PHYSICO-CHEMICAL AND BIOLOGICAL EVOLUTION OF THERMOPHILIC COMPOST AS INFLUENCED BY NITROGENOUS FEEDSTOCK AND DURATION

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other university.

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To Purity, Clare-Fiona, Lance-Jerome, and Laura-Jossie for being the happiness that came across during my studies.

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ABBREVIATIONS AND ACRONYMS

µL	Microlitre
ASV	Amplicon sequence variants
°C	Degree centigrade
CO_2	Carbon dioxide
DADA2	Divisive amplicon denoising algorithm
DNA	Deoxyribonucleic Acid
EDR	Endocrine Disruptors
HUMANn	HMP Unified Metabolic Analysis Network
icipe	International Centre for Insect Physiology and Ecology Institute
ITS	Internal Transcribed Spacer
Ml	Millilitres
PCA	Principal component analysis
PCoA	Principal coordinate analysis
PCR	Polymerase Chain Reaction
pH	Potential of Hydrogen
PICRUSt	Phylogenetic Investigation of Communities by Reconstruction of Unobserved States
rRNA	Ribosomal RNA
SAMSA	Simple Annotation of Metatranscriptomes by Sequence Analysis
CCA	Canonical correspondence analysis
OTU	Operational taxonomic units

ABSTRACT

Thermophilic composting is a source of agricultural enzymes, microbes, and nutrients from organic waste. *Lantana camara*, *Tithonia diversifolia*, and fresh grass (*Pennisetum purpureum*) clippings are readily available supplemental nitrogen sources in compost. However, their influence on microbes and physical-chemical state during the composting period has not been explored. This study sought to enumerate the influence of regimens of these materials and their duration on the evolution of the microbial communities and their physical-chemical and metabolic potential during composting using next-generation sequencing and gravimetric methods. Precisely, the objectives of this study were: (1). To evaluate the influence of composting materials and durations on compost physical-chemical quality; (2). To examine the impact of composting materials and duration on prokaryotic community structure; (3). To evaluate the influence of composting materials and duration on fungal and non-fungal eukaryotic community structure; (4). To enumerate the influence of composting materials and the composting duration on the metabolic potential of compost fauna. A completely randomized block design (CRBD) was adopted, involving the following compost treatments; (1). Cattle manure + Dry maize stalks + *Lantana camara* twigs (L), Cattle manure + Dry maize stalks + *Tithonia diversifolia* twigs (Tithonia), Cattle manure + Dry maize stalks + Grass clippings (G), and a mixture of the 3 treatments (LTG) composts. Sampling from triplicate compost heaps was mainly done at 21, 42, 63, and 84 days of composting as per standard procedures of each objective. Physical-chemical parameters such as temperature, nitrogen pH, and carbon were analyzed from triplicate heaps using standard gravimetric protocols. On the other hand, total community 16S rRNA, ITS, and 18S rRNA were also extracted using the ThermoFisher Scientific® microbiome kit before separate amplification and sequenced under the Illumina platform. Sequence bioinformatics was mainly done using Divisive Amplicon Denoising Algorithm v2 workflow. Total compost ribonucleic acid (RNA) was extracted using the trizol-based method, before shotgun sequencing under the Illumina miseq platform. This was followed by functional profiling based on the Metapro, and SAMSA2 bioinformatics workflows. Complementary functional profiling was also done based on 16S rRNA sequences using PICRUSt2 bioinformatics workflow. There were positive and negative correlations between various physical-chemical parameters, with temperature positively correlated with ammonia and carbon. *Proteobacteria*, *Sordariomycetes*, and *Holozoa* were the most dominant prokaryotic, fungal, and non-fungal eukaryotic classes respectively. The most significant prokaryotic and eukaryotic abundance, richness, and diversity were observed in Lantana-based compost which is attributable to its complexity compared to other materials. PcoA results showed distinct groupings as influenced by composting materials and duration with a total of 73%, 28%, and 66% variation of prokaryotes, fungi, and non-fungal eukaryotes respectively. Composting materials had no significant influence on most metabolic pathways. Composting days had a significant influence, with days 42 and 63 having the most sequences for carbon and nitrogen metabolism respectively. This study recommends that composting practices should focus on extending composting duration rather than varying feedstock types to improve compost quality and stability.

CHAPTER ONE

INTRODUCTION

1.1 Background information

Composting refers to converting organic waste into agriculturally useful humic material by macro and microorganisms (Meena et al., 2021). Composting offers a sustainable alternative to environmentally harmful chemical inputs by supplying similar nutrients as those supplied through these conventionally available synthetic inputs. Application of compost to agricultural soils also serves as a buffer in acidic soils thus optimizing conditions for plant growth, ultimately enhancing productivity. Moreover, compost provides the soil with naturally available plant growth promoters, micronutrients, and free-living antagonistic microbes that help in the reduction of pathogens responsible for crop diseases such as Fusarium wilt (Singh, et al., 2022). Unlike natural decomposition, composting regulates environmental conditions such as temperature, moisture, and oxygen. Composting utilizes locally available materials such as kitchen waste, cattle manure, municipal waste, sawdust, dry maize stalks, wood chippings, grass clippings, and hedge plants such as *Lantana camara* and *Tithonia diversifolia* among others. Recent advances in composting have included pathogen-laden waste such as human waste; though, caution is necessary to ensure that the product is pathogen-free (Effebi et al., 2019; Ezugworie et al., 2021).

Composting occurs through two main methods, namely, thermophilic (aerobic) and non-thermophilic (cold/anaerobic) processes. Anaerobic composting occurs under limited oxygen conditions, slowing carbon breakdown, which would otherwise lead to a spike in temperatures. Due to this, the anaerobic compost heaps remain at around ambient

temperatures throughout the composting period. On the other hand, thermophilic composting is an aerobic (oxygen dependent and abundant) and self-heating process that utilizes carbon polymers present in the organic matter to produce a final humic substance that is nutritionally and microbially stable. This process is faster than the anaerobic process and guarantees a product that is free of pathogens (Ayilara et al. 2020; Meena et al. 2021).

The process of thermophilic composting produces heat and carbon dioxide as by-products; while the humic product contains essential inorganic nutrients for plant growth such as nitrogen, carbon, phosphorus, potassium, and calcium. The humic product also carries micro and macro-organisms that enhance soil nutrient cycling, plant growth, and protection. Thermophilic composting also reduces antibiotic resistance in cattle by curtailing the expression levels of microbial resistomes. These resistomes mainly consist of genes enabling microbial resistance to antibiotics in ecosystems such as manure. Some of the commonly studied resistance genes that have been reduced from manure through composting include those responsible for the breakdown and utilization of aminoglycosides, beta-lactam, fluoroquinolone, tetracycline, and vancomycin (Wang et al., 2013). Additionally, the beneficial microbes borne in the compost applied to the soil serve as biological sequesters of chemicals such as persistent organic pollutants (POPs), Endocrine Disruptors (EDRs), and antibiotics left in ecosystems (Manyi-Loh et al. 2018; Ayilara et al. 2020).

The temperature of a thermophilic compost heap is a direct reflection of the microbial metabolic activity and defines the progress of the composting process. Based on the temperature-based ecosystem occurrences, the thermophilic composting process has four temperature-defined phases. Assorted microbial diversities and abundances dominate these

various temperature-related phases. Soon after materials heaping (day 0), the aerobic compost progressively heats to attain temperatures above 40°C within 2 days. Precisely, the mesophilic microbes and macroorganisms promptly break down the readily available materials, leading to a rapid temperature rise. The temperatures above 40 °C, render the mesophilic organisms less competitive, reducing their dominance in the compost environment. Consequently, they are replaced by thermophilic classes mainly belonging to the bacteria classes such as *Bacilli* and *Clostridia* (Papale et al., 2021). This is followed by sustained temperatures above 60 °C, for about three weeks, before gradually cooling down to the ambient temperature (environmental temperature) after exhaustion of carbon-rich materials. The cooling down of the thermophilic compost heap prompts recolonization by mesophilic micro-organisms which in turn facilitate the maturation of compost and enhance nutrient stabilization through completion of the humification process (Manyi-Loh et al. 2018).

Turning the compost heap is important to ensure uniform biodegradation of material such as total nitrogen, total phosphorus, and total potassium in the resulting compost (Antunes et al., 2016; Zhang, et al., 2019). When thermophilic compost heaps fail to be turned, thus depriving access of oxygen and moisture to the inner levels, cooking of the manure occurs, resulting in whitish material which is associated with “fire fungus” (mainly *Actinomycetes*) that dominates ecosystems that are oxygen and moisture limited but hot (Matheri et al., 2023b).

The type of composting materials (feedstock) has a significant and direct influence on the colonization capacity of micro-and macroorganisms during the composting process (Miao et al.,2022). This ultimately influences the time of composting, the physical-chemical and

biological succession as well as the nature of the final product. Temperature is a key indicator of this influence of feedstock on the nature and process of the composting process. For example, materials containing high levels of carbon polymers are bound to bring about high temperatures and prolonged thermophilic conditions depending on the complexity of the carbon polymers (Azim et al., 2018). This presents such materials as superior in terms of hastening the process of humification and sanitization. Precisely, the thermophilic phase, which occurs at temperatures between 40℃ and 70℃, is the crucial phase that sanitizes the compost by destroying weeds and pathogens as well as the initial breakdown of dry matter (Xiao et al., 2009; Mandpe et al., 2021).

The subsequent cooling and curing phases are characterized by declining pile temperatures and the activation of stabilization and humification enzymes that are relatively heat-sensitive (Ma et al., 2020). At this composting time, the humification substance precursors, which are primarily formed in the thermophilic phase, are polymerized. The cooling and maturation phases are characterized by different categories of micro- and macro-organisms that are responsible for transforming materials into subsequent forms and ultimately the final stable product (compost). These organisms include prokaryotes (bacteria and archaea), algae, and eukaryotes. Microbes and macroorganisms act by secreting enzymes, which serve as catalysts for the process of degradation and sequestration. These two enzymatic routes of the utilization of materials are biochemically referred to as catabolism and anabolism respectively (Van-Fan et al. 2018; Abtahi et al. 2020).

Prokaryotic communities are the dominant microbial categories in all stages of the composting process. This domain, which is constituted by bacteria and archea plays a significant role in carbon cycling, particularly in the thermophilic phase where prokaryotic

thermophiles utilize available sugars to produce stable carbon compounds and carbon dioxide (Papale et al., 2021). Fungal categories are mainly present in mesophilic phases of the composting process and play a role in breaking open recalcitrant carbon-rich material to release simpler forms of sugars that are subsequently utilized by bacteria (Nirmalasari et al., 2023). Non-fungal eukaryotes, such as *Chloroplastida* and *Holozoa*, are also associated with nutrient cycling in the environment and their community structure is closely associated with nitrogen transformation. Bacterial communities are responsible for nitrogen cycling, with some species involved in nitrogen oxidation to forms available for plants to absorb; while others participate in denitrification, which results in nitrogen losses in forms such as ammonia (Li et al., 2019). There is however no existing literature on the influence of composting materials such as a source of green nitrogenous material on the community structure and function of prokaryotes and eukaryotes in the compost ecosystem.

The quality of compost is determined by the input material, carbon-nitrogen ratio, moisture, and oxygen levels. The ideal carbon-to-nitrogen ratio (C: N) for feedstock is 30:1 to 35:1, with 60% moisture and adequate aeration. The forms of carbon and nitrogen polymers in the composting material influence the evolution of nutrients and microbes. For instance, the breakdown of complex materials like lignin, cellulose, and hemicellulose in *Lantana camara* requires more genes responsible for the breakdown of these materials compared to *Tithonia diversifolia*. Such complex materials may also inhibit certain categories of microbes and hinder their ability to colonize compost prepared with materials like *Lantana camara* and *Tithonia diversifolia*. This inhibitory potential varies with feedstock material

and requires further investigation to evaluate its influence on microbial community structure (Mansoori et al., 2020).

Lantana camara and *Tithonia diversifolia* are plants that originated from Central and South America and have become naturalized in East Africa, including Kenya. The plants are common in furrow lands as well as border hedges of farms in most regions of Kenya (Shackleton et al., 2017; Tagne et al., 2018). These plants are commonly found in furrow lands and border hedges of farms throughout Kenya, and farmers have adopted them as a part of their soil fertility regimen due to their ubiquity and high nitrogen content. Studies have shown that *Lantana camara* enhances soil enzymatic activity, particularly cellulases in the soil rhizosphere, while in vitro experiments have shown that *Tithonia diversifolia* has antimicrobial properties against human pathogens such as *Salmonella typhi*, *Staphylococcus aureus*, and *Pseudomonas aeroginosa*. However, there is no research on the influence of these two plants on proliferation and metabolic potential in ecosystems such as compost environments. (Shackleton et al. 2017; Opala, 2020; Wang et al. 2020).

Microbial interactions in the ecosystem can be positive or negative. Positive interactions, also known as mutualism, involve cooperation between individual microbial species rather than groups. Negative interactions include parasitism, ammenalism, competition, and predation. An example of such an interaction is when a category/species of microbes hunts another species in the ecosystem where Vampirovibrio species feeds on chlorella cells of eukaryotes (Lee et al., 2022). In some instances, certain microbial categories feed on others for nutrients and survival, which has the potential to manage harmful microbial species and enhance useful ones (Tshikantwa et al. 2018; Lee et al. 2022). For example, compost application can reduce harmful pathogens such as plant parasitic nematodes (Maina et al.

2020; Atandi et al. 2022). Parasitism is another form of microbial interaction in the ecosystem, where one organism benefits while the other does not. A common example is Bacteriophages, which can halt fermentation by inactivating dominant fermentation strains such as lactic acid bacteria (Sturino and Klaenhammer, 2006).

The bacteria-fungi interaction is a unique interactive association within the ecosystem. Bacteria and fungi co-exist in similar ecosystems, particularly in biofilms attached to solid surfaces. The two microbial categories, and interact through different assorted signaling processes, enabling each category that enables one category to benefit from the metabolites of the other (Carrascosa et al., 2021). For example, ligninolytic bacteria and fungi break down complex carbon-rich materials such as lignin, releasing metabolites that are used by other bacterial categories (Suryadi et al., 2022). Enumerating these interactions in the compost environment can reveal their significance and identify the core microbes in the compost ecosystem. This information can be used to enhance critical microbial categories in the compost environment and for further studies.

Culture-dependent approaches have been used for years to study microbial profiles in ecosystems, such as compost (Anguita-Maeso et al. 2020; Phulpoto et al. 2021). These methods, which have been developed since 1873, have enabled ecologists to access microbes by cultivating them. However, this approach has limitations, such as the presence of unculturable organisms and limited information on microbial activity and metabolic capabilities (Phulpoto et al. 2021; Youseif et al. 2021). To overcome these limitations, high-throughput sequencing techniques of nucleic acids have emerged, allowing for community structure analysis based on environmental DNA sequencing and activity and functional potential based on RNA sequencing (Chialva et al. 2019; Bang-Andreasen et al.

2020). These techniques have been improved to increase sequencing depth, and bioinformatics approaches, such as PICRUSt2, have been developed to predict functional capabilities based on DNA sequences (Douglas et al., 2020). Although metagenomics can provide information on community diversity and metabolic and functional profiles, its high costs have limited its application. Nevertheless, tools like PICRUSt have been customized to predict environmental microbial population genomes and functional capabilities based on targeted sequence data outputs from pipelines such as DADA2 (Douglas et al., 2020).

This study sought to explore the impact of diverse green composting supplements on the succession of physical-chemical parameters and microbial community structure and metabolic potential as influenced by assorted sources of compost nitrogen. The study utilized the analysis of 16S rRNA, 18S rRNA, Fungal ITS amplicons, and non-amplified mRNA shotgun under the Illumina platform to create high-resolution taxonomic diversities, abundances, and functional profiles of compost microbial communities. In addition, different physical-chemical parameters of compost were evaluated during the composting period. The study was conducted at the Thika site of the ongoing long-term farming systems comparison (SysCom; www.system-comparison.fibl.org) trials in Kenya. The influence of these parameters on respective microbial categories was also evaluated using a stepwise statistical model. The findings of this study are essential in improving compost production in terms of sanitization of harmful pathogens, enhancing populations of beneficial microbes, and physical-chemical stability of compost.

1.2 Statement of the problem

The rise in the global human population has resulted in a heightened demand for food, which has led to the overuse of soil fertility to maximize production (Sun et al., 2023).

Recent studies have focused on establishing organic farming systems as stand-alone production systems managing soil fertility and pests solely using organic inputs (Brust, 2019). Such organic amendments for soil fertility include compost which is one of the main components of organic farming and a means to supply nutrients and biological activity to soils (Ma et al., 2020).

Compost has however been faced with challenges of nutrient insufficiency despite good nutrient levels of the material used for composting. The low nutrient levels in compost are attributable to the initial quality of manure or losses during composting. The low nutrient quality of the initial manure is brought about by poor upstream manure handling techniques or poor nutrition to cattle (Ayamba et al., 2021). This has necessitated the supplemental application of materials such as fresh leaves and twigs during composting to ensure quality compost (Mwangi et al. 2020; Kauser and Khwairakpam, 2022). Despite studies by many authors on aspects of nutrient level in the product of the composting process, only a handful of studies have focused on the actual succession of these nutrients along the composting period (Meng et al. 2019; Xie et al. 2021).

1.3 Justification

Composting is an essential process in agricultural systems, and various composting durations have been suggested for different experiments using diverse sources of feedstocks (Yang et al. 2021; Mahapatra et al. 2022). It is crucial to determine the maturity of compost to ensure its effectiveness on farms. However, there is a gap in understanding the composting process, as the functional capabilities of compost microbial populations and the impact of green feedstock on these capabilities have not been explored (Yang et al. 2021).

Green materials, such as fresh grass clippings and fresh twigs of *Lantana camara* and *Tithonia diversifolia*, have been used to supplement nitrogen levels in soils and compost (Liu et al. 2018; Antonangelo et al. 2021). However, some of these materials have been reported to have inhibitory properties on prokaryotic, fungal, and non-fungal organisms under *in vitro* studies (Das et al. 2017; Guilabert et al. 2021). The phytochemical composition of *Lantana camara* and *Tithonia diversifolia* has been previously studied. *Lantana camara* has been reported to contain more complex polymers, requiring more microbial categories to break down into agriculturally useful material. For example, the phytochemical composition analysis of *Lantana camara* showed 23.3% crude fiber, 26% cellulose, 16.2% lignin, and 21% hemicellulose, while *Tithonia diversifolia* contains 11.2%, 17%, 7%, and 16% of these elements, respectively (Getachew and Zeleke, 2019; Cadena-Villegas et al. 2020; Dongmo et al. 2021).

It is necessary to identify the strategic metabolic pathways and microbes involved in nutrient cycling, which are critical for composting. In this study, metagenomics and metatranscriptomics were used to analyze nucleic acids extracted from compost samples to determine the community structure and functional potential of prokaryotes and eukaryotes in different manure samples affected by co-composting of green nitrogenous feedstock and composting days. Most studies have focused on the physical-chemical quality of manure after the composting process, neglecting the critical aspect of the changes that occur over time. Biological activity and community structure have not been adequately investigated in the compost ecosystem (Yu et al. 2019; Mazumder et al. 2021).

1.4 Hypotheses

1. The use of different green composting feedstocks and varying composting durations does not significantly affect the physical and chemical characteristics, or the stability, of cattle manure during composting.
2. Different green composting feedstocks and composting durations do not influence the evolution of the prokaryotic community structure during the composting of cattle manure.
3. The evolution of fungal and non-fungal eukaryotic community structures during the composting of cattle manure is not influenced by different green composting feedstocks or composting durations.
4. Green composting feedstocks and composting durations do not have a significant effect on the microbial metabolic potential of compost microbes during the composting of cattle manure.

1.5 Objectives

1.5.1 Main objective

To evaluate the physical-chemical and biological evolution of manure as influenced by thermophilic composting materials and duration.

1.5.2 Specific objectives

i) To evaluate the physical and chemical shifts in manure during composting, focusing on how different green composting feedstocks and varying composting durations affect manure stability.

ii) To investigate the evolution of the prokaryotic community structure during cattle manure composting in response to varying green composting feedstocks and composting durations.

iii) To examine the evolution of fungal and non-fungal eukaryotic community structures during the composting process, influenced by different green composting feedstocks and durations.

iv) To evaluate the metabolic profiles of compost microbes during cattle manure composting, as influenced by green composting materials and composting durations.

1.6 Significance of the study

The significance of this study lies in its comprehensive examination of the composting process, particularly in relation to cattle manure. By addressing both the physical and chemical transformations during composting, the research provides essential insights into how different green feedstocks and composting durations influence manure stability. This is critical for optimizing composting techniques to enhance the quality and environmental sustainability of manure-based fertilizers.

Additionally, the study delves into the evolution of microbial communities, focusing on both prokaryotic and eukaryotic organisms. Understanding how these communities shift in response to feedstocks and composting durations is key to unraveling the biological mechanisms that drive compost maturation. This knowledge could be pivotal in advancing microbial management practices during composting, ultimately improving nutrient recycling and soil health.

Furthermore, by evaluating the metabolic profiles of compost microbes, the study offers valuable data on microbial functions and activities throughout the composting process. These insights could inform the development of targeted composting strategies that enhance microbial activity, leading to more efficient compost production and better-quality end products. Overall, this research has the potential to contribute to more sustainable agricultural practices, reducing reliance on chemical fertilizers and improving soil fertility management.

CHAPTER TWO

LITERATURE REVIEW

2.1 The concept of composting

Composting is a controlled process in which microorganisms transform organic materials into a safe and agriculturally useful product (Wu et al., 2017). The resulting humic substance has an earthy smell and is formed through decomposition and biosynthesis (Ayilara et al., 2020). Composting can be achieved through thermophilic or anaerobic methods, with thermophilic composting being the most commonly used method that involves turning the compost heap to circulate oxygen for organic matter breakdown (Alkoaik, 2019; Soni and Devi, 2020).

The process of organic materials composting can be apportioned into two phases: mineralization and humification (Liu et al., 2021). During the mineralization phase, readily fermentable organic materials such as sugars and amino acids are rapidly broken down by microorganisms (Ayilara et al., 2020). The biosynthesis of complex tri-dimensional polymers occurs through secondary metabolic pathways, including shikimic acid and malonic acid pathways, leading to the formation of humic substance precursors that are further polymerized through enzymatic action (Zhao et al., 2021).

Good agricultural practices recommend supplementing cattle manure with other materials to improve nutrient levels, with supplemental carbon sourced from dry organic matter and nitrogen from green nitrogenous materials, such as leguminous plants (Greff et al. 2022; Matheri et al. 2023a). Non-leguminous materials, such as fresh grass clippings, have also been used as nitrogenous supplements (Khan et al., 2009). Manure sources include poultry

litter, cow manure, swine manure, and wastes from municipal units (Kadir et al. 2016; Cesaro et al. 2019).

