ratio fell between 14 and 16. Salinity was slightly high but acceptable and the germination index indicated low phytoxicity. Complete values for all parameters are reported in Table 5.1.

5.4. Discussion

The results of this study demonstrate that *Bv* is a potential inoculant for compost bioaugmentation, although its effects should be investigated more. Specifically, monitoring the population density of *Bv* could be of great benefit to better understand which are the composting phases in which the inoculant is more active. Unfortunately, this was not possible during this study because bioreactors were sealed and their opening would compromise the measurement of other factors (e.g. CO_2). Effects on microbial activity, thermophilic phase and OM degradation were detected; however, these did not significantly affect the chemical properties of the final product.

The tolerance of *Bv* to high temperatures was confirmed up to 55 °C, ensuring the survival during the peak temperatures of composting. The sharp drop in CFU concentration from the liquid culture (7.6×10^7 CFU/mL) to the inoculated substrates (3,000 CFU/g in S1, 6,600 CFU/g in S2) indicates that the transition from a nutrient rich substrate to organic substrates which contain more complex molecules, had a strong selective pressure of *Bv*. The low CFU concentration in S2 probably did not allow *Bv* to clearly establish in the feedstock and likely suffered competition from the indigenous microbial community, which is one of the main problems reported from other studies (Hart et al., 2003; Jurado et al., 2014; Mironov et al., 2024).

Temperature and CO_2 emissions across replicates show that the microbial activity is enhanced in bioaugmented treatments, although difference exist in the first and second trial due to changes in the environmental conditions. The results indicate that bioaugmentation enhanced carbon consumption solely in November while in October, when degradation was already efficient, there was no improvement. In both trials, *Bv* inoculation led to higher peak temperatures and longer thermophilic phases. Prolonged high temperatures are favoring lignocellulose degradation (Meng et al., 2024), indicating that *Bv* can contribute to a faster and more robust degradation of the OM during the early composting phase, where lignocellulosic materials are attacked most by microorganisms (Ryckeboer, Mergaert, Coosemans, et al., 2003). Furthermore, a longer thermophilic phase is beneficial for pathogens eradication (Meng et al., 2024).

The correlation between CO_2 emissions and temperature profiles was expected, because both parameters are highly associated with microbial activity and respiration (Ghaly et al., 2011; Straatsma et al., 2000). Bioaugmented trials showed higher carbon dioxide emission and total carbon consumption, likely linked to an enhanced breakdown of the OM.

Ammonia emissions followed the classical pattern of composting and are usually associated with the degradation of organic nitrogen (Q. Wang et al., 2024). During the initial phase of the process organic nitrogen (proteins, peptides, nucleic acids) are mineralized through ammonification and produce NH_4