The composting process is influenced by several key environmental factors, including oxygen, moisture, pH, temperature, C/N ratio, particle size, and nutrient levels of feedstock material. These factors have a direct impact on the composting duration, nutrient levels, and pathogenic loads (Jain et al., 2019).

2.2 The environmental and economic value of compost

Composting is a sustainable way of converting organic waste into useful agricultural material, which reduces the amount of waste that could harm the environment (Kadir et al., 2016). This process provides economic and environmental benefits, especially for resource-limited farmers in sub-Saharan Africa, by addressing issues such as soil degradation, unemployment, and energy scarcity, as well as reducing greenhouse gas emissions (Galgani et al., 2014).

Furthermore, composting can reduce antibiotics and antibiotic resistomes in manure, which has become a significant environmental and human health concern, by managing antibiotics through xenobiotic degrading microbes that break down foreign materials (Awasthi et al., 2019). Compost also serves as a sink for greenhouse gases such as methane, which ultimately sequesters carbon for application to soil (Chen et al., 2022). Consequently, farmers and compost-producing companies have focused on improving the quality of compost to make it nutritionally competitive with synthetic fertilizers (Ayilara et al., 2020).

2.3 Source of feedstock material used for composting

Composting materials can be broadly classified into three main categories. The first category is plant-based materials, which include grass clippings, shrub leaves, dry grass, wheat straw, rice husks, and woody plant material. The second category is biosolids, which are derived from municipal waste such as sewage sludge. The third category is manure-based compost, which is derived from animal waste such as cow, poultry, or swine manure (Cesaro et al., 2019).

Composting is commonly done using biosolids or manure, which can be composted alone (de Mendonça-Costa et al., 2015). However, manure alone may have low nutrient levels and require plant-based material to improve compost quality (Gao et al. 2015; Mahapatra et al. 2022). The plant-based materials used in composting are classified as carbon-rich (dry grass, maize stalks, wheat straw, and rice husks) or nitrogen-rich (grass clippings and shrub leaves) (Mahapatra et al., 2022). Shrubs like *Tithonia diversifolia* and *Lantana camara* have been recommended and used as nitrogen sources in composting (Mucheru-Muna et al., 2014).

2.4 Description and Geographical Distribution of *Lantana camara* (Linn)

Lantana camara is a thorny shrub growing up to 2-3 m tall and belongs to the family *Verbenaceae:* Figure 2.1. It is indigenous to South and Central America and was introduced to East Africa in the 1950s, where it has been classified as an invasive species that negatively impacts native biodiversity. Additionally, *Lantana camara* poses a serious threat to grazing cattle. However, farmers have adopted this plant as a nitrogen source in farms through the direct addition of leaves to soils or compost, despite its detrimental effect

on the environment such as being an invasive weed (Pfadenhauer et al. 2020; Bukombe et al. 2021; Gillela et al. 2022).

Figure 2. 1: **A photo of *Lantana camara* (Linn).**
Credit:dreamstime.com

The effects of Lantana camara on soil health and mostly the composting process remain understudied, particularly in terms of its influence on microbial dynamics, nutrient cycling, and compost stability compared to other green feedstocks. Additionally, little is known about how varying composting durations affect its role.

2.5 Description and Geographical Distribution of *Tithonia diversifolia* (Hemsl.) A. Gray

Tithonia diversifolia is a flowering plant from the *Asteraceae* family, commonly known as Tithonia or Mexican sunflower; Figure 2.2. It is native to Mexico, Central America, and the West Indies but has now spread to tropical parts of Asia and Africa. The plant was

introduced to Kenya as an ornamental plant and is now commonly found in the Western, Central, and Coastal regions, and parts of Rift Valley, where it is a common shrub on land boundaries and grasslands. Tithonia has agricultural value as a natural source of nitrogen, and its leaves can be used directly on soil or as composting material or plant teas (Ngosong et al. 2015; Matheri et al. 2023a).

Figure 2. 2: **A photo of *Tithonia diversifolia* (Hemsl.) A. Gray.**
Credit: powo. science.

Despite its widespread use, there are notable gaps in understanding the effects of *Tithonia diversifolia* on composting and soil health. Research has yet to fully examine how it influences microbial dynamics, nutrient cycling, and compost stability compared to other materials. Additionally, there is limited insight into how different composting durations affect its interaction with compost properties.

2.6 Physical parameters during composting

2.6.1 Moisture

Moisture plays a critical role in composting as it is essential for the growth and enzymatic activity of microbes that decompose organic matter (Li et al., 2021). In addition to providing a medium for nutrient transport, moisture levels affect the production of leachate, porosity, and oxygen levels in the compost environment (Jain et al., 2019). For optimal decomposition, moisture levels should be maintained between 50% and 70% on a wet basis (Richard et al., 2002). High moisture levels facilitate the attainment and maintenance of high temperatures in the composting heap. Moisture content is a convenient proxy for other factors affecting microbial enzymatic activity (Makan et al., 2013). Extremely low moisture levels can halt biological activity in the compost and result in a physically stable but biologically unstable product, while very high levels can lead to waterlogging and anaerobic conditions, limiting oxygen movement and affecting particle aggregation, porosity, and permeability (Makan et al. 2013; Li et al. 2021).

2.6.2 Temperature

Pile temperature is both an influence of composting and a resultant effect of the process (Matheri et al., 2023b). Temperature arises from the intense microbial activities focused on carbon cycling in the compost ecosystem where CO_2 is also a derivative (Bohacz, 2018). High temperatures are important in the promotion of rapid decomposition of organic matter, weed suppression through the killing of weed seeds, and sanitization by killing disease-causing organisms. The high temperature during thermophilic composting also effectively helps in the decomposition of recalcitrant organic materials such as lignin which are difficult to break down under the natural rotting process (Sołowiej et al., 2021).

There are three main temperature-related phases (thermophilic, cooling, and curing phases) in thermophilic composting (Meena et al. 2021). Some authors include the early brief mesophilic phase (Li et al. 2019; Sołowiej, et al. 2021) while others merge the cooling and curing phases; Figure 2.3. The early mesophilic phase begins immediately after the heaping of material for up to about 8 hours before the thermophilic phase sets in (Liu et al., 2021). The phase is characterized by temperatures closer to ambient temperature (up to 40°C), thus allowing heat-sensitive compost micro and macroorganisms to colonize the heap, albeit briefly (Chinakwe et al., 2019). Such organisms include earthworms, termites, and collembolla among others which actively break down complex material such as lignocellulose, leading to an increase in temperature. These high temperatures are not favorable for the mesophilic organisms hence their populations decline in the compost environment and most of the remaining organisms colonize the peripheries of the heap (Matheri et al., 2023a).

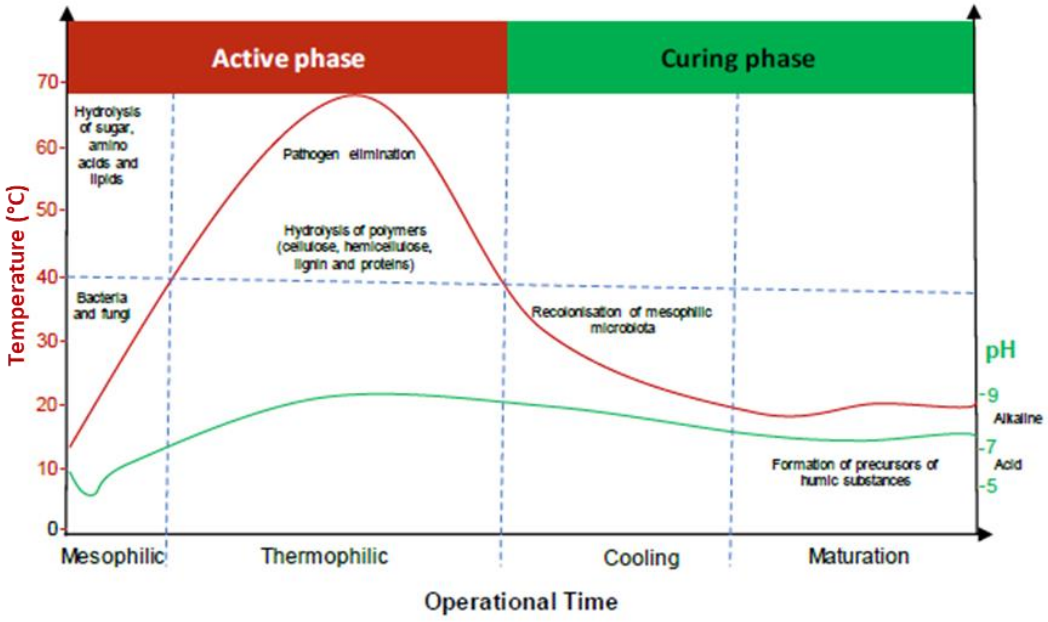


Figure 2. 3: **Temperature profile along the thermophilic composting process of organic waste**
Image adapted from Sánchez et al. (2017).

The pile temperature influences microbial abundance and diversity in the compost environment. Bacteria are the most dominant microbial category in the overall composting process and are dominant during the thermophilic phase. There are however bacterial classes that are sensitive to high temperatures and only colonize during mesophilic phases of the composting process or on the peripheries of the compost pile (Alegbeleye and Sant'Ana, 2020).

Most fungal categories cannot survive the high temperatures in the thermophilic phase of composting and are therefore mostly absent in the pile core and only reside on the cooler outer layers. These fungal categories are present during the earlier brief mesophilic phase and recolonize the compost environment during the cooling and maturation phases (Moreno et al., 2021). Most eukaryotic classes are also mainly heat sensitive and are present in the composting feedstocks and are quickly eliminated during the thermophilic phase of composting only to recolonize the compost environment in the maturation phase. Keeping the pile temperature in the range of 55-65°C during the thermophilic phase provides thermal comfort for thermophilic microbes. This however results in higher CO_2 emissions from higher microbial activity (Sołowiej et al., 2021).

2.6.3 Particle size

The particle size of the organic matter has a direct influence on the composting process as the size of the input material has a direct influence on microbial colonization and enzymatic access (Koniuszewska et al., 2020). The smaller the particles, the larger the surface area and hence the easier and faster access of the material by compost microbes. Conversely, the large particle size of the organic matter will reduce access by microbes and could negatively affect the decomposition rate (Castillo, 2020). Therefore, it is necessary to cut

the feedstock material into small pieces during the preparation of the compost heap. Very small particle size is however not recommended as it will reduce the porosity. The optimum particle size of composting feedstock is recommended to be in the range of 0.3 to 5 cm in diameter (Matheri et al., 2023b).

2.6.4 Aeration

Aeration is a critical factor in thermophilic composting. Oxygen is essential for microbial proliferation as most microbes involved in the thermophilic composting process are obligate aerobes (Castillo, 2020). Oxygen is also critical in the cycling of important soil nutrients which require oxygen for transformation and stabilization. For example, the breakdown of carbon into simpler polymers which are precursors for humic material requires its oxidation to add an oxygen atom or molecule to form oxidized carbon which combines with other elements to form humus (Koniuszewska et al., 2020). Nitrogen in the plant material requires oxidation to form N_2O, NO_2, and ultimately NO_3 which is the nitrogen form that plants can readily absorb (Castillo, 2020). Precisely, higher oxygen levels are required during the early phases of composting, which is the period that microbes especially carbon-metabolizing bacteria breakdown carbon into other forms in the compost environment, producing precursors for humic material biosynthesis (Koniuszewska et al., 2020).

Turning frequency is critical in these early days of composting to ensure oxygen penetration and circulation in the compost heap for ease and sufficient access by the anaerobic microbes. Oxygen uptake rate was found to be most intensive with an increase in pile temperature and was related to the microbial-specific growth rate (Miyatake and Iwabuchi, 2006). Despite the critical influence of oxygen, its monitoring and optimization

in the compost environment are however difficult without having a real-time online gadget to monitor levels in the composting pile (Zheng, et al., 2018).

2.6.5 pH

Compost pH is a critical factor influencing microbial colonization and function hence the decomposition period and the quality of the product. During the early days of thermophilic composting, short-chain organic acids are produced by bacteria and fungi resulting in a decrease in the pH (Sundberg et al., 2004). This decreased pH in the early mesophilic stage provides acidic conditions that particularly active fungi degrade organic matter. During the thermophilic phase that follows shortly, the volatilization of the short-chain organic acids and mineral nitrogen release ammonia which in turn contributes to a progressive increase in pH. The optimal pH for good microbial function and colonization is in the range of 5 to 8, with values outside this range being reported to lead to a decline in microbial activity and prolonged organic matter decomposition (Sundberg et al., 2004).

2.6.6 Germination index as a measure of compost maturity

The germination test is a useful metric in assessing compost maturity and phytotoxicity. The Germination Index (GI) evaluates seed performance by considering germination rate, radicle length, and shoot length. It compares treatments against control, usually distilled water, to assess their impact on seed germination and early plant growth, with longer radicle and shoot lengths indicating better development. Compost with germination indices values above 80% is deemed to have reached maturity and is free from phytotoxins and mature. Studies have shown earlier composting days as having poor germination indices and thus not ready for use in soil (Yang et al., 2021). However, composting material has a clear influence on the maturity of compost.

2.7 Chemical parameters of composting

2.7.1 Mineral composition of organic wastes used for composting

The main components of organic waste are carbohydrates whose building blocks are carbon, hydrogen, and Oxygen molecules. The organic wastes also contain proteins (carbon, hydrogen, oxygen, nitrogen, sulfur, and phosphorous). Proteins are amino acids polymers, with carboxyl and amino groups. Proteins are important because they are utilized by microbes for growth and enzymatic functions (Cesaro et al., 2019). The availability of these two main composting categories in different forms ensures proper microbial growth conditions and ultimately leads to humification and the quality of the product of the process (Castillo, 2020).

2.7.2 Carbon to Nitrogen ratio

The initial ratio of carbon to nitrogen (C: N) is a major factor influencing composting (El-mrini, et al., 2022). This is in turn dictated by the type of composting raw material (feedstock). The international technical standards put the ideal C/N ratio between 20 to 30. A high C/N ratio at the beginning of the composting leads to a slower start of the process and a longer composting period than usual. On the other hand, a low C/N ratio at the beginning of the process will result in high ammonia (NH_3) losses from the compost pile (Vochozka et al., 2017).

The C/N ratio of the raw materials (feedstock) significantly influences the levels of other nutrients such as phosphorous and magnesium and informs the amounts of nitrogen lost during composting (Tripetchkul et al., 2012). The initial C/N ratio also has a major influence on compost maturity (Zhu, 2007). The aggregate C/N ratio of the raw materials

has also been reported to influence the microbial population in the compost environment (Macias-Corral, et al., 2019).

2.8 Microbial ecology during composting

2.8.1 Composting microbiome

For a long time, interactions between microbes were only considered to be inhibitory. Developments in microbiome research have shown that multiple categories of microbes occur and produce different forms of products upon interaction with other microbes and the environment, utilizing a wide scope of valuable facets beyond common antibiosis within environments (Tshikantwa et al., 2018). This trend has led to a deeper understanding of inter and intra-category microbial interaction and roles in colonization and nutrient cycling in ecosystems such as the compost environment.

There are diverse characteristics and functions of these biological categories in the ecosystem. Microorganisms in categories such as bacteria, archaea, fungi, algae, protists, and viruses vary in shape, surface morphologies, feeding preferences, and functional capabilities (Castillo, 2020). These categories form complex interactive webs within the ecosystem and do not exist individually. The interactive webs are among microorganisms within the same, different, or completely different categories such as genera, and species. These intricate microbial interactive webs are either for gain, loss, or neutral to the interacting category thus providing the basis for diversity and interaction patterns (Tshikantwa et al., 2018). For example, bacteria belonging to different taxa can associate, resulting in the formation of a biofilm. This association may ultimately lead to a biological complex that enables individual members of this interaction resistant to antibiotics. Antibiotic resistance has been a key ecosystem bottleneck, especially where antibiotic

resistance genes are cycled within an ecosystem to affect animals and human beings (Tshikantwa et al., 2018).

Biological stability is also a metric for the maturity and stability evaluation of compost. The compost ecosystem is largely an amorphously heterogeneous environment, with diverse feeding guilds and functional categories (Antunes, et al., 2016). Some microbial categories cycle and provide sinks of nutrients by feeding on the composting materials (herbivores) while others feed on other microbes in the compost environment (carnivores). Due to the microbial complexity and diversity, the roles of community members require further investigation into prevailing environmental conditions. These interaction webs are a critical factor in the composting process, compost quality, and safety. Precisely, the intricate associations ensure that a certain microbial category breaks down certain types of material in the compost ecosystem to release compounds that are metabolites for utilization by other categories.

An understanding of how microbial communities interact in the ecosystem in the breakdown of complex organic material such as lignocellulose has industrial importance. Moreover, this understanding of specific ecosystem roles of different microbial categories has the potential to provide biocatalysts that are useful in the conversion of agricultural wastes into industrially important chemicals (Alessi, et al., 2018).

2.8.2 Prokaryotic community in compost

Prokaryotes have been enumerated by most studies as the dominant microbial category during composting. Composting studies have shown some prokaryotic classes as dominant during certain phases of composting. For example, Papale et al. 2021 reported Firmicutes

as the dominant class during the early thermophilic composting phase in coffee compost samples, accounting for most of the total sequences, followed by *Proteobacteria* and *Actinobacteria*. Wang, et al. 2022 also reported similar trends with *Proteobacteria, Bacteroidetes, Firmicutes, Actinobacteria,* and *Chloroflexi* being the predominant prokaryotic communities during composting of dairy waste. This composition is however dependent on the type of composting feedstock. There is therefore the need to examine the influence of green feedstock on the prokaryotic community structure.

2.8.3 Eukaryotic community in compost and role in nutrient cycling

The eukaryotic domain has diverse community structures, and metabolic and association profiles in the compost environment. Fungi are the most prevalent kingdom of the eukaryotic domain in most ecosystems. Fungi perform a vital role in natural nutrient cycling in agroecosystems, by acting as decomposers and arbuscular mycorrhizal mutualists (Yang, et al., 2019). Despite Fungi and other eukaryotes being reported to be heat sensitive and mostly not present during the thermophilic phase of the process; some studies have enumerated some phyla that are present in all stages of composting (Papale et al., 2021). *Ascomycota* for example was reported as the dominant phylum over the composting period, especially in the thermophilic phase (Biyada et al., 2021). Fungi are mainly responsible for compost maturation with higher fungal populations being observed in the mesophilic phases of cooling and maturation i.e. after 56 and 63 of composting (Rouhullah, et al., 2012).

Previous studies based on different composting materials have shown the dominance of certain species of fungi in the composting process. In their study on medical waste, Rouhullah, et al. 2012 reported that *Aspergillus spp, Microsporium spp,* and *Mucor spp*

were the dominant fungal species in compost. The study, however, employed culture-dependent techniques that effectively leave out the unculturable fungal categories. Moreover, there is a need to evaluate the impact of other substrate options such as *Lantana camara*, *Tithonia diversifolia,* and Pennisetum *purpureum* on eukaryotic community's profile.

In their study on the influence of diverse composting material on fungal communities, Neher, et al. 2013 reported that *Pezizomycetes* and *Microascales* were the dominant fungal classes in a compost based on dairy manure. Their study also deduced that hay recipes had higher populations of *Thermomyces, Arthrobotrys*, and *Myriococcum* classes while composts based on hardwood wastes contained a relatively higher population of *Sordariomycetes.* This implies that *Sordariomycetes* are more present during the degradation of recalcitrant organic material such as lignocellulolytic material such as hardwood. The succession of fungi along the composting period points to the composting process following temporal dynamics prompting the degradative succession of composting material. Neher et al. 2013, further pointed out that the initial compost community had *Phycomycetes, Ascomycota,* and *Basidiomycota* as the dominant classes in that order. On the other hand, other studies have shown *Basidiomycota, Ascomycota,* and *Mortierellomycota* were predominant in all treatments and accounted for almost all the population of fungi in the compost; 97.5% (Wang et al., 2022).

Ideally, thermophilic composting is meant to eliminate human and plant pathogenic microorganisms by the end of the composting process. This sanitization of harmful pathogens is done by the high temperatures. Most pathogenic microbes do not survive at

temperatures above 45° and thus achieving pile temperatures above this level is a critical element of this composting method (Mengqi et al., 2021).

Elimination of pathogenic microbes from compost is also achieved through biological processes such as cannibalism and competition by indigenous beneficial microbes. Improper compost management practices such as failure to turn the heap can lead to the final compost product harboring pathogenic microbes along with beneficial ones (Ezugworie et al., 2021). The presence of pathogenic microbes at the beginning of the composting process is however dependent on the source of the feedstock. For example, food waste might contain pathogenic bacteria like *Campylobacter, Shigella, Vibrio,* and *Salmonella* (Awasthi et al., 2019). Wastes from sources such as poultry, associated with *Salmonella spp.* While E. coli can be found in beef waste. On the other hand, sewage sludge has been reported to contain both *Salmonella* spp. and *E. coli* (Fijalkowski et al., 2017).

2.9 By-products of the composting process

The main product of the composting process is the humic substance which is a polymer of organic acids that is rich in nitrogen, carbon, phosphorous, and trace elements as well as inoculum of microbes that are important in nutrient cycling and pathogen management in soils (Ayilara et al., 2020). There are however other products that evolved in the composting process that account for losses of nutrients in the compost ecosystem. These by-products are mainly gaseous losses of carbon dioxide, nitrous oxide, and ammonia, although nutrients are also lost by the leaching of water through the compost pile (Kataya et al., 2023).

Carbon dioxide is lost through the metabolism of carbon in the compost pile, especially from dry matter. This loss points to fewer populations of CO_2 utilizing microbes such as blue-green algae in the compost environment, although the losses are inevitable due to the intensity of the thermophilic phase (Yang et al., 2019). Singh et al. (2018), also showed that the carbon levels decreased during the composting process, and differences in carbon levels were observed among the composting treatments. In another study by Rahman et al. (2017), similar results were reported, where the carbon levels decreased during the composting process, and differences in carbon levels were observed among the composting treatments.

Nitrogen loss like nitrous oxide and ammonia points to the presence or high population of denitrificans which are a category of microbes responsible for nitrogen losses in the compost environment. These micro-organisms metabolize nitrates or precursors of nitrates such as nitrites to nitrous oxide and ultimately ammonia which is a gaseous form of nitrogen (Meng et al., 2019). Rahman et al. (2017) also found no significant differences in nitrogen levels as influenced by composting materials, except for the treatment that used poultry manure, which had higher nitrogen levels compared to the other treatments.

The losses of CO_2, nitrous oxide, and ammonia from the compost environment have been pointed out as the biggest shortcomings of the composting process, leading to nutrient losses and greenhouse gas emission levels from the compost ecosystem. Furthermore, the type of composting process, materials used, and the period of composting can have a significant impact on greenhouse gas emission levels and subsequent loss of carbon and nitrogen in the compost (Wu et al. 2017; Liu et al. 2018). Notably, some studies are

currently focusing on mitigating these losses through the introduction of inert materials such as biochar and gypsum during composting (Meng et al. 2019; Yang et al. 2019).

2.10 Evolution of physical-chemical properties of compost leading to maturity

Most composting studies have shown the first three weeks as the most critical days of nutrient cycling in the aerobic composting process. This has technically been regarded as the rate-limiting stage for microbial and nutrient synergies for successive composting phases. This calls for an investigation of the nutrient and microbial potential at this point since it critically informs the evolution of nutrients in the pile. Vazquez et al. (2020) intimated those 21 days of composting are critical as they were the process "loading" stages where material builds up temperature after which they retain an almost constant high temperature. Mengistu et al. (2018) reported that the evolution of total nitrogen showed a rapid decline in nitrogen levels after 20 days of composting and a gradual rise in subsequent samplings. This was attributed to a resultant concentration effect from the degradation of organic carbon compounds. This in turn leads to a loss of mass and therefore, a relative increase in nitrogen concentration (Antara et al., 2012).

In their study on a mixture of anaerobic digestate (derived from the anaerobic digestion of municipal food waste), green wastes, and a screened compost (green waste/kitchen waste compost), Franke-Whittle et al. (2014) composted for 63 days. Sampling was done in their study at 20 and 63 days showing microbial diversity as a significant driver of nutrient cycles and stability, with nitrogen peak reported at day 63. Steel (2011) also indicated significant shifts in temperatures, especially at around 21 days and 42 days. The following feedstock materials were composted: 43% on a volume-to-volume base (%, v/v) fine wood chips, 43% (v/v) dry hay, and 14% (v/v) fresh grass. Sobratee et al. (2007) also showed

these days among others to record temperature and moisture changes. The study utilized chicken manure (spent broiler litter composting).

2.11 Microbial functional potential in the compost ecosystem

Understanding of functional potential in compost as influenced by diverse composting material is an important tent of composting knowledge. This is informed by known losses of important nutrients during composting. For example, Nitrogen has been listed as one of the most susceptible elements to environmental loss varying between 19% and 77% (Maeda et al., 2011). Most of these losses are majorly aided by microbial action with denitrificans enabling the reverse nitrogen cycle from nitrates to nitrites and for instance ammonia. The deposit of nitrogen-based greenhouse gas ammonia into the environment causes eutrophication. Any form of N losses from agroecosystems is attributable to constrained crop production, soil sustainability, and environmental safety (Nan et al. 2019; Mahmud, et al. 2021).

Carbon loss is also another greenhouse gas concern during composting, with losses mainly in the form of methane (CH_4) and carbon dioxide (CO_2) with carbon dioxide accounting for most of the carbon loss (Nan, et al., 2019). An increase in CH4 losses has been linked to moisture levels above 60%. This is through the creation of anaerobic zones in the compost heap (Ba et al., 2020). There is, therefore, a need to understand the influence of the nitrogenous material on the microbial pathways responsible for the greenhouse gas losses of elements such as nitrogen to inform mitigation measures.

2.12 Evolution of approaches to identify microbiome structure

Ecosystem microbiomes have vast phenotypic and genotypic diversities with most microbes being unculturable using classical microbiological strategies such as growth media cultures. Less than 0.1% of the ecosystem microbes are accessible with these classical approaches (Hill, et al., 2000).

Advances in molecular ecology have seen the development of techniques such as denaturing gradient gel electrophoresis (DGGE) to study diversity based on the PCR amplification of environmental DNA (Wang et al. 2013; Ongagna-Yhombi et al. 2019). In recent years, ecologists have adopted molecular methods that have increased access to the pool of eco-biomes and provided a platform for more in-depth studies. The newer approaches: commonly referred to as high throughput sequencing methods have better resolution and are also capable of working on mixed nucleic acids such as those from environmental systems (Farmer et al., 2017).

One of these methods focuses on the small subunit rRNA genes which are amplified from total-extracted nucleic acids. This method has enabled the detection of microbial rRNA genes directly from environmental samples through sequencing (Allaband et al., 2019). Precisely, these techniques provide enhanced ways of studying environmental microbial diversity. Molecular techniques have undergone improvements over time with scientists adopting newer sequencing platforms. The transformations have seen the change from the pioneer Sanger approach to pyrosequencing, followed by the Illumina platform. Newer sequencing platforms include the Oxford nanopore which has the capacity for long-read sequencing (Allaband et al. 2019; Heng et al. 2020).

2.13 Enzymatic influence on nutrient cycling and microbial survival in compost

Enzymes are critical in microbial processes within composting ecosystems, facilitating the breakdown of organic material and recycling of nutrients, ultimately contributing to compost stabilization. Microbial enzymes catalyze the decomposition of complex organic substances, enhancing nutrient availability and the maturation of compost (Nannipieri et al., 2003). These enzymes fall into six major classes: oxidoreductases, ligases, transferases, hydrolases, isomerases, and lyases (Burns et al., 2013).

Oxidoreductases, such as pyruvate dehydrogenase, play a vital role in electron transfer during the oxidation of organic matter, such as converting pyruvate to acetyl coA. Ligases, exemplified by DNA ligase, catalyze the binding of large molecules, while transferases, like transaminase, transfer functional groups between compounds (Nannipieri et al., 2003). Hydrolases, including enzymes like pepsin, are involved in the hydrolysis of compounds, critical for organic matter degradation during composting (Insam and de Bertoldi, 2007). Isomerases and lyases contribute to rearranging and cleaving molecules, facilitating further degradation of complex organic compounds like lignocellulose.

Despite significant knowledge of these processes, there remains a substantial gap in understanding how microbial metabolic potential, particularly enzyme production, varies across compost feedstocks like Tithonia diversifolia. Studies on microbial communities, such as those involving *Bacillus, Pseudomonas,* and *Aspergillus,* have highlighted the role of enzymes in nutrient cycling during composting, yet the impact of specific green feedstocks and varying compost durations on enzyme activity remains poorly understood (Insam and de Bertoldi, 2007; Awasthi et al. 2019).

2.14 Advances in ecological sequence data exploration

Bioinformatics pipelines offer ecologists a platform to pre-process sequence data and visualize important aspects of diversity. Such pipelines include *obiclean*, DADA2, SWARM, VSEARCH, LULU, and QIIME for DNA-based sequences. On the other hand, workflows such as SAMSA, METAPRO, and HUMANn are available to ecologists for the exploration of messenger RNA-based diversity and metabolic profiles. Advancements in existing workflows have seen the availability of superior functions such as chimera removal steps in programs such as VSEARCH and DADA2. Furthermore, newer approaches to these workflows have helped reduce errors by adding workflow steps that filter based on abundance (Sigsgaard et al., 2020).

Messenger RNA sequence data workflows (metatranscriptomics) have also been improved in the recent past and have helped unravel intricate metabolic potential in the ecosystems. However, utilization of these workflows has been faced with challenges such as the requirement of high computational power. To overcome this, ecologists are developing pipelines that require less computational space (Taj et al., 2021), although this has not been largely achieved. Furthermore, metatranscriptomics has been faced with the challenge of high costs associated with the sequencing of mRNA. To overcome this, ecologists have developed pipelines that enable functional prediction based on metagenomic sequences. This has however been only developed and validated based on 16S targeted sequences or near full genome sequences of prokaryotes. Such pipelines include the Phylogenetic Investigation of Communities by Reconstruction of Unobserved State (PICRUSt2) which has been widely utilized to explore the functional capabilities of environmental DNA relying on 16S rRNA gene sequences (Douglas et al., 2020).

2.15 Association networks in ecosystems

There are assorted interaction patterns and strengths of different microbial communities informed by feeding and metabolic guilds. The degradation of complex material such as lignocellulose in ecosystems like compost is driven by a synergistic microbial action of oxidative and hydrolytic enzymes that break the linkages within the material (Wu et al. 2017). Some microbial categories are involved in upstream metabolic actions that release metabolites utilized by other microbes in the ecosystem. This process requires a variety of interactions among different categories of microorganisms. For example, fungi are known to be upstream eco-degraders in ecosystems such as compost environments.

The major role of fungi and other eukaryotes in compost is the degradation of recalcitrant materials such as cellulose and lignin, breaking open these complex materials and exposing simpler forms that are degradable by prokaryotes. Through this degradation of recalcitrant carbon-rich material, these microbes contribute to carbon cycling in the compost environment. Specifically, fungi break down complex plant polymers enabling bacteria to continue with the decomposition process (Antonangelo et al., 2021).

CHAPTER THREE

GENERAL METHODOLOGY

3.1 Field site and compost treatments rationale

The field experiment (Compost preparation and heaping) was done at the Longterm Farming Systems Comparison in the Tropics; Kenya (SysCom Kenya) site at Thika, Kenya (01˚0.231' S 37˚04.747' E) which hosts the long-term farming systems comparison trials that have been ongoing since 2007 (SysCom; www.system-comparison.fibl.org; Karanja et al. 2020).

Treatment design was done as per Table 3.1 with the source of green composting feedstock adopted from common farmer practice in the East African region and availability (Kihanda, 2000; Adamtey et al. 2016). Each heap involved 400kg of cattle manure, 200kg of dry maize stalk, and 100kg of green matter (Lantana/Tithonia/Grass/mixture). *Pennisetum purpureum* is the grass species that was used in the experiment. A ratio of 4:2:1 was adopted for cattle manure: dry maize stalks: green materials. This ratio was used as per standard farmer's practice in Kenya.

Table 3. 1: **Compost preparation, experimental treatments, and ratios of associated feedstock.**

Source of variation	Feedstock	Ratio	Treatment/sample description
Lantana (L)	Fresh cow dung manure, dry maize stalks, Lantana twigs	(4:2:1 w/w)	L1- Lantana-based compost at 21 days of composting L2- Lantana-based compost at 42 days of composting L3- Lantana-based compost at 63 days of composting L4- Lantana-based compost at 84 days of composting
Tithonia (T)	Fresh cow dung manure, dry maize stalks, Tithonia twigs	(4:2:1 w/w)	T1- Tithonia-based compost at 21 days of composting T2- Tithonia-based compost at 42 days of composting T3- Tithonia-based compost at 63 days of composting T4- Tithonia-based compost at 84 days of composting
Grass (G)	Fresh cow dung manure, dry maize stalks, Grass clippings	(4:2:1 w/w)	G1- Grass-based compost at 21 days of composting G2- Grass-based compost at 42 days of composting G3- Grass-based compost at 63 days of composting G4- Grass-based compost at 84 days of composting
Lantana, Grass, Tithonia (LTG)	Fresh cow dung manure, dry maize stalks, Lantana twigs, tithonia twigs, and Grass clippings (in the ratio of 1:1:1)	(4:2:1 w/w)	LTG1- Lantana + Grass + Tithonia-based compost at 21 days of composting LTG2- Lantana + Grass + Tithonia-based compost at 42 days of composting LTG3- Lantana + Grass + Tithonia-based compost at 63 days of composting LTG4- Lantana + Grass + Tithonia-based compost at 84 days of composting

Wood ash (1kg) and soil (5kg) were sprinkled after every layer was heaped

Therefore, the sources of variation in this experiment were;

1. The source of green nitrogenous composting material (Lantana, Tithonia, grass, and mixed/combination (Lantana + Tithonia + Grass) and 2. Composting days (21, 42, 63, and 84).

3.2 Experimental setup and sampling procedure

A completely randomized block design was used to set up triplicate heaps of each nitrogenous material-based treatment. Heaps were prepared on a flat, sheltered composting surface, devoid of direct sunshine and surface run-off. A sharp, precleaned shovel, was used to individually sample from each of the windrow piles from the peak of the pile down to the bottom as described by Matheri et al. (2023a).

CHAPTER FOUR

THE VARIABILITY OF COMPOST PHYSICAL-CHEMICAL CHARACTERISTICS AS INFLUENCED BY FEEDSTOCK AND COMPOSTING TIME

4.1 Introduction

Nitrogen, phosphorus, and potassium are essential nutrients that are enriched by adding green matter during the composting process of cattle manure. Typically, green materials such as *Tithonia diversifolia*, *Lantana camara*, and grass are co-composted with cattle manure and dry matter by combining them in heaps. However, green materials decompose more rapidly, with *Tithonia diversifolia* breaking down within ten to thirty days, in contrast to the slower decomposition of carbon-rich dry matter. Understanding how these green materials influence the physical-chemical properties of compost can enhance nutrient availability and improve compost quality (Matheri et al. 2023a; Matheri et al. 2023b).

The limiting parameters for microbial establishment and activity are related to the physicochemical nature of compost such as carbon-to-nitrogen ratio, ambient temperature, and pH (Gómez-Silván et al., 2020). Therefore, variation in the quality and nutrient level of feedstock material directly influences microbial diversity, structure, and activity; eventually leading to variation in the product of the process (Chandna et al., 2013). Among the physicochemical properties influencing composting, temperature reflects microbial metabolism within a thermophilic compost heap (Holman et al., 2016). Naturally, aerobic compost piles gradually heat up to temperatures above 50°C. This is followed by sustained high temperatures above 60°C and a final gradual heap cooling (Antunes et al., 2016).

4.2 Materials and Methods

4.2.1 Collection of samples for physical-chemical characterization

Daily pile temperature data were collected at 10 a.m from each triplicate pile per compost treatment with a compost thermometer (model: WIKA 110824862-EN 13190). The thermometer was inserted at three locations. The length of the thermometer was inserted into the pile from the top and through the sides towards the core of the compost heap to the maximum probe depth (45 cm). Recording of this data was done over the 84 days of composting.

The pH of each compost sample was measured by first sieving ground compost samples, air-drying, and passing through a two-millimeter sieve. Thereafter, 10.0 g of the ground samples were put into a 50 ml beaker, and 50 ml distilled water was added. This was followed by stirring the suspension several times with a glass rod for ten minutes and being allowed to stand for 30 minutes. Two buffer solutions with pH 4 and pH 7 were used to standardize the pH meter. The buffer solution of pH 4 was prepared by dissolving 19.12 g potassium hydrogen phthalate; previously dried for 2hrs at 110 ºC and volume made to 1.0 L with deionized water. The pH 7 buffer was prepared by dissolving 2.721 g potassium dihydrogen phosphate; previously dried at 130 ºC for 2hrs and 3.904 g anhydrous disodium hydrogen phosphate; previously dried at 130 ºC for 2hr topped to 1.0 L with deionized water. The electrode of the pH meter was dipped into the partly settled suspension (supernatant) and the pH for the various samples was measured one after the other (Adamtey, 2005).

On the other hand, for moisture quantification, 100 g of fresh samples from each compost treatment and sampling time were put on moisture cans and oven-dried at 108 ºC for 72

hours when an initial dry weight was recorded. The samples were then dried for a further 24 hours, and a final dry weight was recorded.

The germination index of various compost samples was done using tomato seeds because they germinate early (within three days). Moreover, tomato seeds have been reported to have a wider range of tolerance to ammonia toxicity compared to other available plant species such as cabbage, cucumber, spinach, onion, and kale (Adamtey, 2005). The samples were air-dried and passed through a 2.0 mm sieve after which, twenty (20) grams of each of the samples were put into a bottle containing 50ml of distilled water and shaken for one hour. The resultant solutions were then filtered under Whatman paper number 42 and replicated three times per extract. Afterward, twenty viable tomato seeds were placed in 50mm Petri dishes lined with filter papers for each sample and labeled accordingly. Two milliliters of filtrate from each sample solution were pipetted into each of the corresponding Petri- dishes with distilled water being used for the control treatment. The treated seeds were then placed under room conditions in the laboratory.

Germination was stopped on the third day by adding 1ml 50% ethanol to each of the dishes, followed by counting the germinated seeds and measuring the length of the roots (radicle). Seeds that failed to germinate were regarded to have zero root length. The germination index was calculated by multiplying the number of germinated and root growth, both expressed in percent values (Adamtey, 2005).

$$\text{Germination index} = \frac{\text{mean germination in treatment x mean root length in treatment}}{\text{mean germination in distilled water x mean root length in distilled water}} \times 100$$

4.2.2 Carbon dioxide evolution measurement

The laboratory setup was done at the Kenya Agricultural and Livestock Research Organisation (KALRO); Muguga centre's, Soil chemistry laboratories. Measurement of carbon dioxide emission during sampling days (mg CO_2 g-1d-1) was done by measuring 20 g of each compost sample into a beaker and moistening it to about 60% moisture. Twenty (20) ml of 2M NaOH were then put into a 50 ml beaker and placed together with the moist compost into a bigger container and covered and incubated at 25ºC for 24 hours. A control was similarly set up but with distilled water replacing compost. After incubation, 10-20 ml of $BaCl_2$ were added to the NaOH in the beaker. Then, the precipitate was left to settle after which, titration was done against 2M HCl using a phenolphthalein indicator (Adamtey, 2005).

The calculation of CO_2 was as per the following equation:

A milligram of CO_2 evolved per day = (B-V) NE weight of the sample

where B= volume (millilitres) of acid is needed to titrate the NaOH in the blank jar to the endpoint.

V= volume (millilitres) of the acid needed to titrate the NaOH in the jars exposed to the compost.

M= Molarity of the acid.

E= Equivalent weight

The total nitrogen, phosphorous, and potassium amounts of the different compost treatments were analyzed by first grinding 10 g of each sample. Thereafter, 0.3 g of each sample was individually completely digested with 4.4 ml of a digestion mixture. Total N

was determined by Kjeldahl distillation and titration (Okalebo et al., 2002), phosphorus, and potassium (K) by an Atomic Absorption Spectrophotometer (Okalebo et al., 2002).

4.2.3 Statistical analysis of physical-chemical parameters

All statistical analysis was conducted under R software version 4.3.1 (R core team, 2023). The resulting measurements of physical-chemical analysis were individually subjected to a normality test using the Shapiro test before means separation using ANOVA under the agricolae package (version 1.3-5). Comparing the compost treatments was done per composting day for each physical-chemical parameter, followed by a Tukey's HSD posthoc analysis. Principle component analysis to show relationships among the physical-chemical parameters was done by first standardizing the raw data using the "decostand" function in the Vegan version 2.6-2 package. Then the Euclidean distance between samples was calculated based on the principal component 1 (PC1) and principal component 2 (PC2) values. The permutation analysis of variance (PERMANOVA) under Adonis was used to calculate the significance and R2 of the sample PC1 and PC2 distribution according to compost treatment and composting days before plotting. This was followed by the computation of the Pearson correlation matrix and plotting using the function "corrplot" in the Corrplot package (version 0.92).

Triplicate daily temperature readings were entered in an MS Excel spreadsheet and means were computed before plotting the daily temperature curves of the different compost treatments. Where the date of compost sampling for physical-chemical and molecular analysis coincided with turning day, compost turning was done after sampling was done.

4.3 Results

4.3.1 The evolution of the physical-chemical state of cattle manure during composting

There were no significant differences (P> 0.05) in pH among compost treatments during the early stages of composting (21 days). Still, apparent differences were reported at the end of the composting period (84 days). Grass-based compost (G) had the highest pH (8.72) among the different compost types at the end of the composting period (Table 4.1).

Carbon levels decreased during the composting period. Differences in carbon levels in the composting treatments were observed from the initial to later stages. There were no differences (P> 0.05) among the various treatments regarding nitrogen levels at the end of the composting process. However, Lantana-based compost (L) recorded higher nitrogen levels (0.60%) compared to other treatments (Table 4.1).

Nitrogen levels were highest in Tithonia-based compost at 21 days of composting (0.97%). However, this was not significantly different (P> 0.05) compared to Lantana and mixed (LTG) composts. The regimen, however, recorded the lowest nitrogen levels at 42 days and the highest decline in nitrogen levels (0.97% at 21 days to 0.59% at 42 days), Table 4.1.

Temperature and moisture levels were not significantly different among the composting treatments at 21, 63, and 84 days. Significant differences were recorded for these two parameters at 42 days, with mixed compost (LTG) recording the highest temperature levels (26.3 °C). Tithonia-based compost had the highest moisture content at 42 days of composting (51.6%), which was not significantly different from that of Lantana-based compost (51.1%), Table 4.1.

Table 4. 1: **Physical-chemical characteristics of compost treatments on various composting days.**

Parameter	Sampling Time (day)	G	L	LTG	T	Significance
pH	21	8.41^{a}	8.50^{a}	8.48^{a}	8.48^{a}	**Ns**
	42	8.95^{ab}	9.00^{a}	8.90^{b}	8.99^{a}	**
	63	8.37^{a}	8.36^{a}	8.25^{a}	8.47^{a}	**Ns**
	84	8.72^{a}	8.62^{c}	8.55^{d}	8.67^{b}	***
Organic Carbon (%)	21	22.73^{c}	28.00^{a}	26.40^{b}	25.77^{b}	***
	42	17.67^{a}	17.17^{ab}	16.07^{bc}	15.33^{c}	**
	63	16.40^{a}	15.57^{ab}	15.20^{ab}	14.47^{b}	*
	84	13.75^{a}	13.67^{a}	12.87^{c}	13.27^{b}	***
Potassium (%)	21	1.42^{b}	1.52^{b}	1.58^{ab}	1.78^{a}	**
	42	1.26^{a}	1.16^{b}	1.19^{ab}	1.21^{ab}	*
	63	1.07^{a}	1.10^{a}	1.10^{a}	1.09^{a}	**Ns**
	84	1.09^{a}	1.04^{b}	1.01^{b}	1.09^{a}	***
Total Nitrogen (%)	21	0.77^{b}	0.96^{a}	0.82^{ab}	0.97^{ab}	*
	42	0.75^{a}	0.68^{ab}	0.67^{ab}	0.59^{b}	*
	63	0.62^{a}	0.58^{a}	0.59^{a}	0.49^{a}	**Ns**
	84	0.55^{a}	0.60^{a}	0.54^{a}	0.55^{a}	**Ns**
Total Phosphorous (%)	21	0.18^{b}	0.21^{ab}	0.24^{a}	0.22^{ab}	*
	42	0.18^{a}	0.14^{b}	0.13^{b}	0.14^{b}	***
	63	0.16^{a}	0.15^{a}	0.15^{a}	0.15^{a}	**Ns**
	84	0.17^{a}	0.16^{b}	0.14^{c}	0.16^{ab}	***
Temperature (°C)	21	41.3^{a}	39.7^{a}	38.3^{a}	38.3^{a}	Ns
	42	24.3^{ab}	23.0^{b}	26.3^{a}	25.0^{ab}	**
	63	25.0^{a}	25.0^{a}	25.0^{a}	27.0^{a}	ns

	84	22.7[a]	22.7[a]	23.0[a]	22.3[a]	ns
Moisture Content (%)	21	72.2[a]	73.9[a]	70.9[a]	72.3[a]	ns
	42	46.7[b]	51.1[a]	44.9[c]	51.6[a]	***
	63	17.1[a]	18.8[a]	19.6[a]	18.8[a]	ns
	84	18.3[a]	19.3[a]	19.3[a]	19.8[a]	ns

Values with the same superscripts were not significantly different, ns-not significant; * P ≤ 0.05; ** P ≤ 0.01 and *** P ≤ 0.001. G is Grass-based compost; L is Lantana-based compost; T is Tithonia-based compost; LTG is the compost-based mixed green materials (Lantana, Tithonia, and Grass).