and NH_3 (S. Wang & Zeng, 2018). Low pH prevents the volatilization of NH_3, but as soon as pH increases, ammonia starts to be emitted (L. Zhang et al., 2023). At the same time, part of NH_4 is transformed into nitrate and nitrite (Sun et al., 2019).
The difference in NH_3 emissions between the bioaugmented trial and the not bioaugmented trial of October are not sensible but could reflect slightly more intense protein degradation in the bioaugmented, however, more tests should be carried out to confirm this hypothesis. The second set of replicates of November showed lower and more stable NH_3 emissions, likely due to the limited microbial activity.
Chemical analyses at day 60 showed that most parameters were similar across treatments and that bioaugmentation did not radically change the properties of the final product. On day 0, moisture content was around 60%, which is an ideal condition for starting composting (Kuwahara et al., 2009; Lin et al., 2008). Water loss was expected to be high during the thermophilic phase, but moisture started to decrease mostly during maturation, when compost was in contact with external air. Probably the enclosed environment of the reactor prevented water loss.
pH is a critical factor for composting because if it's too low at the beginning, it might inhibit microbial activity (Paradelo et al., 2013). A suitable pH for composting should range between 6.5 and 8 (Hachicha et al., 2009). Despite the acidic environment, which is common for bio-waste (Adhikari et al., 2009; Oviedo-Ocaña et al., 2023; Turan & Ergun, 2008; F. Yang et al., 2013), both treatments started their thermophilic phase rapidly, demonstrating the capability of the indigenous microbial community and *Bv* to tolerate low pH. In general, the results of the chemical analysis are not extraordinary and are in line with the values reported from most of the studies on bio-waste composting (Adhikari et al., 2009; Kim et al., 2008; Martínez-Blanco et al., 2010; Tognetti et al., 2007). Only the slightly higher TOC in bioaugmented samples (40% and 37%) compared to controls (34% and 35%) raises an interesting point: although bioaugmentation enhances degradation of OM, it also seems to preserve a bigger fraction of carbon, which can potentially be represented by humic substances. There might be a possibility that bioaugmented samples started composting with a higher percentage of TOC, however, the attention in respecting the exact proportion of bio-waste and woodchips during mixture preparation, it makes us think that TOC content should have been very similar. Nonetheless, the same humic acids content was detected in all treatments. The differences in CO_2 emissions and TOC content were probably not big enough to sensibly affect the final content of humic acids. This dual effect of stronger OM degradation and higher TOC residue is notable for compost quality and requires further investigation because a higher content of humic acids would significantly increase the product's quality and is related to carbon sequestration effect (Meng et al., 2024).
The C/N ratios (14–16), low heavy metal content, moderate salinity, and germination indices (68%–73%) are common parameters used for assessing compost maturity and they all indicate mature, non-toxic compost which is suitable for agricultural use. A GI value above 80% is preferred, however, only when GI is under 50% a compost is considered phytotoxic (Zucconi et al., 1981).
Salinity tends to increase during composting due to water loss and OM degradation and there is no full consensus regarding the maximum threshold of salinity in compost (M. K. Awasthi et al., 2014; Oarga Mulec et al., 2016; Turan, 2008). In general, it should not be above 4 to 5 mS/cm by the end of composting otherwise compost might inhibit plant growth due to salts accumulation (Gao et al., 2010; Hoekstra et al., 2002; Onwosi et al., 2017). High EC is however usually related to a better degradation of OM (Q. Wang et al., 2024). In our case, EC can be considered within the accepted range.
In conclusion, bioaugmentation with *Bacillus velezensis* proved effective in stimulating microbial activity during the thermophilic phase, under both favorable and unfavorable environmental

conditions. The small differences recorded between treatments are probably caused by the low concentration of inoculum that was added at the beginning of composting. A bigger amount of inoculated substrate, a higher CFU concentration or the inclusion of an additional and complementary inoculant organism would probably result in more marked differences between treatments.
The lower performance of the second trial in November highlights the importance of seasonal variability in composting performance. Future work should be focused on testing different inoculation strategies to increase the magnitude of the beneficial effects of *Bv* for composting.