Physical-chemical status of different compost samples revealed significantly distinct compost properties for the assorted feedstock and composting days (p-value < 0.05). The first principal component (PC1) contributed to 41.7% of the total variance while, the second principal component (PC2), contributed 22.1%. Together, the first two principal components accounted for 69.8% of the total physical-chemical variation of different compost treatments. Notably, day 21 of composting was highly distinct from other composting days. The most variability within groups was recorded in the Tithonia-based compost and composting day 42; Figures 4.1A and B. On the other hand, most compost physical-chemical properties were correlated. The compost temperature was positively correlated with Carbon, phosphorus, and nitrogen but negatively correlated with pH and Nitrates (Figure 4.1C). The daily temperature plot showed that all compost treatments achieved temperatures of 70° on the 5th day of composting. Notably, all compost treatments consistently maintained a temperature near ambient beginning the 75th day of composting. Moreover, all compost treatments attained the pathogen sanitization temperature (40°C) by the 21st day of composting (Figure 4.1D).

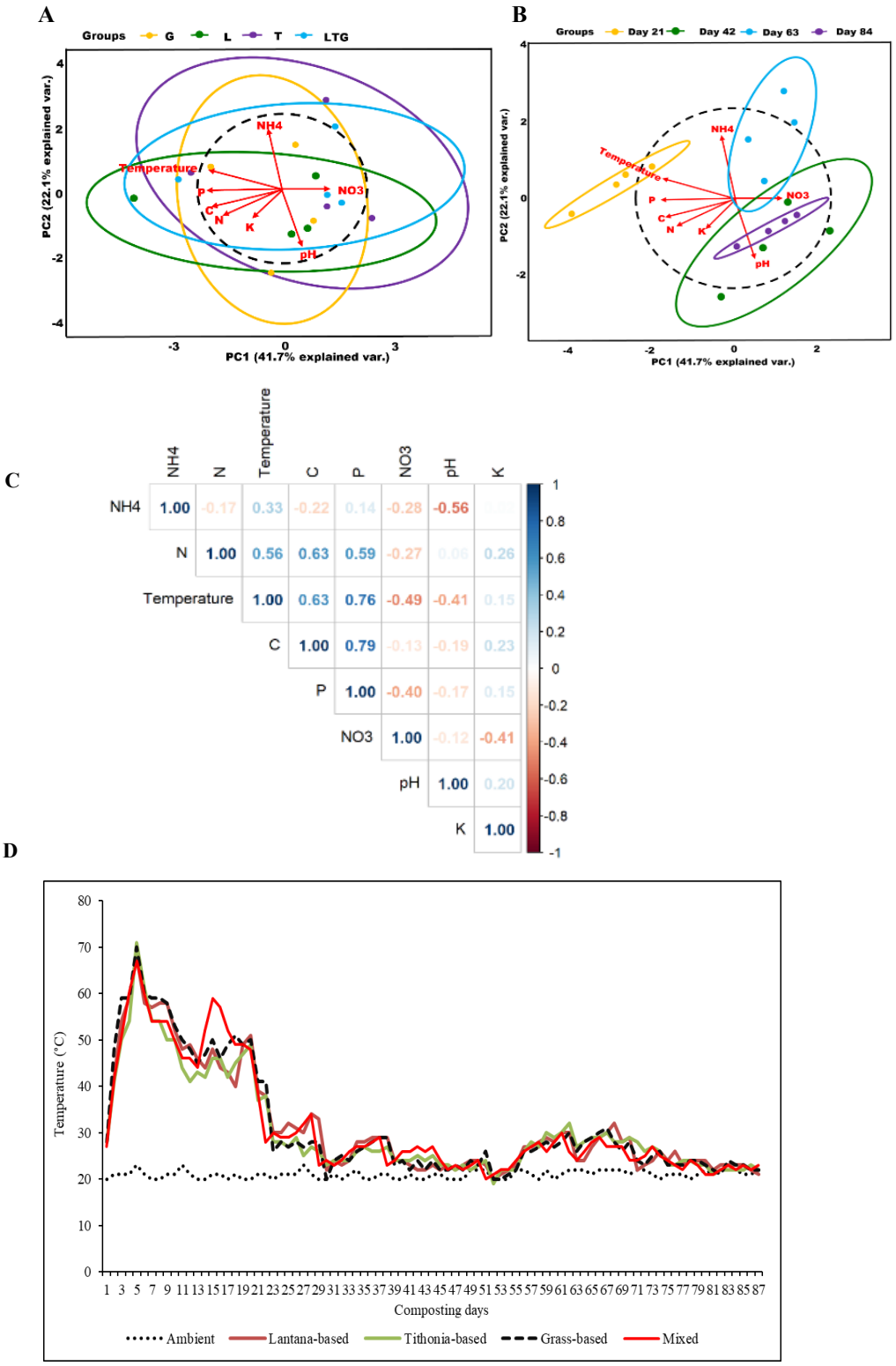


Figure 4. 1: **Principal component analysis (PCA) biplots of compost.** *(A)- compost treatments and (B)- composting days. Pearson correlation matrix between different physical-chemical variables (C); Daily temperature plots of respective compost treatments (D)*

4.4 Discussion

Composting has been associated with nitrogen losses due to the related high temperatures despite the characteristic high nitrogen content in composting materials such as Tithonia and Lantana (Rameshwar and Argaw, 2016). The high loss of total nitrogen in the compost environment is attributable to the high pile temperatures.

The results from this indicate a notable decrease in carbon levels throughout the composting period, suggesting effective decomposition across all treatments. While there were no significant differences in nitrogen levels at the end of the composting process ($P > 0.05$), Lantana-based compost emerged with the highest nitrogen concentration at 0.60%, highlighting its potential as a nutrient-rich amendment (Matheri et al., 2023b). Interestingly, Tithonia-based compost initially recorded the highest nitrogen levels at 21 days (0.97%), although this was not statistically different from Lantana and mixed composts. By 42 days, Tithonia-based compost experienced the greatest decline in nitrogen content, dropping to 0.59%, indicating a rapid depletion of available nitrogen in this treatment.

Temperature and moisture levels remained largely consistent across treatments, except at 42 days, where mixed compost (LTG) exhibited the highest temperature (26.3 °C). Additionally, Tithonia-based compost maintained the highest moisture content at this stage (51.6%), closely followed by Lantana-based compost (51.1%). These findings suggest that while Tithonia shows promise for early nitrogen retention, the dynamics of nutrient availability may vary significantly over the composting period, necessitating further investigation into the optimal management of these materials for enhanced compost quality (Matheri et al., 2023b).

Physical-chemical conditions are indicators of the humification rate that ultimately bring biological and nutritional stability to compost (Estrella-González et al., 2020). Humification is preceded by rapid biogeochemical phases which are microbially driven, breaking down complex polymers into organic acids (Zhu et al., 2021). The high variability within Tithonia-based compost can be attributed to the significant physical-chemical changes within this treatment compared to other treatments along the composting process. The strong positive correlation between factors such as Carbon and temperature points to the co-dependence of these two elements in the physical-chemical nature of compost. The breakdown of Carbon by microbes leads to a temperature increase in the compost environment (López et al., 2021). This temperature increase is responsible for nutrient mineralization and, ultimately, humification of compost (Estrella-González et al., 2020). The attainment of pathogen sanitization temperatures (40°C) by all compost treatments affirms the overall suitability of the technology in the management of pathogens in manure and the environment (Manyi-Loh et al., 2016). This also likely coincides with the highest turnover of carbon which prompts such temperature rise.

CHAPTER FIVE

IDENTIFICATION OF POPULATIONS AND DIVERSITY OF DIFFERENT COMPOST PROKARYOTIC TAXA AS INFLUENCED BY COMPOSTING FEEDSTOCK AND DAYS

5.1 Introduction

Composting is a natural process that involves the transformation of organic matter into a nutrient-rich soil amendment. This process is facilitated by a diverse community of microorganisms, including prokaryotes. Prokaryotes are unicellular organisms that lack a membrane-bound nucleus and are ubiquitous in compost (Sun et al., 2021). The diversity of prokaryotes within compost is important to understand, as it can provide insight into the ecosystem's functioning and potential applications in agriculture and waste management.

Several studies have investigated the prokaryotic diversity within compost using molecular techniques such as 16S rRNA gene sequencing. For example, Wang et al. (2020) identified a high abundance of *Firmicutes* and *Proteobacteria* (*Pseudomonadota*) in compost using 16S rRNA gene sequencing. Another study by Sun et al. (2021) analyzed the microbial community structure of compost samples using high-throughput sequencing and found that the dominant phyla were *Proteobacteria*, *Actinobacteria*, and *Firmicutes*.

Several studies have investigated the influence of the type and nature of composting materials on the prokaryotic community in composting systems. In one study, Liu et al. (2021) analyzed the bacterial community structure and diversity in composting systems using different feedstocks, including food waste, garden waste, and sewage sludge. The study found that the bacterial community structure varied significantly between different

feedstocks, with food waste compost having the highest bacterial diversity and sewage sludge compost having the lowest bacterial diversity.

Similarly, Al-Kaabi et al. (2021) investigated the impact of using different feedstocks, including date palm waste and vegetable waste, on the microbial community in composting systems. The study found that the bacterial community composition varied significantly between different feedstocks, with the date palm waste compost having a higher bacterial diversity and richness than the vegetable waste compost.

The type and nature of composting materials can also affect the abundance of specific prokaryotic groups. For example, in a study by Zhao et al. (2020), the abundance of *Actinobacteria* was significantly higher in chicken manure compost than in swine manure compost, while *Firmicutes* were more abundant in swine manure compost than in chicken manure compost.

On the other hand, several studies have investigated the influence of composting time on the prokaryotic community in composting systems. For example, Antunes et al. (2016), found that the bacterial community structure and diversity changed significantly during the composting process, with the relative abundance of *Proteobacteria, Actinobacteria*, and *Firmicutes* increasing significantly during the initial stage of composting and decreasing during the later stages in a 90-day composting period.

Chen et al. (2022), found that the bacterial community structure and diversity changed significantly during the composting process, with the relative abundance of *Firmicutes* and *Proteobacteria* decreasing, while the relative abundance of *Actinobacteria* and

Bacteroidetes increased during the later stages of composting over a 180-day composting period.

In addition to changing the prokaryotic community structure, composting time can also affect the functional diversity of the prokaryotic community. Sun et al. (2021), found that the functional genes in pig manure composting were changing over the composting period. The study reported that the relative abundance of nitrogen cycling genes increased during the initial stage of composting and decreased during the later stages.

Canonical correspondence analysis (CCA) is a useful tool for investigating the influence of physical and chemical parameters on prokaryotic communities in composting. Several studies have shown that temperature, pH, moisture, and the C/N ratio are the most important factors that shape microbial community structures during composting. According to Li et al. (2019), CCA was used to analyze the effect of physical and chemical parameters on prokaryotic communities in composting. The study found that temperature, pH, and the C/N ratio were the main factors influencing the composition of prokaryotic communities in composting. Zhang et al. (2019) also used CCA to investigate the impact of physicochemical parameters on bacterial and fungal communities during composting. The results showed that moisture, temperature, and pH were the main factors affecting the microbial communities during composting.

Furthermore, Sun et al. (2019) applied CCA to explore the influence of temperature, pH, moisture, and the C/N ratio on bacterial communities in vermicomposting. The study revealed that temperature and pH were the most significant factors that shaped the bacterial community structure during vermicomposting. Additionally, moisture and the C/N ratio

were also found to have a significant impact on the bacterial community. The diversity of prokaryotes in compost has significance in understanding the composting process and identifying factors that affect microbial community structure. In addition, this knowledge can lead to the development of more efficient and sustainable composting practices, as well as potential applications in agriculture, such as enhancing soil health and plant growth.

To explore the prokaryotic diversity in compost from different sources, this study used 16S rRNA gene sequencing to identify the dominant bacterial phyla and genera in each sample. Environmental factors, including temperature, moisture, and nutrient availability, were also evaluated for their influence on the microbial community structure. The findings of this study aid in the comprehension of the prokaryotic diversity within the compost and its potential implications in agriculture and waste management.

5.2 Materials and Methods

5.2.1 Sampling for 16S rRNA gene analysis of compost prokaryotic communities

Compost samples for 16S rRNA extraction were collected simultaneously with those for the physical-chemical analysis described above (at 21, 42, 63, and 84 days of composting). Samples were collected from five different positions of each compost heap using a sharp shovel that was, pre-cleaned with 70% ethanol, making pre-measured cuts into the windrow pile from the peak of the pile down to the bottom (Matheri et al., 2023a). The samples were put into sterile 200g containers and transported to SysCom Kenya's sample room at icipe for storage at -20°C before total DNA extraction at the Kenyatta University Plant transformation laboratories, Kenyatta University.

5.2.2 DNA Extraction and Amplification

Total compost DNA was extracted from triplicate compost subsamples per treatment replicate. Extraction was done using the PureLink™ Microbiome DNA Purification Kit (ThermoFisher Scientific®) as per the manufacturer's instructions. DNA quality and concentration per sample were confirmed using NanoDrop (Maestrogen) and visually under 2% agarose gel. The extracted compost DNA was shipped under dry ice to the Molecular Research DNA Lab (www.mrdnalab.com, Shallowater, TX, USA) for downstream processing.

The 16S rRNA gene V4 variable region PCR primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (GGACTACNVGGGTWTCTAAT) were used in PCR using the HotStarTaq Plus Master Mix Kit (Qiagen, USA). The following conditions were used for 16S rRNA gene amplification: Initial denaturation heating at 95 °C for 5 minutes, followed by 30 cycles of denaturation at 95 °C for 30 seconds, annealing at 53 °C for 40 seconds, and extension at 72 °C for 1 minute, and final elongation at 72 °C for 10 minutes. After amplification, PCR products were checked in 2% agarose gel to determine the amplification success and the bands' relative intensity. Equimolar quantities of PCR amplicons obtained from 16 individual composts were multiplexed using unique indices, pooled, and sequenced using Illumina MiSeq next-generation technology at MR DNA (www.mrdnalab.com, Shallowater, TX, USA).

5.2.3 Bioinformatics and statistical analysis of 16S rRNA sequence data

After sequencing, barcodes and amplicon primer sequences were trimmed, after which low-quality sequences were denoised and filtered out. Reads with <200 base pairs after

phred20-based quality trimming, sequences with ambiguous base calls, and those with homopolymer runs exceeding 5bp were removed (Reeder et al., 2010).

The raw 16S rRNA sequences were submitted to the NCBI sequence read archive with accession number PRJNA822850; (https://www.ncbi.nlm.nih.gov/sra/PRJNA822850). The sequence data were analyzed in R (version 4.3.1), Divisive Amplicon Denoising Algorithm 2 (DADA2) version 1.20.0 workflow (López et al., 2021). The "filterAndTrim" function in DADA2 was used to filter the reads (Schloss, 2020), after which error rates for each sample were computed. Subsequently, the "derepFastq" function was used to dereplicate the reads inferring associated amplicon sequence variants (ASVs) and their abundance. Spurious sequence variants were eliminated by merging forward and reverse reads and removing chimeric ASVs using the "mergePairs" and "removeChimeraDenovo" functions (López et al., 2021).

Finally, the "assignTaxonomy" function utilized the SILVA (version 138) reference database obtained from zenodo to assign ASVs to corresponding taxonomies. ASVs are amplicons resulting from the removal of amplification and sequencing errors. They distinguish the variation of sequences by a single nucleotide change (López et al. 2021, Schloss, 2020). Further, classification was done using the Refseq-ribosomal database project (RDP) (Quast et al., 2021). The Refseq-RDP classifications were combined with those from SILVA 138 using the "cbind" function. Multiple sequence alignment was done on ASV sequences not classified to genus level using the "AlignSeqs" function (Prodan et al., 2020). The taxonomy table was merged with the abundance table.

Taxa prevalence was done based on the absolute abundance data to determine the predominant taxa present in the compost samples. The taxa prevalence was plotted by taxonomic sub-setting of phyla counts using the "subset" function followed by plotting using "ggplot" function in the ggplot2 package. The relative abundance and bar plots of the top twenty most abundant prokaryotic classes were plotted using the phyloseq package (version 1.36.0). A table of all prokaryotic genera in the compost samples was also output using the "write.table" function.

Alpha diversity estimate was done from a rarefied phyloseq object, using "Observed" "Shannon", and "Simpson" metrics to determine the diversity and richness of the four compost treatments and sampling days. The Shapiro and significance tests were calculated before plotting the alpha diversity metrics. Venn diagrams were used to estimate the beta diversity among composting treatments and days. Key environmental drivers of prokaryotic communities at the class level were also determined by computing the CCA using the "plot" function of the vegan 2.6-2 package.

The absolute abundance data were used to construct an undirected co-expression network of compost microbes. Using Pearson correlation, this was done by establishing positive co-occurring microbes from the OTU count table and taxonomy file. Then, the Fruchterman Reingold layout algorithm was used for network layouts, such as node placement, before plotting using the "plot_net" function in the ggplot2 package (version 3.3.5) (Prodan et al., 2020).

5.3 Results

5.3.1 Prokaryotic biodiversity and community structures

Preprocessing of the sequence FASTQ files resulted in plots of adapter content, overrepresented sequences, per base N content, mean quality scores, per sequence GC content, per sequence quality scores, sequence counts, sequence duplication levels, sequence length distribution (Appendices 1-8). After demultiplexing, quality filtering, denoising, and chimera removal, 4,198,194 high-quality reads were obtained from 5,957,427 16S rRNA reads (Appendix 9) all the compost samples, which generated a total of 1813 ASVs. Ninety-nine percent (99%) of the resultant ASVs had a sequence length of about 250bp (Appendix 10). Out of these reads, grass (G), lantana (L), tithonia (T), and their combination (LTG) based compost accounted for 622573, 802660, 646500, and 666617 ASV copies, respectively. A total of 25 Phyla, 51 classes, 246 families, and 338 genera were recovered from the 16S rRNA amplicon sequence variants.

Phylum-level classification of prokaryotic communities using the feature prevalence of the 16S datasets of the compost samples showed the overall distribution of prokaryotic phyla among various samples (Figure 5.1). Each point on the plot corresponds to a different or unique taxon per prokaryotic phylum, within the number of samples (x-axis). In this study, *Entotheonellaeota, Halanaerobiaeota, Fermentibacterota*, and T*hermotogota* only appeared once in less than two compost samples. Moreover, the predominant phyla included *Acidobacteriota, Actinobacteriota, Bacteroidota, Chloroflexi, Myxococcota, Planctomycetota, Proteobacteria (Pseudomonadota),* and *Verrucomicrobiota*. These were present in all compost treatments at all stages of composting (Figure 5.1).

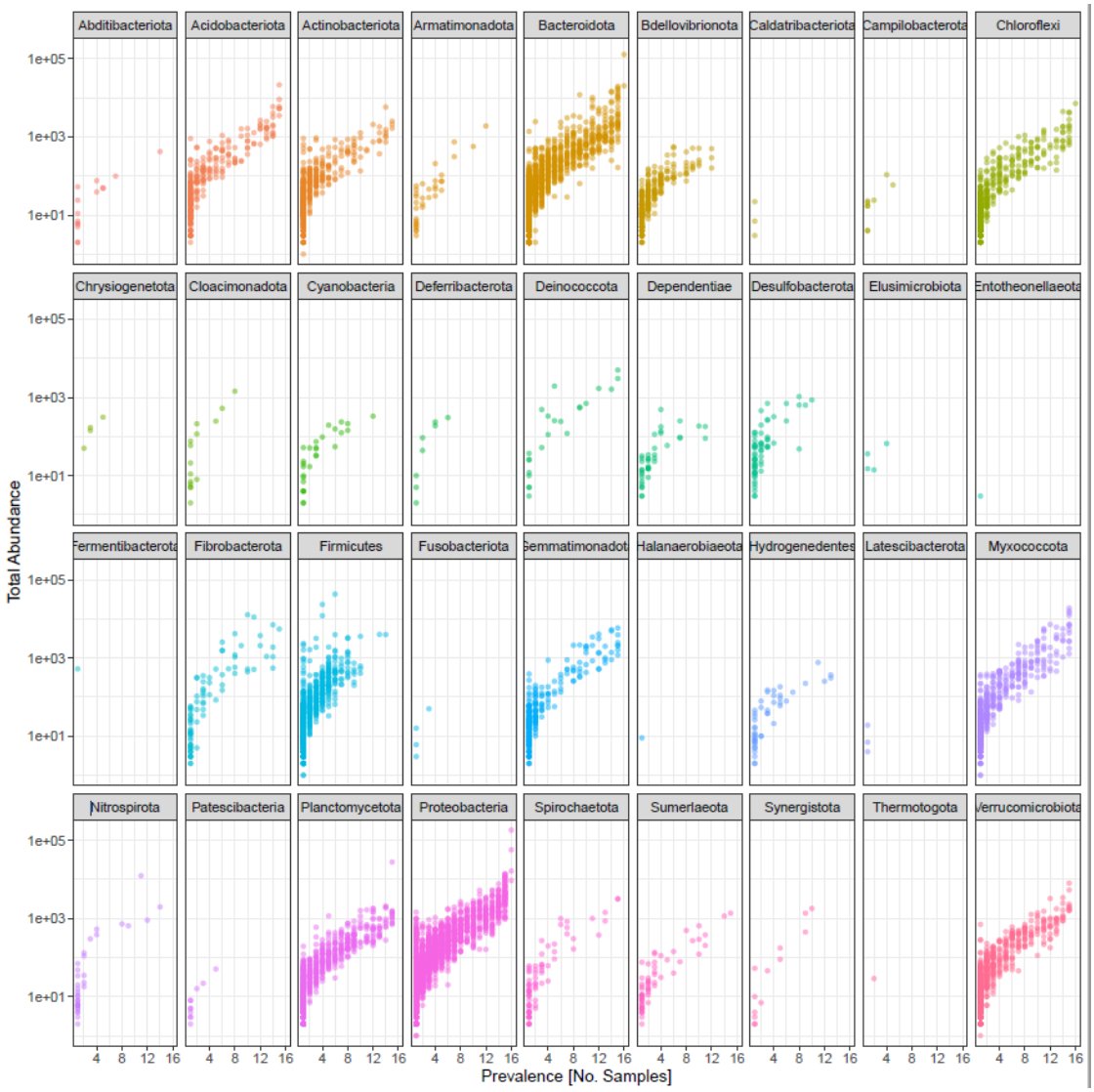


Figure 5. 1: **Feature prevalence of major prokaryotic taxa in different compost samples.**

5.3.2 Taxonomic composition of prokaryotic communities

Bacteroidia was the most abundant and ubiquitous prokaryotic class in all treatments and composting days except grass-based compost at 42 days of composting (G2). It was more abundant in most treatments on the 21st day of composting than on other composting days. During the 21st day of composting, Grass-based compost (G1) had the highest relative

abundance of this class, and Lantana-based compost (L1) had the least relative abundance of this class among all the treatments (Figure 5.2A).

The study observed generally low abundances of taxa known to include pathogenic bacteria across all the compost treatments along the composting period. Grass-based compost recorded the highest overall abundance of Prokaryotic classes, with comparably higher levels of *Gammaproteobacteria* and *Bacteroidia.* On the other hand, Lantana-based compost had the least overall abundance of prokaryotic classes (Figure 5.2B). Conversely, 42 days of composting had the highest relative abundance of prokaryotic classes compared to other composting days (Figure 5.2C).

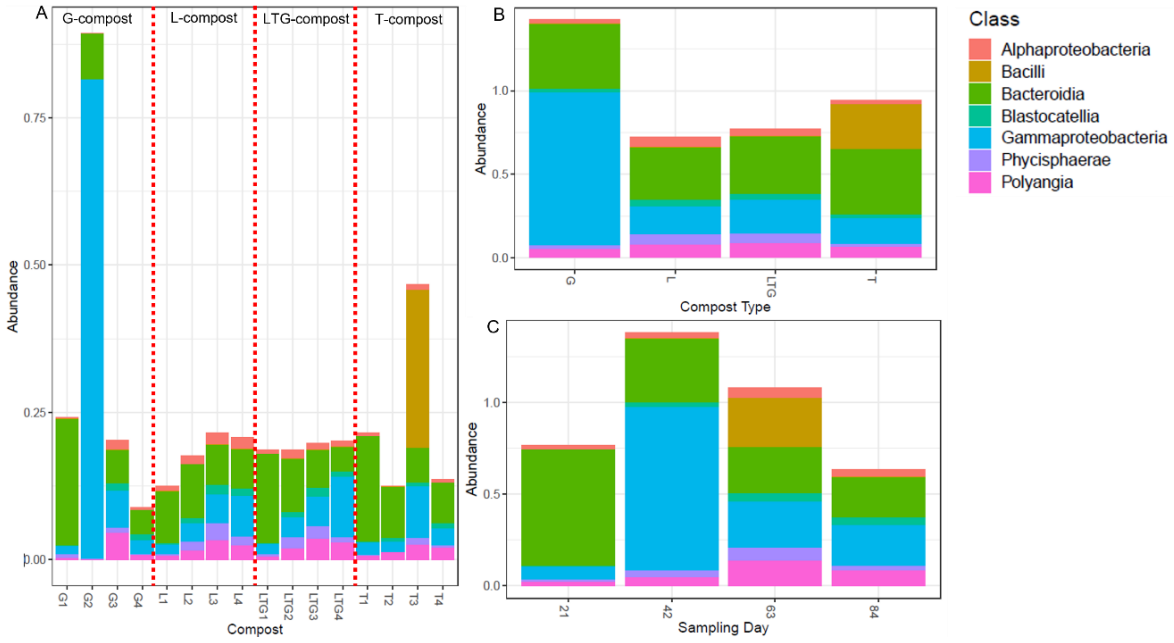


Figure 5. 2: **Relative abundance of the most abundant prokaryotic taxa at class level.**
The samples are from various composting samples (A) treatments (B) and sampling days (C).

5.3.3 Prokaryotic alpha and beta diversity metrics by compost type and composting day

The alpha diversity index showed a significant impact of compost treatment on prokaryotic populations (Figure 5.3A). Minimal variation was observed in L and LTG-based composts (Figure 5.3A). Significant variability was observed on the 42nd day of composting (Observed, Shannon, and Simpson). The least variability was observed at 21 days of composting (Observed). However, Shannon and Simpson diversity indices showed the least variability at 84 days of composting (Figure 5.3B).

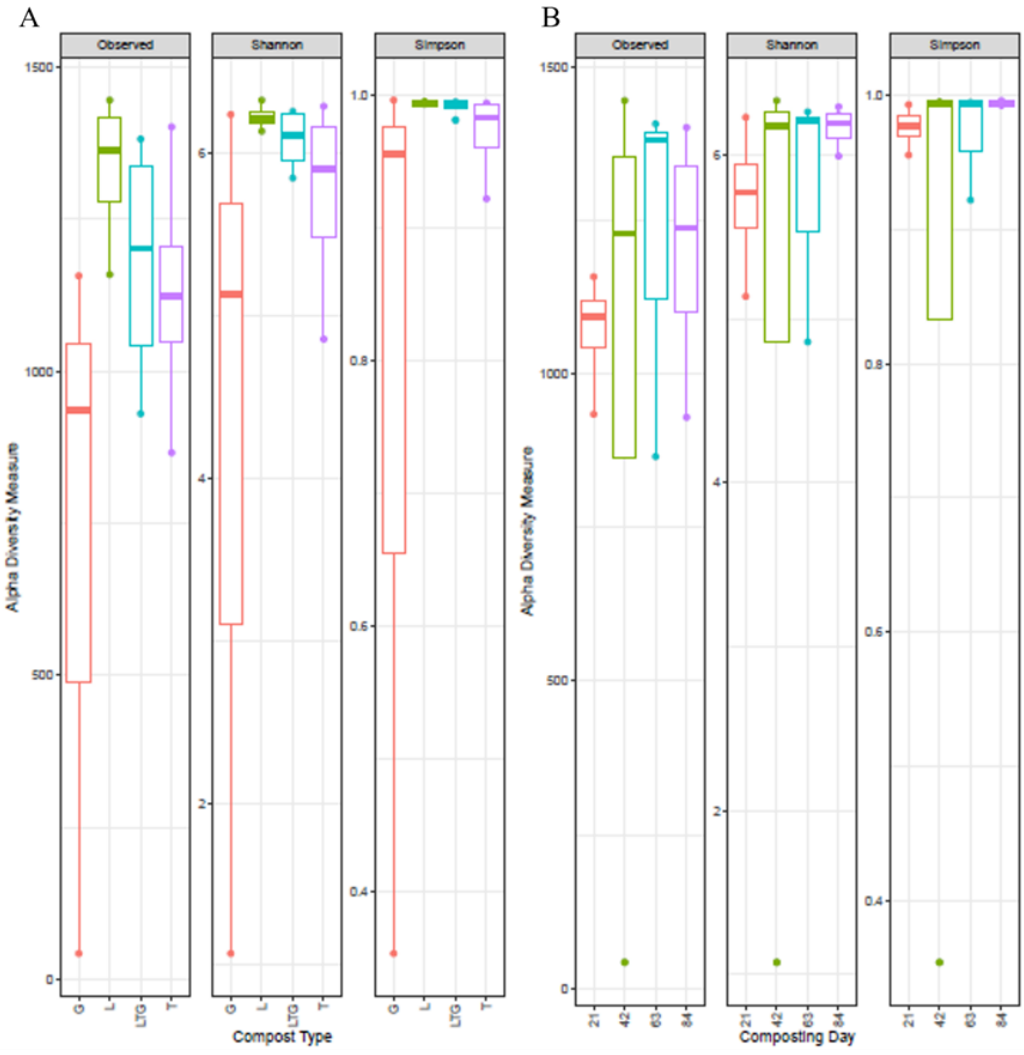


Figure 5. 3: **Alpha diversity of prokaryotic communities under different composting treatments (A) and composting days (B).**

The widest Bray-curtis beta diversity was on the other hand recorded in Grass-based compost (Figure 5.4A) as well as at 42 days of composting (Figure 5.4B). Notably, there was very minimal variation among different treatments at 21 days of composting (Figure 5.4B).

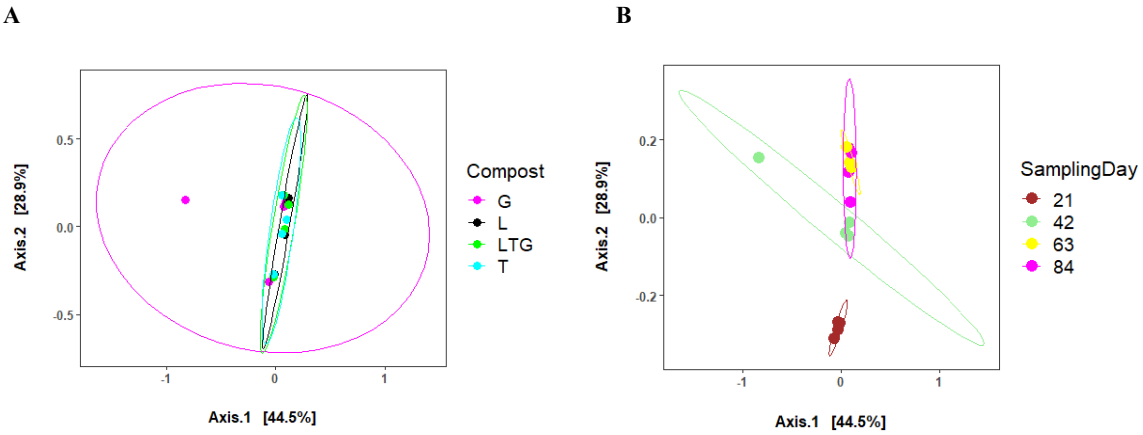


Figure 5. 4: **Principal coordinate analysis (PCoA) ordination plots of compost treatments (A), and composting days (B) based on prokaryotic diversity with the Bray-Curtis index.**
Computation and ordination were done with 95% confidence.

Four prokaryotes were common to all compost regimens and composting days (*Acidibacter spp., Hydrogenophaga temperata, Ruminofilibacter xylanolyticum*, and a prokaryote from the *Saprospiraceae* family). Grass-based compost (G) did not harbor any unique genera. A combination of Lantana, Grass, and Tithonia (LTG) based compost (L) recorded the highest unique genera (7). At the same time, L and T had 5 and 3 unique genera, respectively (Fig. 5.5A). The highest unique ASVs were recorded at 21 days of composting (55), while there were no unique ASVs at 42 days of composting (Fig. 5.5B). There were no shared ASVs between 42 days and 21 days. This was also the case between 42 days and 63 days.

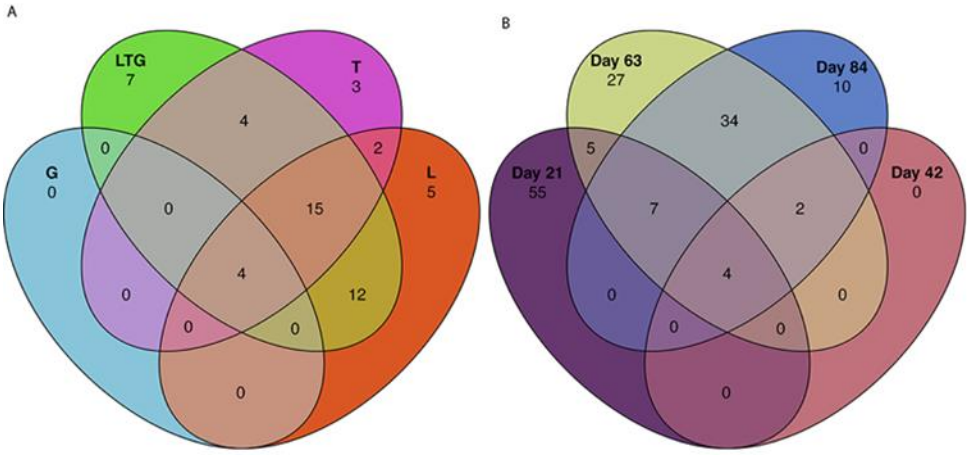


Figure 5. 5: **Venn diagrams showing the distribution of unique and shared ASVs within various composting treatments (A), and composting days (B).**

5.3.4 Physical-chemical properties that drive the Prokaryotic community structure of compost microbiomes

Analysis of physical-chemical variables in combination with prokaryotic diversities among the samples resulted in an ordination plot. As shown in Fig. 5.6, the first two canonical axes explained 44% and 32% of the variation. Class *Bacilli* was the most sensitive taxa to ammonia, while class *Nitrospiria* was the most sensitive to nitrates. Nitrate and pH influenced the grouping of most compost samples closer to each other. Nitrogen (N), Phosphorous (P), Carbon (C), and Potassium (K) had little influence on the grouping of prokaryotes in the various compost samples (Fig. 5.6).

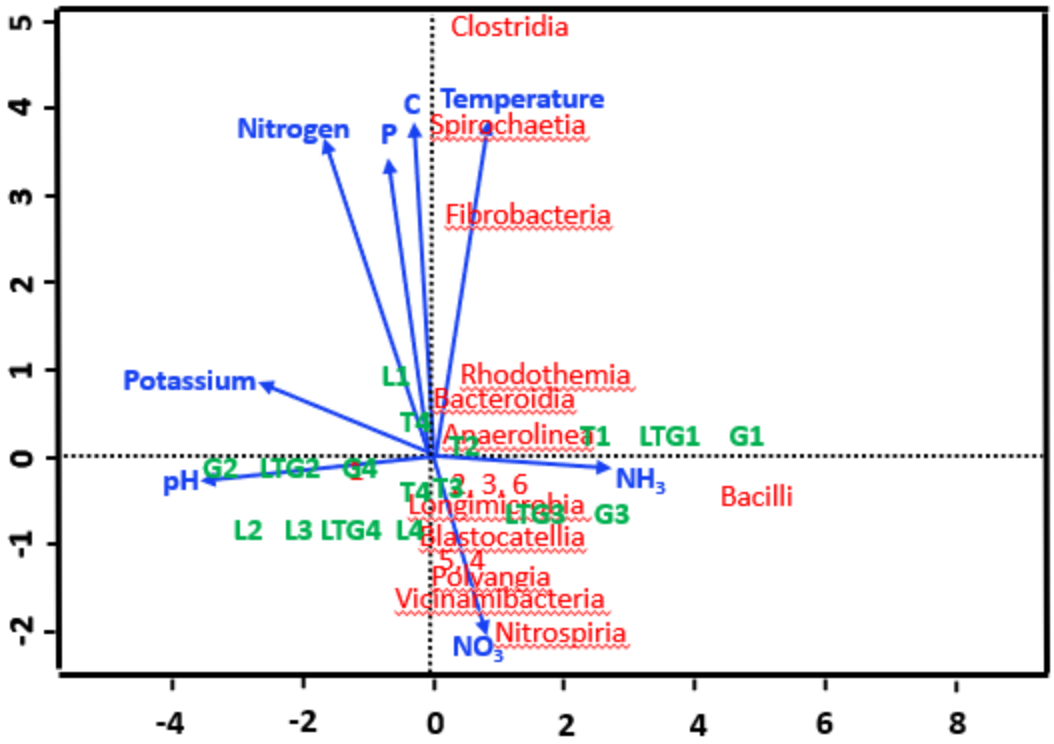


Figure 5. 6: **Canonical correspondence analysis (CCA) of physical-chemical characteristics and top 20 prokaryotic classes in different compost samples.**
K - Potassium, C - Carbon, NO_3 - Nitrates, NH_4 - Ammonia, and P-Phosphorous.

5.3.5 Compost prokaryotic association networks

The relationship between various compost bacterial microbiota is presented in an interaction network based on the correlation of the most abundant bacterial microbiota during compost degradation and maturation (Fig. 5.7). The thick edges (with a distance of 0.0) represent a close interaction between the taxa. The nodes in green and blue colors represent highly ubiquitous and co-occurring prokaryotes of *Bacteroidia* and *Gammaproteobacteria* taxa at the class level. The edges with boldness ranging from 0.0 to 0.5 represent association co-dependence of different classes, where 0.0 and 0.5 show high and insignificant co-dependence, respectively.

Among bacterial communities, *Pseudomonas* of the class *Gammaproteobacteria* had the most interactions with other prokaryotic genera. However, it had the least correlation with *Chrysomicrobium* of the class *Bacilli* (Fig. 5.7). In the prokaryotic interaction network, the class *Alphaproteobacteria* had two (2) genera (*SWB02* and *Tagaea*), and *Bacilli* had one genus (*Chryseomicrobium*) that had major roles in community networks. On the other hand, class *Bacteroidia* had six (6) genera (*Aquimarina, Chryseolinea, Ruminofilibacter, Terrimonas,* and an Unknown genus), while *Blastocatellia* had one genus (*Blastocatellaceae*), *Gammaproteobacteria* class had three (3) genera (*Acidibacter, Hydrogenophaga,* and *Pseudomonas*) involved in major networks in the compost environment; Fig. 5.7.

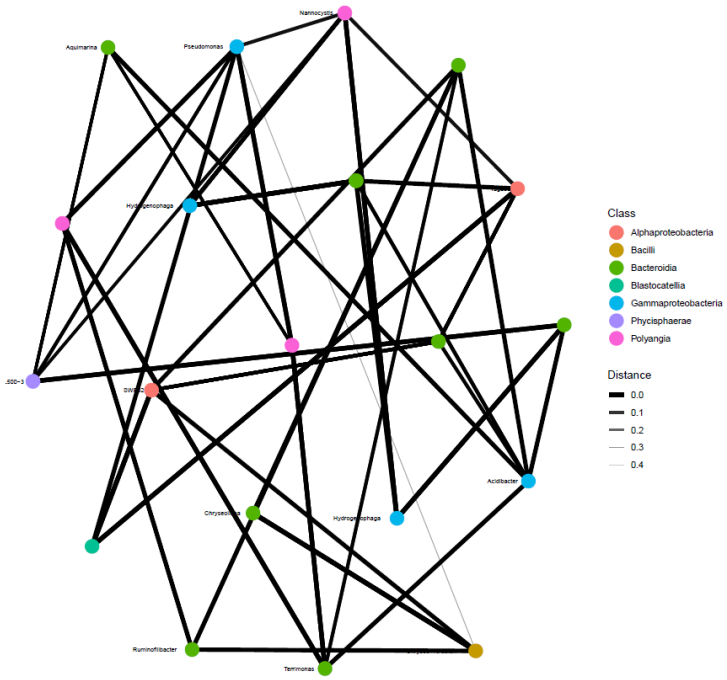


Figure 5. 7: **A correlation network showing interactions among prokaryotic classes driving composting.**

5.4 Discussion

The dominance of *Acidobacteriota, Actinobacteriota, Bacteroidota, Chloroflexi, Myxococcota, Planctomycetota, Proteobacteria*, and *Verrucomicrobiota* has been reported in other studies (Tong et al., 2018). These phyla are also regarded as resident in compost and are also attributed to the nature of composting material. These findings were consistent with other studies considering bacteria categories as residents across the composting period (Galitskaya et al., 2017). These ubiquitous phyla have been reported to have an essential role in nutrient dynamics in the ecosystem (Park et al., 2010). The low dominance of *Entotheonellaeota, Halanaerobiaeota, Fermentibacterota*, and *Thermotogota* has also been reported in other studies (Gonzalez, 2021).

ASVs determine environmental variations by classifying species groups based on their DNA sequence differences (Schloss, 2020). The highest number of ASVs observed in Lantana-based compost indicated the presence of diverse microbial communities attributable to the complexity of *Lantana camara* requiring more categories of prokaryotes to degrade compared to other composting treatments (Rameshwar and Argaw, 2016).

The study enumerated the presence of class *Bacteroidia* which has species such as *Ruminofillibacter*, at all composting stages. *Bacteroiodia* species contribute to composting by degrading macromolecules such as cellulose, agar, and chitin (Tian et al., 2013). The resurgence of *Acidibacter* at the later stages of composting affirms that sufficient composting enhances agriculturally essential microbes. Taxa such as *Acidibacter* have been reported to contribute to the biocontrol of plant diseases, plant growth, and modulation of plant response to abiotic stress (Ezeokoli et al., 2020). The buildup of this

genus towards the end of the composting process guarantees matures compost as a source of agriculturally beneficial microbes for application to the soils. The presence of an unknown genus in all our compost treatments points to composting being a source of agriculturally beneficial novel microbes. The high abundance of taxa in sample T3 can be attributed to the relatively low Carbon and Nitrogen levels in this treatment compared to other samples. The comparatively low levels of these nutrients point to utilization by microbes (Jones et al., 2018).

The general decline in pathogenic prokaryotes, such as *Staphylococcus* and *Treponema*, confirms that extended composting periods are necessary to sanitize the compost of pathogenic microbial communities. Babajide et al. (2012) reported that these categories were present during the first month of composting, with very minimal levels of Treponema.

The least intra-treatment variability in Lantana-based Compost (L) is attributable to the inhibitory influence of Lantana camara on microbial growth and diversity (Sharma and Kumar, 2009; Anand et al. 2018), which did not allow meaningful changes in microbial diversity along the composting period. The relatively low variability in the mixed Compost (LTG) is also attributable to Lantana camara. The application of Lantana camara during composting has been attributed to the reduction of prokaryotic diversity (Karanja et al., 2020).

This study uncovered unique ASVs present in Lantana, LTG, and T-based compost, indicating the complexity of these materials compared to Grass (Bashir et al., 2019). The unique ASVs in mixed regimen compost (LTG) are attributable to the contribution of Lantana and Tithonia material in the mixed compost. It was observed that 42 days had no

unique ASVs compared to other composting days. Therefore, this depicts this composting time as the decomposition process's transition phase.

The high relative abundance of *Pseudomonas* in grass-based compost after 42 days (G2) shows that, at this time, the compost harbored this taxon. *Pseudomonas* is one of the free-living microbial taxa that produce metabolites with specific suppressive potential against soil-borne pathogens (Bashir et al., 2019). However, positive shifts in the abundance of this taxon were observed in the remaining compost treatments after 63 days of composting. This implies that the composting period positively influenced the abundance of this genus. The findings of this study showed that the class *Bacteroidota* was persistent along the composting period across all compost treatments. Previous studies have reported similar trends regarding this taxon (Karanja et al., 2020).