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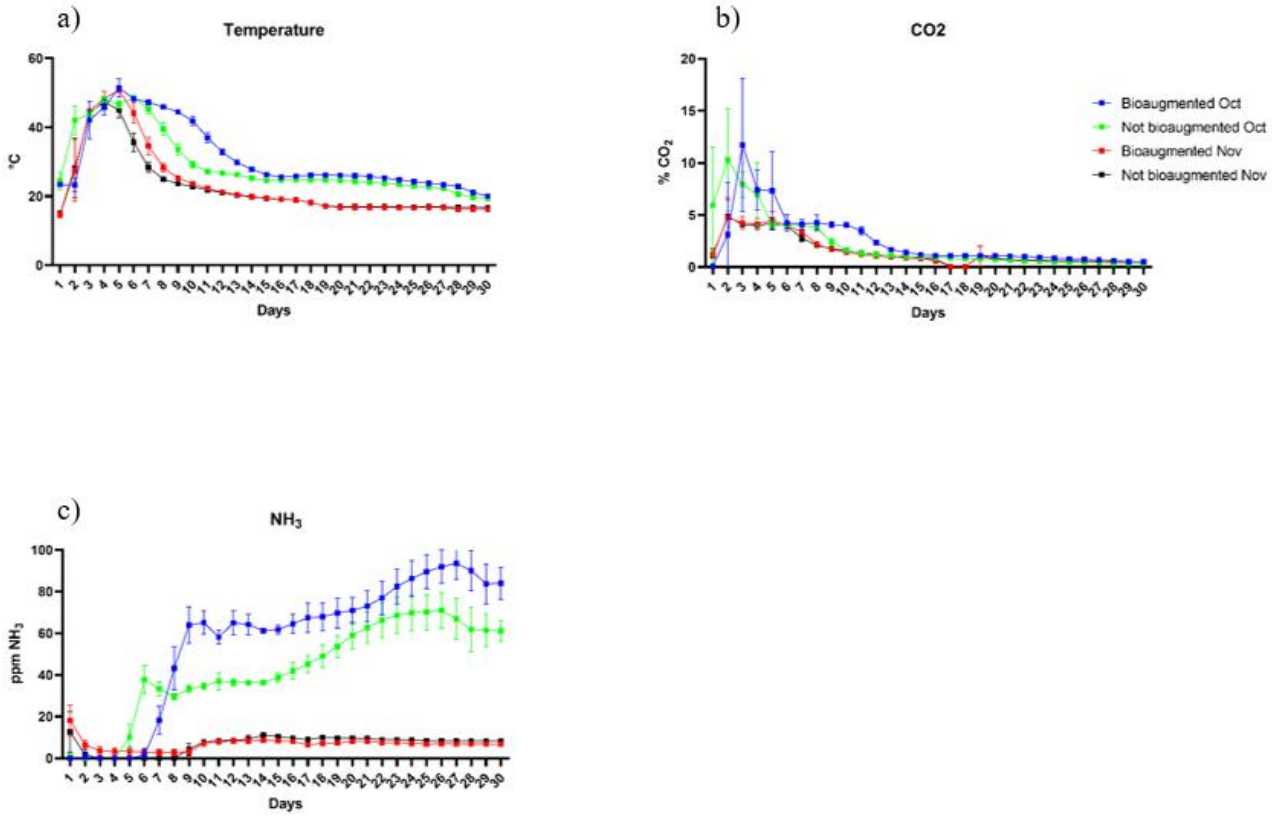

Figure 5.1 Mean±SD of temperature (a), CO2 (b) and NH3 (c) emissions during the 30 days of composting inside the reactors.