The syntrophic interaction between prokaryotes in the compost environment plays a critical role in nutrient cycling, microbial buildup, and persistence in the ecosystem. *Gammaproteobacteria* and *Bacteroidia* are the integral classes driving cattle manure and green material co-composting. *Pseudomonas* species have been shown to have the highest interactions with most prokaryotic species. Studies have shown that *Pseudomonas* species break down complex carbohydrate material such as lignin into simpler biomolecules and substrates utilized by other prokaryotes in the degradation process (Bashir et al. 2019; Karanja et al. 2020). Through this study, it was deduced that *Acidibacter* species, *Hydrogenophaga spp*., and *Pseudomonas spp*. of *Gammaproteobacteria* class; *Aquimarina spp., Chryseolinea spp., Ruminofilibacter spp.,* and *Terrimonas spp.* of *Bacteroidia* class are the major drivers for biodegradation and maturation of compost.

CHAPTER SIX

ENUMERATION OF COMPOST FUNGAL AND NON-FUNGAL EUKARYOTIC COMMUNITY STRUCTURE AS INFLUENCED BY FEEDSTOCK AND COMPOSTING DAYS

6.1 Introduction

The phytochemical complexities of *Lantana camara* and *Tithonia diversifolia* have been previously studied. *Lantana camara* has been reported to contain more complex polymers and thus possibly requires more microbial categories to break down into agriculturally useful material. For example, the phytochemical composition analysis of L. *camara* showed 23.3% crude fiber, 26% cellulose, 16.2% lignin, and 21% hemicellulose, while *T. diversifolia* contains 11.2%, 17%, 7%, and 16% of these elements, respectively (Cadena-Villegas et al. 2020; Dongmo et al. 2021). Furthermore, the different complexities of the composting material could require assorted composting times to produce a biogeochemically stable product. It is, therefore, necessary to evaluate the influence of composting time on the physical-chemical and biological quality of manure during composting.

Numerous experiments have studied compost microbial communities' diversity and abundance using culture-dependent and culture-independent methods (Liu et al. 2020; Dang et al. 2021; Singer et al. 2021). However, there is still a limited understanding of eukaryotic communities' structure in the composting process, concerning the composition and complexity of the composting materials, especially utilizing high-throughput sequencing technology. The effect of the *Lantana camara* and *Tithonia diversifolia* on microbial community structure in complex ecosystems such as the compost environment

has not also been done, despite their widespread adoption by farmers as soil nutrient amendment materials.

This study comprehensively assessed fungal and non-fungal eukaryotic communities associated with the co-composting of assorted nitrogenous green material and composting time. The study hypothesized that different composting materials and days influenced eukaryotic communities differently. The culture-independent, high-throughput sequencing Illumina MiSeq platform was used for library preparation of the fungal and non-fungal eukaryotic communities in the different compost environments. Furthermore, the study evaluated the correlation and co-dependence of compost physical-chemical factors and their influence on fungal and non-fungal eukaryotic communities.

6.2 Materials and methods

6.2.1 Compost microbiome total DNA Extraction and Amplification

Total compost DNA was extracted from triplicate compost subsamples of each treatment for DNA extraction from fungal and non-fungal eukaryotes. The PureLink™ Microbiome DNA Purification Kit (ThermoFisher Scientific®) was used for extraction as per the manufacturer's instructions (www.thermofisher.com/ke/en/home/life-science). Each extracted sample's quality and concentrations were measured under 2% agarose gel and NanoDrop (Maestrogen®). The agarose gel was prepared by adding 7 grams of agarose powder to a conical flask along with 350 mL of Tris-Acetate-EDTA buffer. The mixture was then heated in a microwave until the agarose completely dissolved. Once dissolved, the solution was allowed to cool to approximately 50-60°C. The Sybr® green DNA stain was added at this stage and mixed gently to avoid bubbles.

After cooling, the agarose solution was poured into the gel casting tray, ensuring it filled evenly without trapping air bubbles. The comb was then inserted to create wells, and the gel was left at room temperature for 30-60 minutes to solidify completely. Once set, the comb was carefully removed to reveal the wells, and the gel was transferred to the electrophoresis tank, which was filled with buffer to cover the gel. The purified compost DNA was then shipped under dry ice to the Molecular Research DNA Lab (www.mrdnalab.com, Shallowater, TX, USA) for sequencing under the illumine miseq platform.

The PCR primer sets used were ITS1-F (5'-CTTGGTCATTTAGAGGAAGTAA-3') ITS2 (5'-GCTGCGTTCTTCATCGATGC-3') for fungal ITS amplification while EUK1391F(5'GTACACACCGCCCGTC-3') and EukBr (5'-TGATCCTTCTGCAGGTTCACCTAC-3') for other eukaryotes. Amplification was done in 30 cycles PCR (5 cycles used on PCR using the HotStarTaq Plus Master Mix Kit (Qiagen, USA). The PCR conditions were 95°C for 5 minutes, followed by 30 cycles of 95°C for 30 seconds, 53°C for 40 seconds, and 72°C for 1 minute, with a final elongation step at 72°C for 10 minutes performed.

Resultant PCR amplicons were visually quantified under 2% agarose gel. There were no fungal amplicons associated with grass-based compost sampled at 42 days. Equimolar quantities of PCR amplicons obtained from the remaining 31 samples (16 non-fungal eukaryotic amplicons from individual composts and 15 fungal amplicons) were multiplexed using unique indices, pooled, and sequenced using Illumina MiSeq next-generation technology at MRDNA (www.mrdnalab.com, Shallowater, TX, USA). Barcodes and amplicon primer sequences were trimmed after sequencing. Low-quality

sequences were denoised and filtered out with reads <300 base pairs after phred20-based quality trimming. Sequences with ambiguous base calls and those with homopolymer runs exceeding 5bp were removed (Reeder and Knight, 2010). The raw ITS and 18S sequences were submitted to the NCBI sequence archive with accession number PRJNA822850 (https://www.ncbi.nlm.nih.gov/sra/PRJNA822850).

6.2.2 Bioinformatics Analysis

The Divisive Amplicon Denoising Algorithm 2 (DADA2) version 1.20.0 as described by Callahan et al. 2017 was the primary workflow for analysis. Here, preprocessing such as filtering of the reads, chimera removal, and taxonomic assignment were done separately for the ITS and 18S rRNA gene sequences. The Unite reference database was used for ITS while; Silva 128 and 132 databases were used for non-fungal eukaryotes (18S) referencing. The products of the workflows were ITS and 18S rRNA gene amplicon sequence variant (ASV) tables for the two amplicon sets (Prodan et al., 2020).

6.2.3 Statistical analysis

Data analysis was done using different packages in R version 4.3.1 (R core team, 2023). Alpha diversity metrics were calculated using the "estimate_richness" function in phyloseq. The observed index was estimated to reflect the number of ASVs in each compost sample and values that have a positive correlation with the species richness. Shannon and Simpson Inverse-Simpson indices of the correlation of diverse species abundance in a sample were also computed. Shapiro and significance tests were calculated before plotting the alpha diversity metrics. The taxonomy table and abundance table were merged with the abundance table, and bar plots of compost treatments and sampling day relative abundance were plotted using the "plot_bar" function. Venn diagrams to show the

shared community ASVs among composting treatments and composting days were plotted using the eulerr (version 6.1.1) package.

Beta diversity was computed using the Bray-Curtis index to further explore the influence of different composting treatments and composting days and differences on microbial community profiles. The resulting scores were used for PCoA plotting and compared using the PERMANOVA test of significance. A stepwise modeled CCA was done in a Vegan package (version 2.6-2) to show the effect of explanatory physical-chemical variables on the different compost microbiomes A co-expression network detailing the interaction of abundant composting microbiomes was constructed based on microbial abundance using the "plot_net" function in the ggplot2 package (version 3.3.5).

6.3 Results

6.3.1 Different composting materials and days exhibit distinct biodiversity

Mixed compost (LTG) and composting day 84 recorded significantly ($P<0.05$) higher overall fungal populations ("Observed") among the compost types and composting days. Lantana-based compost and composting da B had the highest fungal community diversity (Shannon, Simpson, and InvSimpson) (Figure 6.1A and B). Non-fungal eukaryotic richness was significantly ($P< 0.05$) more abundant in tithonia-based compost (T) and composting day 21. The most diverse non-fungal eukaryotic biome was in the Tithonia-based compost and composting day 84 (Figure 6.1C and D).

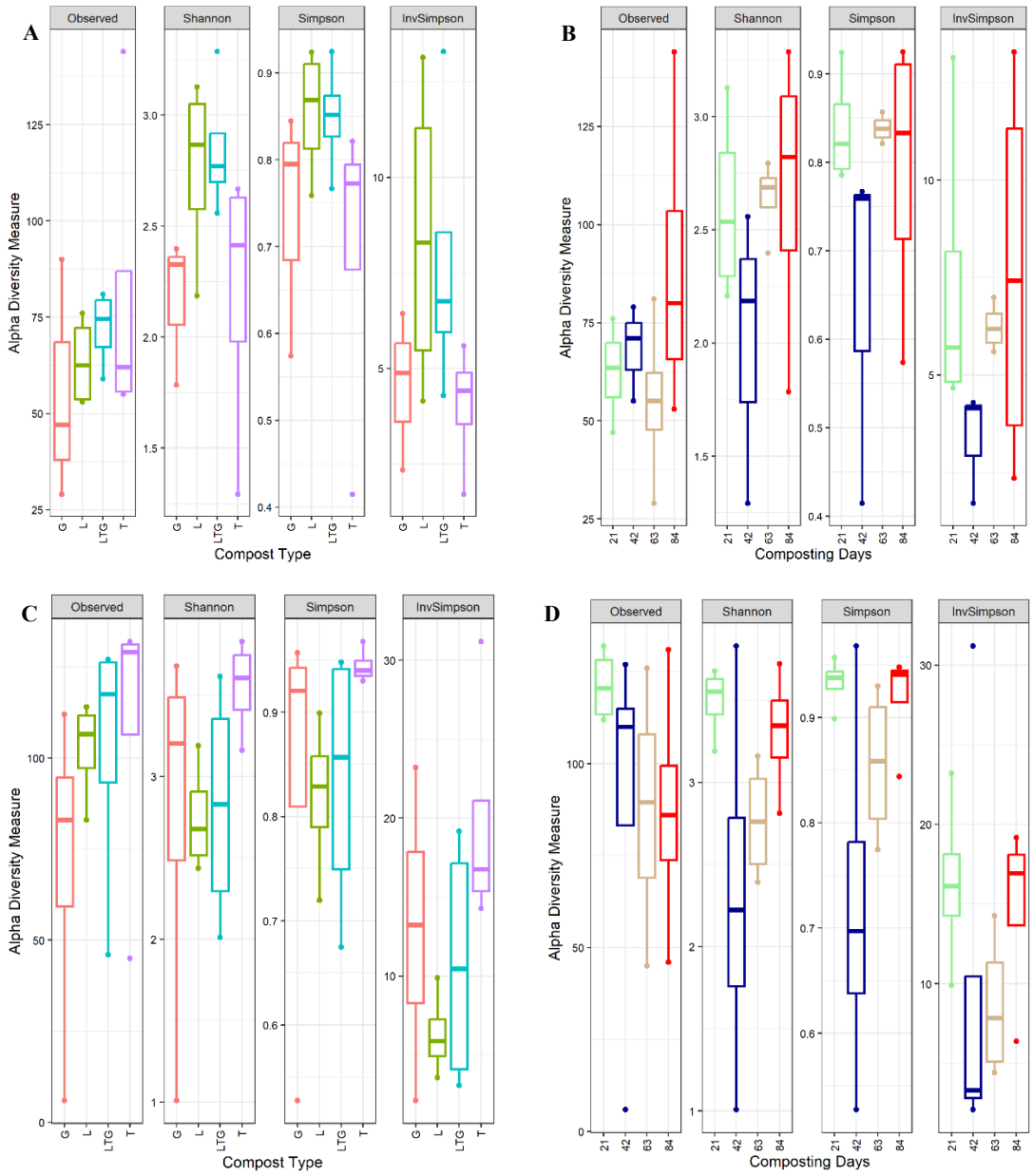


Figure 6. 1: **A-D: Alpha diversity metrics (Observed, Shannon, InvSimpson, and Simpson) of fungal (A and B) and non-fungal (C and D) eukaryotic communities under different composting treatments and composting days.**

6.3.2 Beta diversity of fungal communities distinctly

Overall, the most abundant fungal classes in the compost environment were *Sordariomycetes* (61% mean relative abundance), *Agaricomycetes* (8% mean relative abundance), *Dothidiomycetes* (20% mean relative abundance), *Eurotiomycetes* (4% mean

relative abundance) and *Saccharomycetes* (2% mean relative abundance); Table 6.1A. The fungal class *Sordariomycetes* was the most abundant taxa within the compost treatments, particularly in Tithonia-based composts that had a mean relative abundance of 68%; Fig 6.2A, and Table 6.1A. *Sordariomycetes* was still the dominant fungal class on all the composting days with the highest abundance recorded on composting day 42 (74%). The class *Agaricomycetes* was most abundant on composting day 21 compared to other composting days, recording (Fig 6.2C and Table 6.1A).

The most abundant non-fungal eukaryotic taxa in all the compost environments were *Alveolata* (4%), *Chloroplastida* (13%), *Holozoa* (58%), *Rhizaria* (13%), *Stramenopiles* (8%) and *Tublinea* (2%); Figs 6.2B and D; Table 6.1B. Non-fungal eukaryotic class, *Holozoa* was the most abundant taxa with the highest mean relative abundance recorded in Lantana-based compost (77%); Fig. 6.2B and Table 6.1B. Class *Holozoa* was the most abundant non-fungal eukaryotic taxa across the composting days, with the highest values recorded on composting day 63 (mean relative abundance of 66%); Fig 6.2D and Table 6.1B.

Table 6. 1: **A- Mean relative abundance (%) of the most abundant fungal eukaryotic classes in different compost treatments and composting days**

	Overall relative abundance		Compost treatments				Composting days			
Class	**Mean**	**Standard deviation**	**G**	**L**	**LTG**	**T**	**21**	**42**	**63**	**84**
Sordariomycetes	61	18.3	64	56	57	68	67	74	47	60
Dothideomycetes	20	18.8	19	21	20	20	6	12	33	28
Agaricomycetes	8	7.4	9	8	9	6	18	4	6	3
Eurotiomycetes	4	2.4	3	4	7	3	4	4	5	4
Mortierellomycetes	2	3.3	0	3	3	1	0	3	4	1
Saccharomycetes	2	1.6	3	2	2	1	1	1	3	2
Tremellomycetes	1	3.1	0	4	1	0	3	0	0	1
Pezizomycetes	1	0.8	0	1	1	0	1	1	0	1
Cystobasidiomycetes	0	0.8	1	0	0	0	0	0	1	0
Orbiliomycetes	0	0.8	1	1	0	0	0	1	0	0
Microbotryomycetes	0	0.3	0	0	0	0	0	0	0	0
Leotiomycetes	0	0.2	0	0	0	0	0	0	0	0
Rhizophlyctidomycetes	0	0.2	0	0	0	0	0	0	0	0
Laboulbeniomycetes	0	0.1	0	0	0	0	0	0	0	0
Ustilaginomycetes	0	0	0	0	0	0	0	0	0	0
Total	**100**		**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**

Table 6. 2: **B- Mean relative abundance (%) of the most abundant non-fungal eukaryotic classes in different compost treatments and composting days**

Class	Mean	Standard deviation	G	L	LTG	T	21	42	63	84
Holozoa	58	23.1	33	77	70	53.5	55	56	66	55
Rhizaria	13	11.8	16	9	15	13.5	28	6	8	12
Chloroplastida	13	24.5	33	1	6	14	4	30	7	13
Stramenopiles	8	4.8	7.8	7.5	6	10	7	6	6	12
Alveolata	4	6	8.5	2	1	3.5	2	1	9	4
Discosea	2	1.3	1	2	1.5	2.5	2	1	3	2
Tubulinea	2	1.4	2	1.5	1	3	3	1	2	2
Total	**100**		**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**

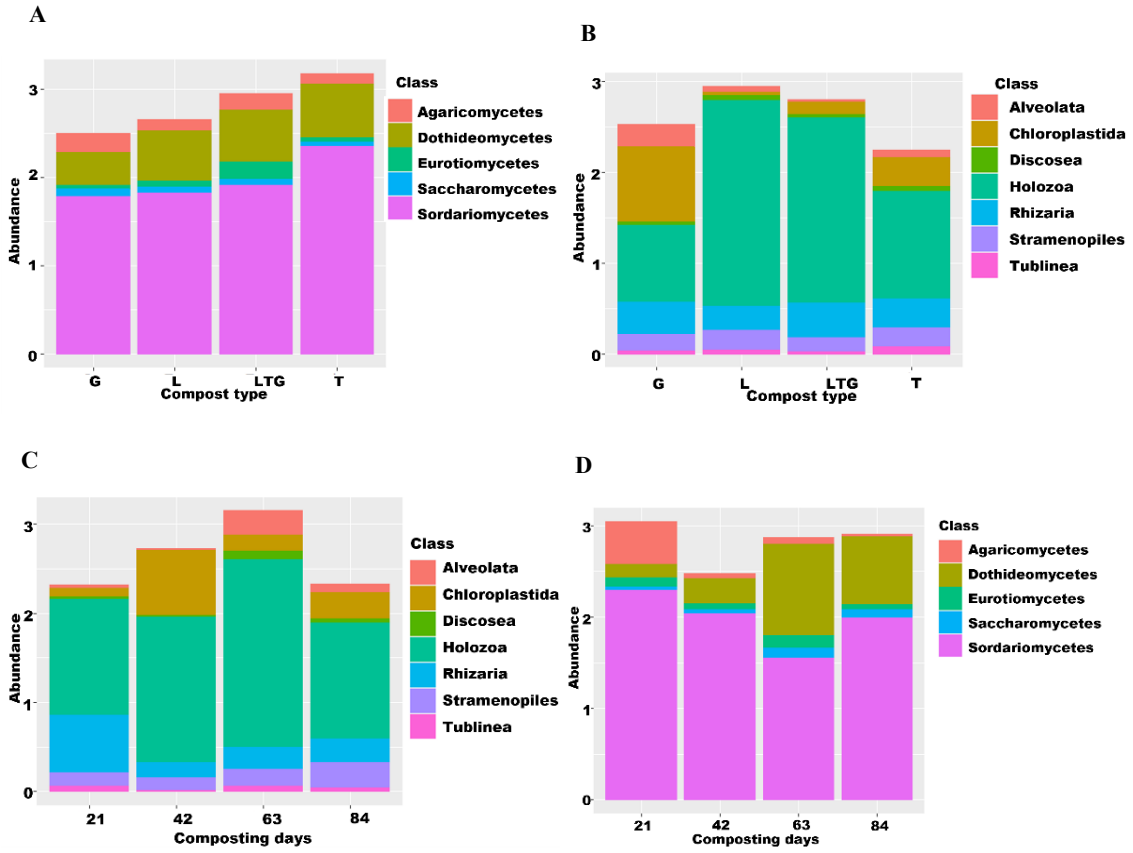


Figure 6. 2: **A-D: Relative abundances of fungal and non-fungal eukaryotic classes in various composting treatments (A and C) and sampling days (B and D).**

6.3.3 Principal coordinate analysis (PCoA)

Bray-Curtis beta diversity of samples from different compost types and days showed distinct groupings (Figs. 6.3A, B, C, and D). Notably, the percentage of variation in fungal community structure attributed to compost type and composting days is relatively small, with about 73% of the unexplained variance (Figs 6.3A, and C). The widest beta diversities for the fungal and non-fungal communities as influenced by compost treatments were observed in Tithonia-based and Grass-based composts, respectively. The least overlap and variation of diversity among the composting days in both fungal and non-fungal eukaryotic communities were observed on day 21 of composting, Figs 6.3C and D.

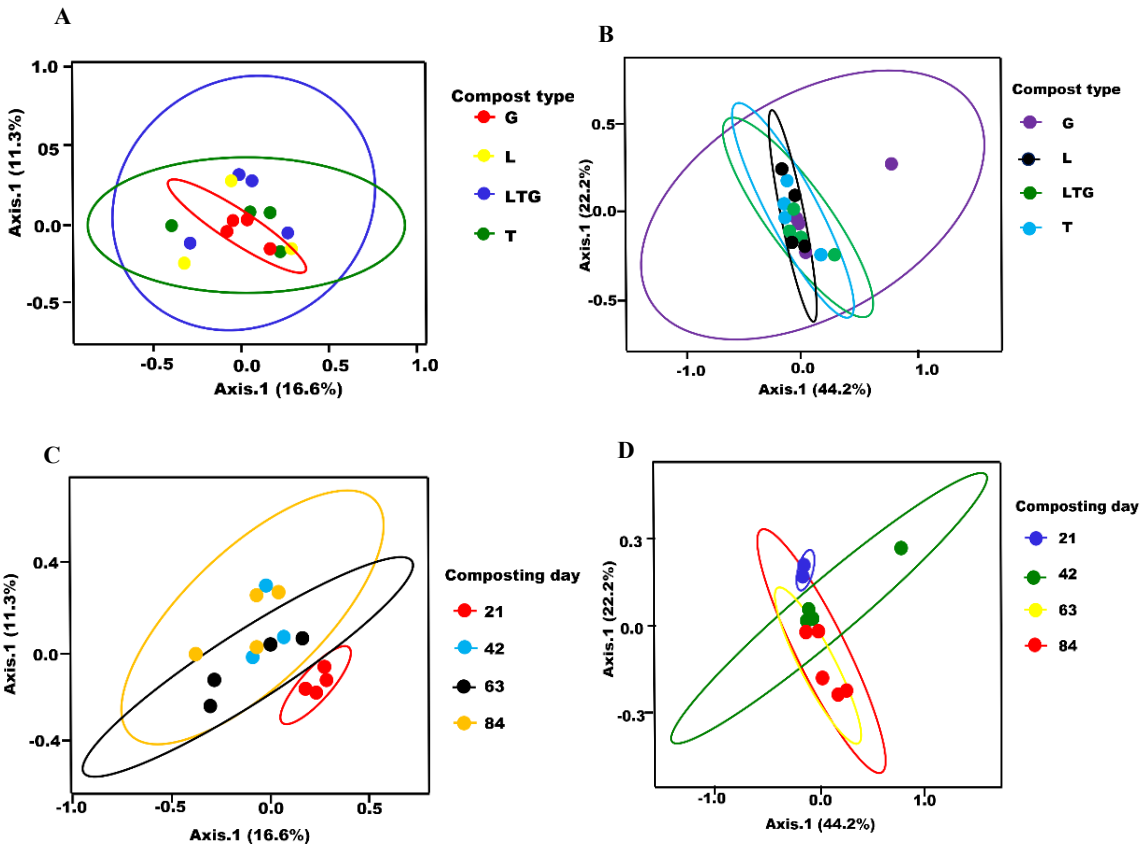


Figure 6. 3: **A-D: Principal coordinate analysis (PCoA) ordination plots based on the Bray-Curtis index at 95% confidence.**
Ellipses highlight the different compost groups.