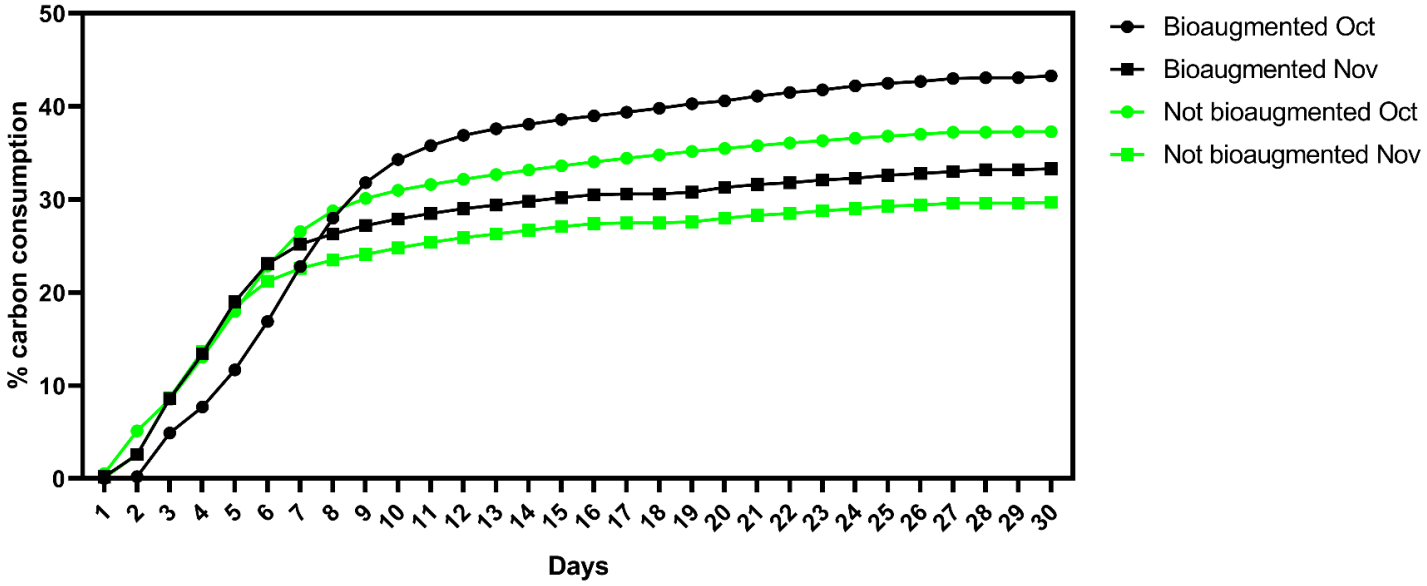

Figure 5.2 Cumulative carbon consumption expressed as percentage for all four treatments derived from CO2 emissions in relation to the total organic carbon present in the feedstock.

Table 5.1 Results of the chemical analysis for compost at the end of the composting process. The column "Dlgs 75/2010" displays the minimum requirements for mature compost according to the Italian Legislative Decree n° 75/2010. U.M.= unit of measure; dS/m = decisiemens per metre; dm=dry matter; TOC= total organic carbon; N_{tot}= total nitrogen; N_{org}= organic nitrogen; GI= germination index; N/A = Not applicable.

		Non-*Bv*	Non-*Bv*	*Bv*	*Bv*	
	U.M.	Oct	Nov	Oct	Nov	Dlgs 75/2010
pH day 0		4	5.5	4	5.5	N/A
pH day 30		9	9.1	8.8	8.9	N/A
pH day 60		8.1	8.6	8.8	8.5	6-8.5
Salinity	dS/m	5.5	4.5	4.3	4.6	N/A
Humidity day 0	%	64	63	64	63	N/A
Humidity day 30		64.4	63.3	62.5	61.3	N/A
Humidity day 60	%	55	56	53	55	≤50%
TOC	%dm	34	35	40	37	≥20%
Humic acids	%dm	10	10	10	10	≥7%
N_{tot}	%dm	2.4	2.4	2.4	2.3	N/A
N_{org}	%dm	2.1	2	2.2	2	N/A
C/N		14	14	16	16	≤ 25
Cadmium	mg/Kg dm	0.4	0.5	0.3	0.3	N/A
Copper	mg/Kg dm	51	71	69	60	N/A
Nichel	mg/Kg dm	23	32	23	24	N/A
Zinc	mg/Kg dm	190	236	252	174	N/A
GI	%	68	69	73	71	≥60%

Conclusions

Throughout this PhD, the primary objective was to study the microbiological mechanisms that regulate composting, focusing on both optimizing the feedstock mixtures and on evaluating the quality of the end product. From the first experiment it became clear that choosing the right proportion of BA had a major impact on the thermophilic phase, humidity and on other chemical parameters such as pH and C/N ratio, but also on the succession of the microorganisms involved in the breakdown of the OM. The mixture containing one-third of woodchips by fresh weight yielded the best results while the other compost piles were either overly compacted and humid or too poor in nutrients for stimulating microbial activity. By combining HTS with pure culture isolation, it was possible to identify the dominant fungal and bacterial communities in the different phases of composting. In particular, some of the isolates showed traits that could be useful for future biotechnological applications like *Bacillus velezensis, Pseudomonas mucidolens* and *Pichia kudriavzevii.* At the same time, other microorganisms were more abundant under poor composting conditions such as *Bacteroidetes* spp. and *Barnettozyma californica* and could serve as early biological markers for problems in the process.