6.3.4 Unique fungal and non-fungal eukaryotic taxa within compost environment

Venn diagrams were generated to show the common OTUs and exclusive OTUs among different compost treatments and composting days. Grass-based and mixed compost recorded the most unique core fungal taxa among the compost types, with three unique taxa for each of the two treatments (Fig. 6.4A). Lantana-based compost had the highest non-fungal eukaryotic core taxa, recording seven unique taxa (Fig. 6.4B). On the other hand, composting day 21 recorded the highest number of unique core fungal taxa among all the composting days, with 12 exclusive taxa (Fig. 6.4C). In contrast, composting day 21 had the most unique non-fungal taxa (Fig. 6.4D).

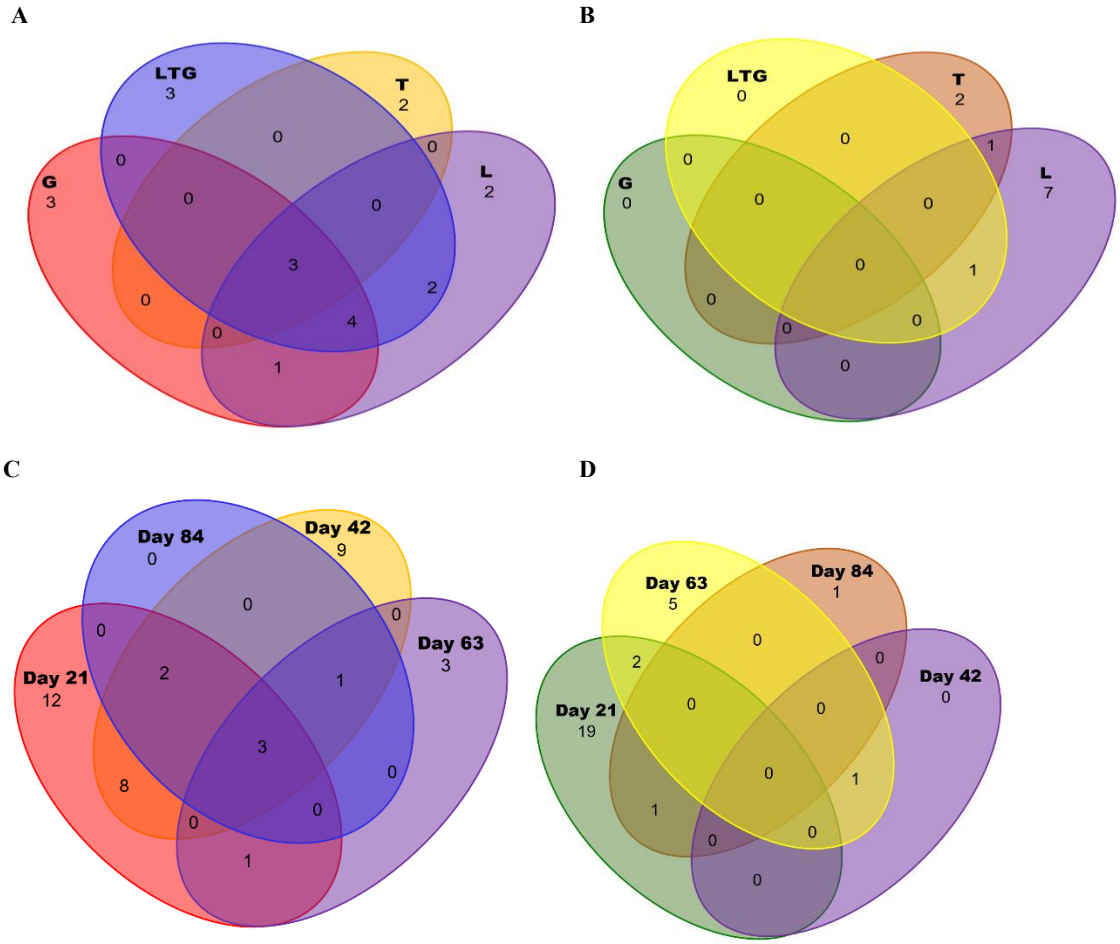


Figure 6. 4: **A-D: Venn diagram based on shared major core taxa of fungal and non-fungal eukaryotic communities under compost treatments (A and B), composting days (C and D).**

6.3.5 Fungal and non-fungal community interactions within the compost environment

Five (5) fungal classes (*Agaricomycete, Eurotiomycetes, Dothideomycets, Saccharomycetes,* and *Sordariomycetes*) were displayed to correlate with each other at different intensities (correlation values between 0.0 and 0.6) as shown in the interaction network. The class *Sordariomycetes* was the hub taxon contributing to the most network nodes, with 10 taxa belonging to the class. Consequently, this taxon had the most interactions with other fungal classes in the compost environment (Fig. 6.5A).

On the other hand, seven (7) non-fungal classes (*Alveolata, Chloroplastida, Discosea, Holozoa, Rhizaria, Stramenopiles,* and *Tublinea*) were shown to bear the most interactions within the compost environment. Class *Holozoa* had the most interactions with non-fungal eukaryotic taxa in the compost ecosystem (Fig. 6.5B). Taxa under this class supported most close interactions (mainly at a correlation of 0.2) within the non-fungal community; Fig. 6.5B.

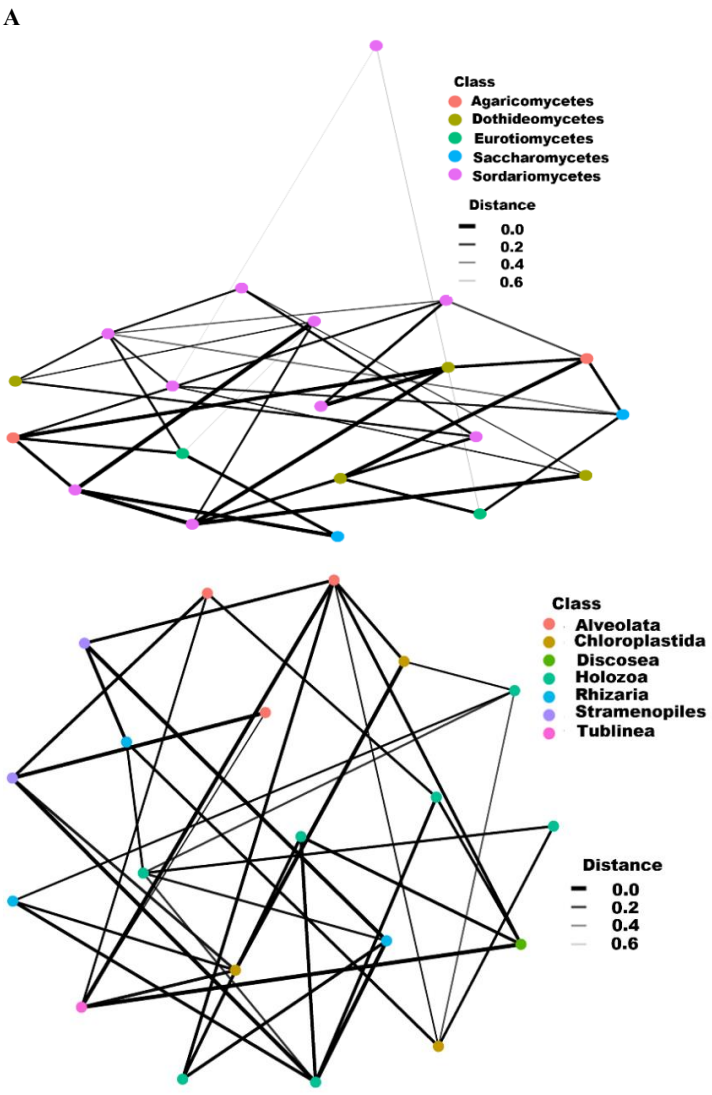
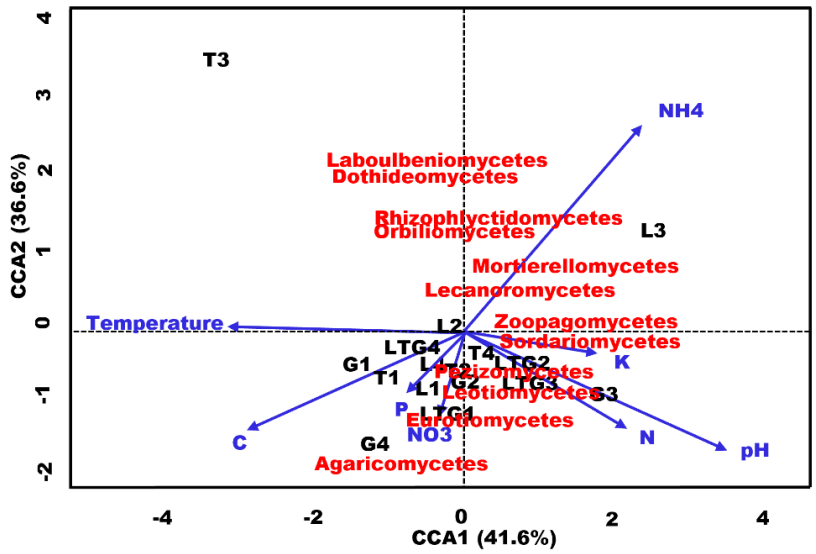


Figure 6. 5: **A-B; Correlation network showing interactions among fungal (A) and non-fungal (B) eukaryotic classes driving composting.**

6.3.6 Physical-chemical drivers of compost fungal and non-fungal eukaryotic communities

Canonical correspondence analysis (CCA) ordination plots of environmental factors showed the significant (adj. p-value <0.01) influence of these factors on fungal and non-fungal eukaryotic biomes of compost (Fig. 6.6A and B). Most fungal classes were responsive to less ammonia, with *Dothideomycetes* and *Laboulbeniomycetes* having the and responded positively to a decrease in other physical-chemical states of compost. The fungal class *Agaricomycetes* was the most sensitive to Nitrates and Carbon. Non-fungal eukaryotic class *Tublinea* was uniquely responsive to decreasing temperature, Carbon, and phosphorus, and increasing Nitrates. The frequency of class *Discosea* is associated with high pH, total nitrogen, and low nitrate levels.

A



B

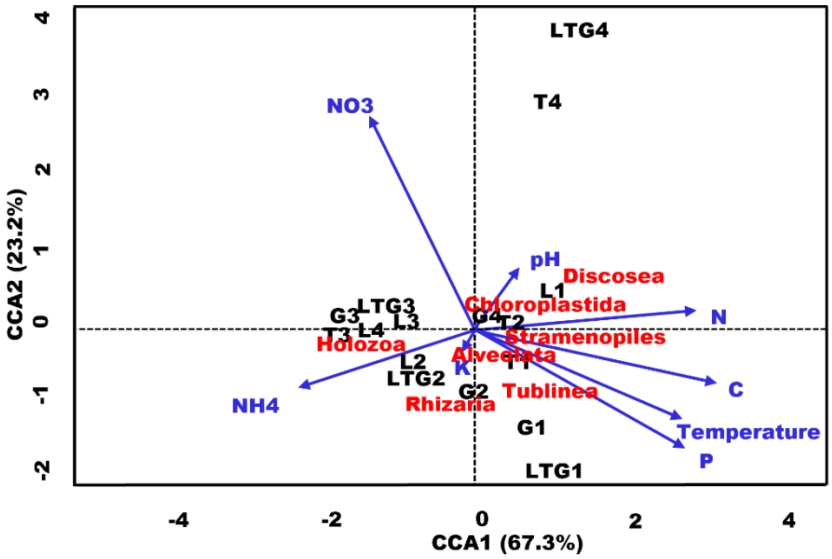


Figure 6. 6: **Canonical correspondence analysis (CCA) plots showing the effect of explanatory physical-chemical variables on the different fungal (A) and non-fungal (B) microbiomes using a significance threshold of 0.05.**

C- Organic Carbon, N- Total Nitrogen, K- Potassium, NO_3- Nitrates, NH_4- Ammonia.

6.4 Discussion

The study of the structure of eukaryotic communities in thermophilic composting is crucial to understanding the categories and succession of fungi that are useful for improved soil health and not harmful to humans, plants, and environmental health (Xie et al., 2021). This knowledge is also worthwhile in the optimization of compost quality standards for safer crop production (Rouhullah et al., 2012). Physical-chemical conditions are indicators of the humification rate that ultimately bring biological and nutritional stability to compost (Estrella-González et al., 2020). Humification is preceded by rapid biogeochemical phases which are microbially driven, breaking down complex polymers into organic acids (Estrella-González et al. 2020; Xie et al. 2021).

The high variability within Tithonia-based compost can be attributed to the significant physical-chemical changes within this treatment compared to other treatments along the composting process. The strong positive correlation between factors such as Carbon and temperature points to the co-dependence of these two elements in the physical-chemical nature of compost. The breakdown of Carbon by microbes leads to a temperature increase in the compost environment (Estrella-González et al. 2020; Xie et al. 2021). This temperature increase is responsible for nutrient mineralization and, ultimately, humification of compost (Estrella-González et al., 2020).

The high fungal biodiversity (richness) at 84 days of composting compared to preceding composting days implies that more extended composting periods are necessary for compost stability (Xie et al., 2021). Several authors have reported the recruitment of more fungal categories responsible for the maturation and, ultimately, humification of compost (Rouhullah et al. 2012; Estrella-González et al. 2020; Xie et al. 2021). The diversity of the

fungal community in Lantana-based compost is attributable to the complexity of *Lantana camara* compared to other materials and hence ecosystem recruitment of diverse fungal categories with the capacity to metabolize this material (Xie et al., 2021).

The ubiquitous fungi taxa in all composting treatments and days (*Sordariomycetes, Agaricomycetes, Dothidiomycetes, Eurotiomycetes,* and *Saccharomycetes*) were reported as resident classes in compost (Rouhullah et al. 2012; Estrella-González et al. 2020; Xie et al. 2021). The dominance of class *Sordariomycetes* in the compost environment indicates its critical role in the evolution of compost and persistence in the compost ecosystem. This class has been reported as having a superior metabolic capability to other fungal classes (Estrella-González et al. 2020; Xie et al. 2021).

Classes *Alveolata, Chloroplastida, Discosea, Holozoa, Rhizaria, Stramenopiles*, and *Tublinea* have been reported as resident non-fungal eukaryotes that colonize organic wastes (Xie et al., 2021). These eukaryotes have been reported as active soil protists (Geisen et al., 2019) with metabolic functions that are essential nutrient cycling pathways. The presence of non-fungal eukaryotic class *Chloroplastida* in all compost treatments and days affirms the suitability of compost as a soil health input bringing in novel microbes with the ability to improve soil physical-chemical state. Algal biota such as *Chloroplastida* have been reported as having broad metabolic capabilities in fixing nitrogen, acting as plant growth promotion, and disease control (Lee and Ryu, 2021).

Fungal class *Agaricomycetes* has been significantly associated with Carbon and Nitrogen cycling in the ecosystem (Dang et al., 2021) hence their dominant association with nitrates and Carbon during composting. This primes this class as a potential soil Carbon and

nitrogen cycling microbial category as a suitable candidate for soil improvement inoculants.

The change of unique fungal ASVs in the different composting days implies a clear evolution of fungal communities as influenced by the changing nature of materials in compost as well as physical-chemical parameters and vice-versa (Galitskaya et al. 2017; Xie et al. 2021; Zhu et al. 2021). The high unique core taxa in Lantana-based compost points to the role of the eukaryotes in the unique degradation of material in this composting treatment. Lantana has been reported as inhibitory to some classes of microorganisms (Ribeiro et al. 2017; Zhao et al. 2017; Tortosa et al. 2020), therefore requires specialized classes of microbes to break down. Hub taxa have a direct inhibitory or facilitative role in the proliferation and survival of other microbes affecting overall interconnected communities (Kong et al., 2022).

The genera in the class *Sordariomycetes*, which is the major fungal hub taxon, have been reported as the first line of colonizers of recalcitrant material in soil, therefore breaking this material into simpler forms available for other fungal and prokaryotic communities (López et al. 2021; Zhu et al. 2021). The degradation of complex material such as lignocellulose in ecosystems like compost is driven by a synergistic action of oxidative and hydrolytic enzymes that break the linkages within the material (Estrella-González et al. 2020; López et al. 2021). This process requires a variety of interactions among different categories of microorganisms (Rouhullah et al., 2012).

CHAPTER SEVEN

IDENTIFICATION OF THE DIVERSITY AND ABUNDANCE OF MAJOR COMPOST METABOLIC PATHWAYS AS INFLUENCED BY COMPOSTING FEEDSTOCK AND DAYS

7.1 Introduction

The complexity of the composting materials and the process of their breakdown involves assorted microbial communities with diverse colonization capacities and metabolic potential. The assorted abundances and diversities of these microbial populations are influenced by the time/phase of composting as well as the nature of composting feedstock (Matheri et al. 2023a). Consequently, these microbial species communally and individually set up assorted assemblages and feeding guilds with diverse feeding preferences and modes which in turn drive the composting process (Gupta et al., 2022). The feedstock used for composting and the duration of composting can influence the diversity and abundance of microbial populations and therefore the metabolic potential responsible for the utilization of these materials. This ultimately has a direct influence on the efficiency and quality of composting (Matheri et al., 2023b).

The cascade with which each element of nature is utilized by micro and macro-organisms happens first by the utilization of complex forms of materials but certain categories thus presenting products and by-products that are precursors for other microbial categories in the ecosystems. The enzymatic-mediated cascade constitutes the metabolic pathways within which nutrient cycling and colonization occur in the compost ecosystems. These metabolic pathways include those mediating the degradation and stabilization (catabolism and anabolism) of carbohydrates, proteins, lipids, and nucleic acids ultimately leading to the utilization of complex materials by microbial categories such as fungal and non-fungal

eukaryotes, before utilization of the simpler material forms mainly by prokaryotic classes leading the production of the stabilized humic substances (Duran et al., 2022).

The duration of composting can also influence the diversity and abundance of microbial populations and the efficiency and quality of composting. Several studies have reported changes in microbial community structure and function over time during composting (Kumar et al., 2012). For example, the degradation of complex organic compounds, such as lignocellulose, increases over time, leading to changes in the composition of microbial communities (Duran et al., 2020). Recent advances in sequencing technology, such as 16S rRNA gene sequencing and mRNA sequencing, have provided new insights into the microbial diversity and metabolic potential of compost (Sun et al., 2023).

One promising tool for predicting the metabolic potential of microbial communities is PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) (Douglas et al., 2020). PICRUSt uses 16S rRNA gene sequencing data to predict the functional potential of microbial communities, providing insight into the metabolic pathways involved in various biological processes. This tool has been applied to diverse environmental systems, including composting, to predict the metabolic potential and functional diversity of microbial communities.

Together, the use of PICRUSt and mRNA sequencing can provide a comprehensive view of the metabolic pathways, enzymatic profiles, and microbial contributors to these profiles in composting (Chen et al., 2019). The complementary approach of the two pathway prediction methods has the potential to improve our understanding of the composting process and to identify ways to optimize compost quality and efficiency.

7.2 Materials and methods

7.2.1 Total compost RNA extraction

A modification of the RNA extraction protocol described by Chomczynski, (1993) was adopted for purification. The total compost RNA was extracted from triplicate compost samples from each compost heap, including mRNA, rRNA, and tRNA, based on the trizol protocol. A pool of aseptically sampled combinations from five sampling points of each compost heap was used, and 250 µg of each compost sample was taken. The samples were then transported under liquid nitrogen to the Kenyatta University plant transformation laboratories for RNA extraction.

To extract the RNA, 750 µl of Trizol LS solution was added to each sample in a 2 ml eppendorf tube containing 0.5g (0.1mm) glass beads and vortexed for 5 seconds. This was followed by incubation for 10 minutes at room temperature allowing complete lysis of microbial cells. The samples were then centrifuged for 30 seconds at 12000 revolutions per minute to get the liquid down the tube. Next, 200 µl of chloroform: isoamyl alcohol (24:1) was added to the supernatant from each sample, and vortexed. The mixture was then incubated for 10 minutes at room temperature before centrifugation at 12000 rpm at 4°C.

The upper aqueous phase (500-550 µl) from each tube was then transferred to a sterile 1.5 ml eppendorf tube. The contents of the tube were then added 200 µl of chloroform: isoamyl alcohol (24:1) and incubated at room temperature for 10 minutes before centrifugation at 12000 revolutions per minute for another 10 minutes at 4°C. The upper aqueous phase (500-550 µl) from each tube was then transferred to a new 1.5 ml eppendorf tube. This was followed by the addition of 500 µl of isopropanol, before vortexing for 30 seconds and centrifugation at 12000 rpm for 10 minutes at 4°C.

At this stage, the RNA precipitate formed a gel-like pellet on the bottom of each eppendorf tube. After centrifugation, the supernatant was discarded, and 500 µl of 75% ethanol was added to the RNA precipitate. The tubes were gently inverted, and centrifuged for 2 minutes at 12000 rpm at 4°C. The supernatant was then removed, and the RNA pellet was air-dried at room temperature for 10 minutes. The dry pellets were shipped under dry ice to the Molecular research DNA laboratories for cDNA synthesis and library preparation.

7.2.2 Messenger RNA (mRNA) sequencing

The triplicate RNA samples of each treatment were resuspended in 30 ul RNase-free water and were pooled in 4 groups (L, T, G, and LTG). Pooled samples were cleaned using RNeasy PowerClean Pro Cleanup Kit (Qiagen) and concentrations were determined using the Qubit® RNA Assay Kit (Life Technologies). RNA samples were treated to remove the DNA contamination using Baseline-ZERO™ DNase (Epicentre) following the manufacturer's instructions followed by purification using the RNA Clean and Concentrator-5 columns (Zymo Research). Because of low RNA concentration, amplification of total RNA was performed by using QuantiTect Whole Transcriptome kit (Qiagen). This was followed by transcript library preparation using Illumina DNA Prep, (M) Tagmentation library preparation kit (Illumina) following the manufacturer's user guide.

The concentration of double-strand cDNA was evaluated using the Qubit® dsDNA HS Assay Kit (Life Technologies). 50 ng dscDNA was used to prepare the libraries. The Qubit® dsDNA HS Assay Kit (Life Technologies) was also used to determine the final concentration of all the prepared libraries. On the other hand, Agilent 2100 Bioanalyzer (Agilent Technologies) was used to measure the average library size.