Building upon these insights, a more detailed study was conducted comparing four different feedstock mixtures and their fungal community: two of them deemed optimal and two deliberately suboptimal. Despite the differences in process parameters and physiochemical properties, HTS analysis showed the existence of a shared core fungal microbiota across all the mixtures. Still, specific indicator species were found in each mixture, mostly during the maturation phase. For example, the order Hypocreales was mainly found in the mixture with perlite and cardboard, probably due to easily accessible carbon sources, while *Thermomyces lanuginosus* thrived where the thermophilic phase was stronger. The presence of the yeast *Pichia kudriavzevii* was linked to a more acidic environment. These observations highlight the importance of studying biomarkers alongside traditional physiochemical parameters for assessing composting performance and quality.

Another part of the project focused on whether the optimized composting protocol designed in the previous studies could eradicate plant pathogens harbored by infected feedstock, such as waste from plant nurseries. Our findings showed that maintaining temperature above 55 °C for four days and by mixing daily the composting material was enough to achieve the eradication of *Phytophthora cinnamomi*. HTS demonstrated also the removal of other plant pathogens, confirming the final product was pathogen-free.

In the final phase of this research, the attention was focused on testing a bioaugmentation strategy aimed at accelerating compost maturation and OM degradation. *Bacillus velezensis* was selected from our isolate collection for its production of biotechnologically relevant enzymes like laccase and peroxidase and for its resistance to high temperatures. When introduced into pilot-scale bioreactors, it demonstrated to speed up the degradation of OM by increasing peak temperatures, CO_2 emissions and carbon degradation. These findings, however, did not translate into superior chemical properties of the final product, which showed little or no differences between the bioaugmented and the non-bioaugmented treatments. Probably, the inoculum concentration was not high enough to cause sensible changes in the quality of the end product, for this reason, other tests should be carried out for optimizing the inoculum dosage.

The emerging evidences from these studies have several practical and scientific implications. They provide a framework for the design of compost feedstocks that ensure the optimal balance of moisture and nutrients. The feedstock ratios tested here can be adopted by household and small-scale composters. Second, the detection of biomarkers opens up the possibility for the development of *in situ* monitoring tools that would allow operators to assess compost maturity or the efficiency of the process in real time. These biological parameters might be coupled with the traditional chemical ones.

The confirmation that *P. cinnamomi*, together with other plant pathogens, can be efficiently removed from infected material, has implications for both waste reduction and biosecurity. Several nurseries struggle with the management of their green waste, often piling the material somewhere for months or burning it. With these findings nurseries and similar facilities can safely recycle this material to produce soil amendment, reducing costs and reliance on commercial and unsustainable products like peat. Moreover, the detection of several potential plant pathogens via HTS underscores the importance of integrating molecular tools in quality control workflows.

That said, some limitations must be acknowledged. All the composting trials were carried out on a pilot scale, hence, their applicability in large scale composting plants, where tons of material are treated, should be validated. Additionally, this work focused on bio-waste and woody materials, however, many other types of feedstocks exist like sludge and manure, for which the conclusions drawn in this PhD project might not apply. Finally, seasonal variations were not fully explored, though in the last study we saw how temperature differences of external air can influence the microbial activity.

Future research should further explore compost microbiomes. The results obtained in this thesis are just a little contribution to a topic that still needs a lot of study. In our case, the analyses have focused on taxonomic composition and abundance. The next step might be the application of meta-transcriptomic and meta-proteomic for the investigation of genes and enzymes that are expressed during different composting conditions. The integration of these technologies would give us a refined understanding of how microbial communities contribute to the process.

To conclude, this doctoral research brought together microbiology, chemistry and agronomy to design a practical and science-based approach for composting bio-waste efficiently and safely. This project contributes to scientific research on composting and provides tools and guidelines for small scale composting scenarios. As the world shifts towards more challenging conditions for waste management and European Union regulations become stricter, the findings here support composting as a key solution and promote an approach based on merging traditional methods with molecular biology.