7.2.3 Metatranscriptome analysis

Two metatranscriptome analysis pipelines were developed for this study based on the Parkinson lab tutorials (https://github.com/ParkinsonLab/Metatranscriptome-Workshop) and Simple Analysis of Metatranscriptome Sequence Annotations (SAMSA). Raw reads quality analysis was done under FastQC software (version 0.11.9) before downstream bioinformatics analysis. The quality analysis included deducing the number of reads, length of reads, GC content of the sequences, and the distribution of sequences before downstream analysis. Firstly, the removal of sequence adapters, which are normally added during RNA library preparation and sequencing was done. Trimming of low-quality bases and sequencing reads was also done using Trimmomatic v.0.39 module. This was followed by the removal of duplicate reads using the software tool CD-HIT and abundant rRNA using Infernal (http://infernal.janelia.org/), before the re-duplication of the duplicate reads. Afterward, the classification of reads to known taxonomic groups based on the Kaiju reference database (https://github.com/bioinformatics-centre/kaiju). Visualization of the taxonomic composition was done using Krona tools (https://github.com/marbl/Krona/wiki).

The SAMSA pipeline was run by first downloading dependencies such as DIAMOND (https://github.com/bbuchfink/diamond), PEAR (https://sco.h its.org/exelixis/web/soft - ware/pear/), and SortMeRNA (http://bioinfo.lifl.fr/RNA/sortmerna/). The RefSeq bacteria database, and the SEED Subsystems database, were downloaded for specific functional results and hierarchical functional ontology respectively. Preprocessing was done by first merging mate pairs of the paired-end sequences using PEAR before adaptor and low-quality reads removal as described above. SortMeRNA was used to remove ribosomal

sequences, as they do not bear any functional capacity. Afterward, the abundance summaries of the DIAMOND output from each metatranscriptome file were grouped, before summarizing and plotting the volcano plots of the top 15 functional profiles (enzymes/proteins) from the respective treatments, using DESeq package. The clustering of assorted proteins into the six major classes of enzymes and associated proteins was done manually using the Expasy database (https://enzyme.expasy.org/).

7.2.4 Metagenomic-based functional prediction

Phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt2) (version 2.4.1) tool was used to predict assorted functional potentials based on ASVs marker gene sequences of prokaryotes (16S). This was only done for 16S since the protocol is not fully developed and validated for the remaining amplicons. The Kyoto Encyclopedia of Genes and Genomes; KEGG Orthology (KO) database was used to predict gene function based on homology searches against known genes. The KO assignments were then used to build gene families for predicting the functional potential of microbial communities. Pathway referencing was done under the METACYC database for prokaryotic pathways (https://biocyc.org/). The resulting PCA, heat maps, and boxplots of pathways responsible for composting were plot using statistical analysis of taxonomic and functional profiles (STAMP) (version 2.1.3) software.

7.3 Results

7.3.1 The concentration of double-stranded complementary RNA

The resulting double-stranded DNA of the respective treatments ranged between 127.80 ng/uL and 143.00 ng/uL while the average library size ranged between 654bp ng/uL and 807bp: Table 7.1.

Table 7. 1: **The concentration of dscDNA, final library concentration, and average library size**.

Sample	dscDNA Concentration (ng/µL)	Library Concentration (ng/µL)	Avg Library size (bp)
L	143.00	11.90	782
T	134.00	12.50	807
G	127.80	7.80	789
LTG	115.00	17.60	654

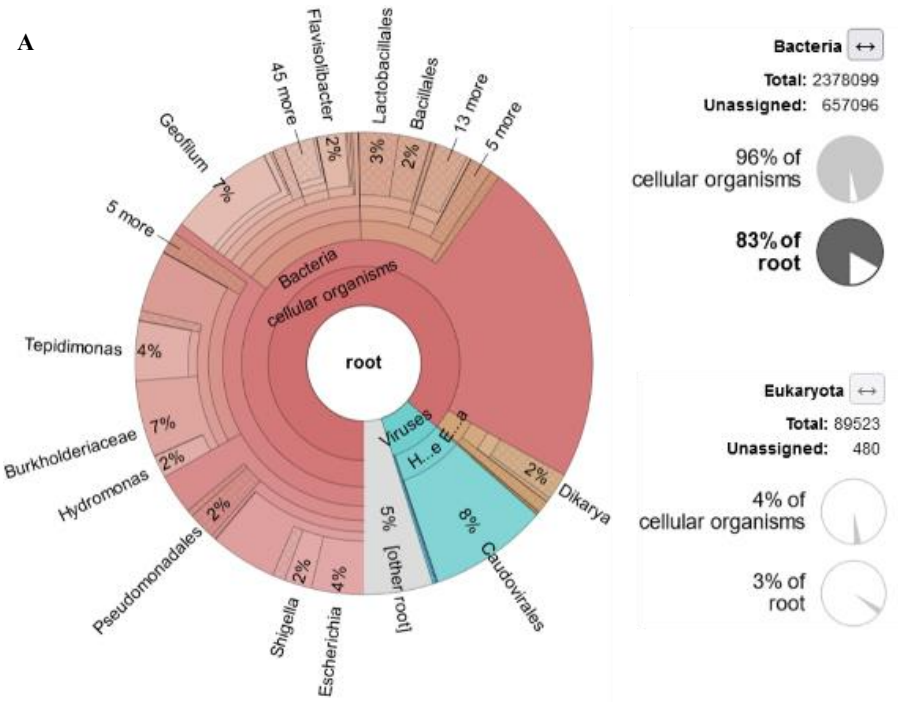
7.3.2 Active microbes contributing to assorted metabolic pathways

The bacteria population accounted for 96% of all cellular organisms in Lantana-based compost and 74% in Tithonia-based compost. On the other hand, this category accounted for 37% of all cellular organisms in Grass-based and 98% in mixed compost (LTG). Present bacteria classes across all compost treatments were mainly from *Proteobacteria* (*Pseudomonadota*), *Terrabacteria, PVC group, Spirochaetes*, and unidentified taxa**.** Lantana-based compost had *Proteobacteria* (*Pseudomonadota*) 43% of total bacteria, Tithonia-based compost had 37%, G had 44%, and mixed compost (LTG) had 22%. It was observed that mixed compost had a higher relative abundance of *Spirochaetes* compared to other composting treatments. *Proteobacteria (Pseudomonadota)* was the dominant prokaryotic category of the bacteria group in this sample **(**Figure 7.1A-D**).**

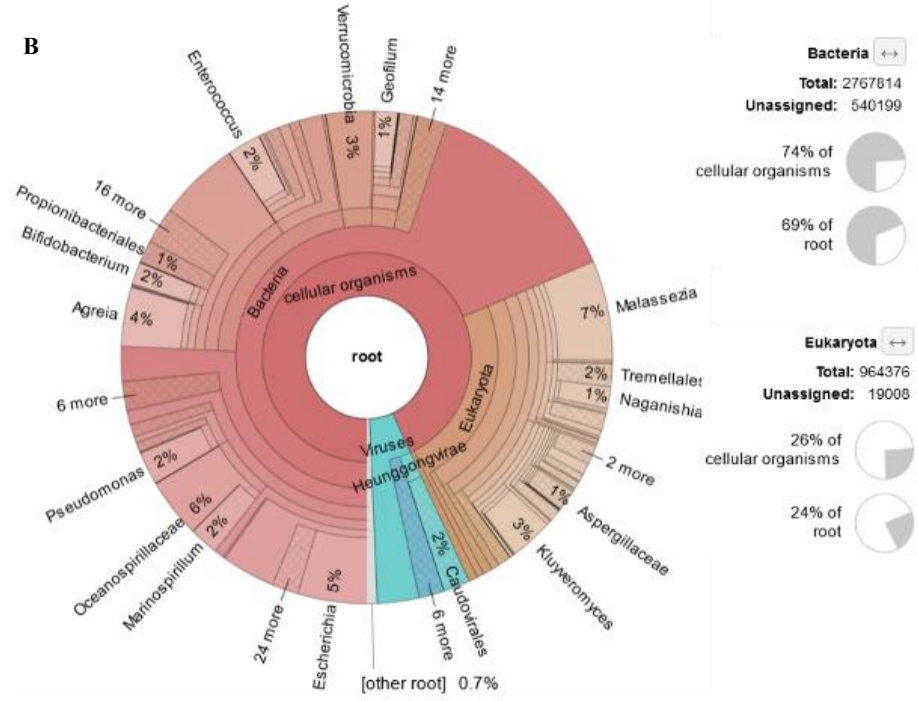
Eukaryotes on the other hand accounted for 4%, 26%, 62%, and 2% of all the active cellular organisms in Lantana-based, Tithonia-based, Grass-based, and mixed compost (LTG) respectively (Figure 7.1A-D). Fungal class *Ascomycota* was the most dominant active fungal population in all compost treatments accounting for 39% in Grass-based, 53% in

Lantana-based, and 41% in Tithonia-based. *Saccharomyceta* was on the other hand the dominant fungal class in mixed compost (LTG) accounting for 50% of all the active fungal classes. *Dikarya* was the predominant active fungal class in Lantana-based compost, accounting for 86% of all active fungi in the compost. Overall, this class included clades such as *Ascomycota* and *Basidiomycota* (Figure 7.1A-D).

A


Flavisolibacter
Lactobacillales
Bacillales
13 more
5 more
45 more
Geofilum
5 more
Bacteria
cellular organisms
root
Tepidimonas
Burkholderiaceae
Hydromonas
Pseudomonadales
Shigella
Escherichia
[other root]
Caudovirales
Dikarya
Bacteria
Total: 2378099
Unassigned: 657096
96% of cellular organisms
83% of root
Eukaryota
Total: 89523
Unassigned: 480
4% of cellular organisms
3% of root

B


Verrucomicrobia
Geofilum
14 more
Enterococcus
16 more
Propionibacteriales
Bifidobacterium
Agreia
6 more
Pseudomonas
Oceanospirillaceae
Marinospirillum
24 more
Escherichia
[other root] 0.7%
6 more
Caudovirales
Kluyveromyces
Aspergillaceae
2 more
Naganishia
Tremellales
Malassezia
Bacteria
cellular organisms
root
Eukaryota
Viruses
Heunggongvirae
Bacteria
Total: 2767814
Unassigned: 540199
74% of cellular organisms
69% of root
Eukaryota
Total: 964376
Unassigned: 19008
26% of cellular organisms
24% of root

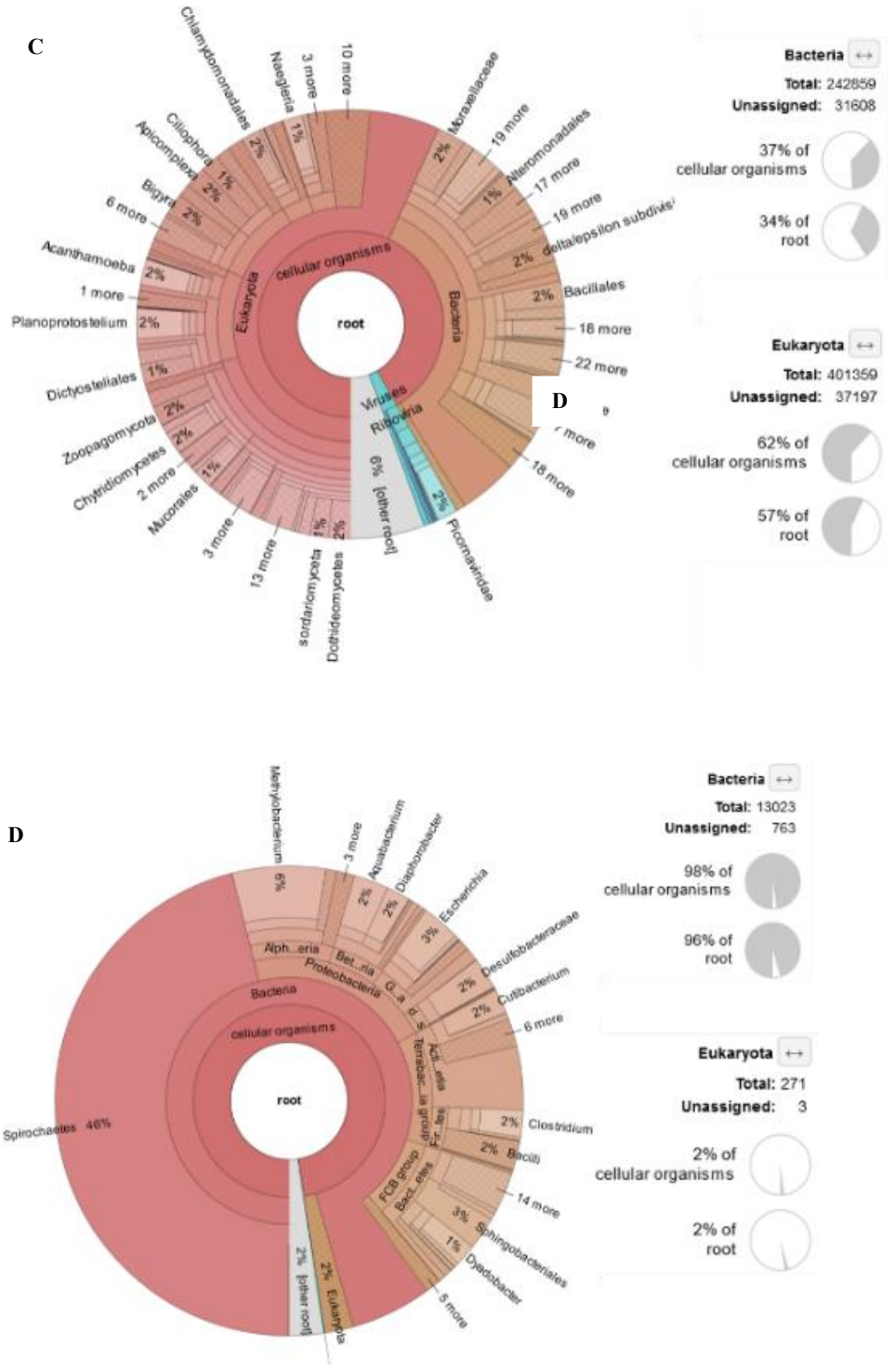


Figure 7. 1: **A-D; Graphical representation (Krona plot) of the cellular organisms contributing to pathways in various compost treatments.**
Further details including the interactive Krona outputs are available at: file:///E:/PhD%20data%20outputs%20and%20manuscripts/mRNA/Krona%20results

7.3.3 Different green feedstock materials had a significant influence on compost

A total of 7860 transcripts were identified as significantly and differentially enriched between compost treatments (L, T, and LTG) and control (G) datasets. There was higher upregulation of the total significantly ($p < 0.01$) regulated genes compared to the downregulated genes (Figure 7.2).

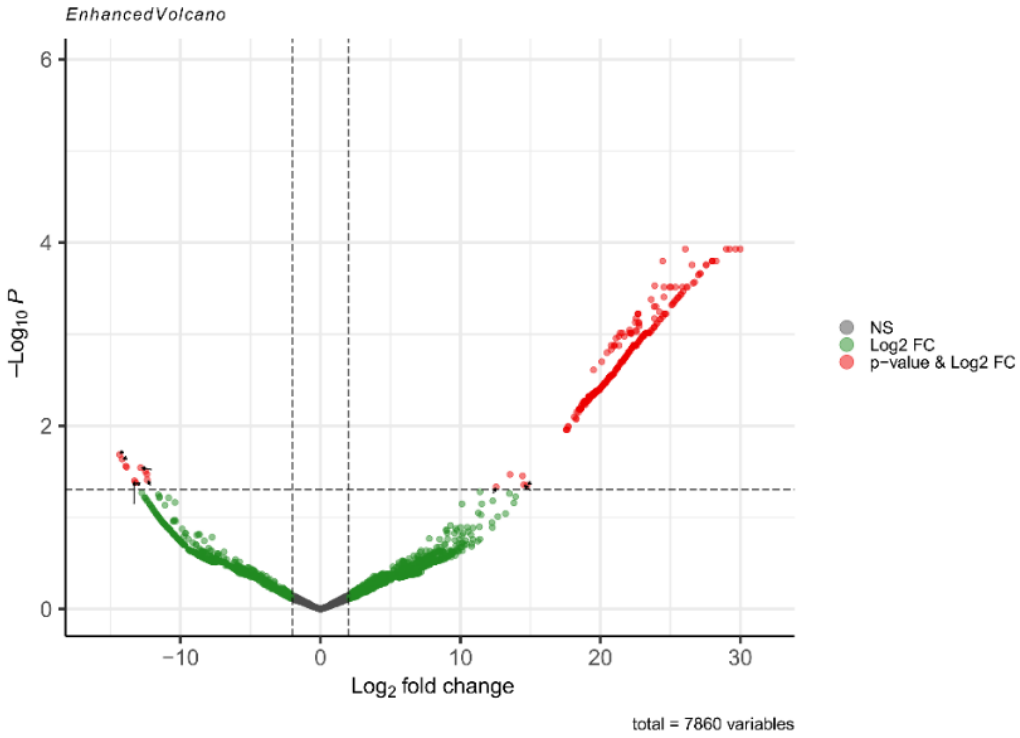


Figure 7. 2: **DESeq volcano plot of overall gene expression in the compost environment.**
Each dot on the plot is a single gene/transcript/feature. Horizontal axis: fold change; vertical axis: p-value (in log10 scale). Colour coding is based on the fold change. Thick vertical lines highlight fold changes of -2 and +2, while a thick horizontal line represents a p-value of 0.01. NS represents sequence abundances that had no significance.

7.3.4 Different green feedstock materials influence compost functional pathways similarly

Prediction of sequences associated with major functional metabolic pathways revealed the five most abundant pathways: nucleoside and nucleotide biosynthesis, cofactor, carrier and

vitamin biosynthesis, amino acid biosynthesis, energy biosynthesis, and fatty acid and lipid biosynthesis. Carbohydrate biosynthesis and cell structure biosynthesis also ranked high in terms of abundance. Based on this hierarchical clustering, the various compost types and days were clustered into two main clades. G2 (Grass-based compost at 42 days) clustered alone, while LTG1, T1, L2, LTG2, L1, T2, and T4 clustered on the second clade. LTG4, G3, G4, L3, L4, and LTG3 clustered together. T3 and G1 clustered on one unique clade (Figure 7.3).

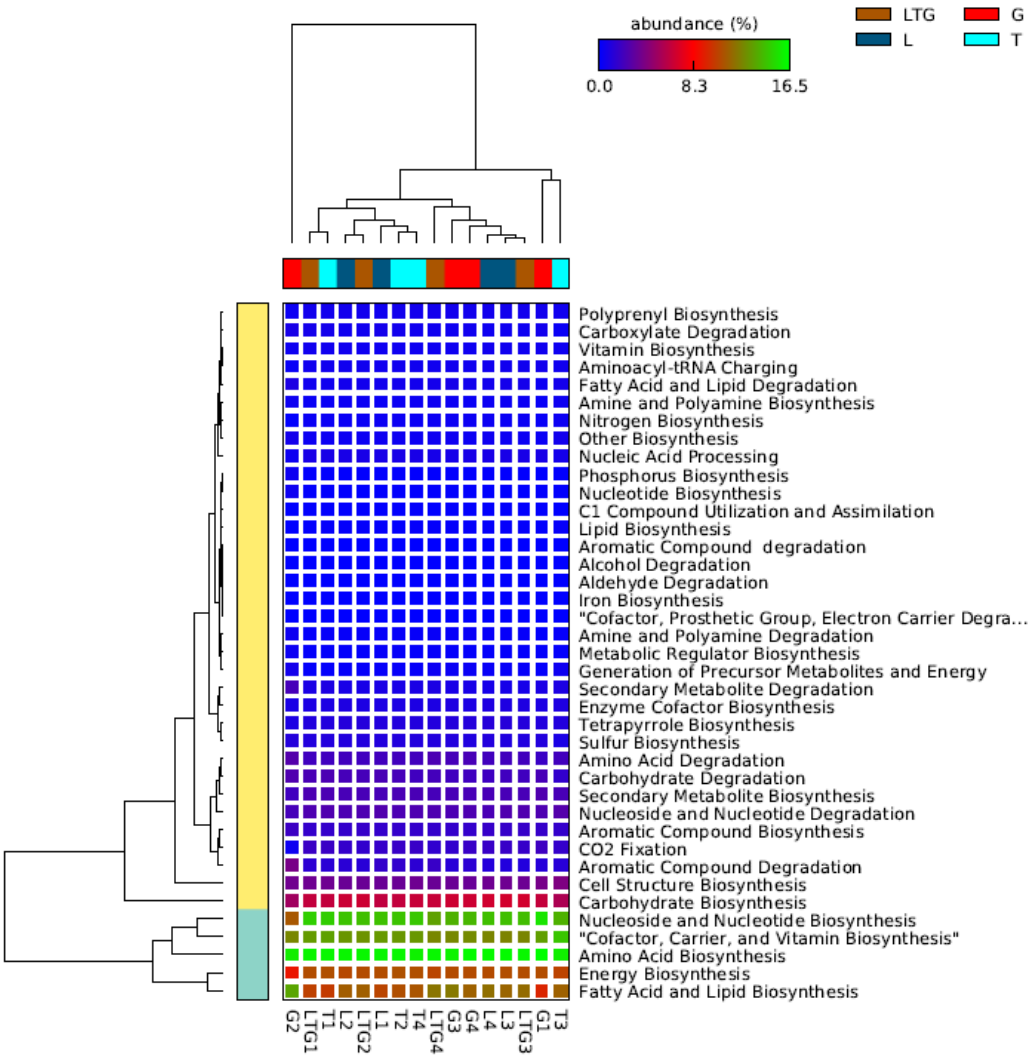


Figure 7. 3: **Heatmap of the normalized relative abundances of the predicted functional categories of microbiomes associated with various composting treatments and days**

This study revealed no significant (P= 0.353) difference among the treatments on carbohydrate biosynthesis; Fig. 7.4A. There was also no significant difference (P=0.649) in carbohydrate biosynthesis among the composting days; Fig. 7.4B. There were no significant differences observed among compost treatments concerning carbohydrate degradation. However, Lantana-based compost recorded the highest number of sequences responsible for carbohydrate degradation (Fig. 7.4C). There were significant differences among the composting days, with the highest carbohydrate degradative sequences recorded on the 42nd day of composting (Fig. 7.4D). Despite the notable differences and shifts in nitrogen levels recorded among the compost treatments and composting days, the abundance of sequences responsible for nitrogen biosynthesis showed no significant difference (Fig. 7.4E and F).

Most other pathways did not have significant differences as influenced by composting materials or duration (Appendices 11-17). This is except for the aromatic compounds degradative pathways which were significantly different among compost days (appendix 17).

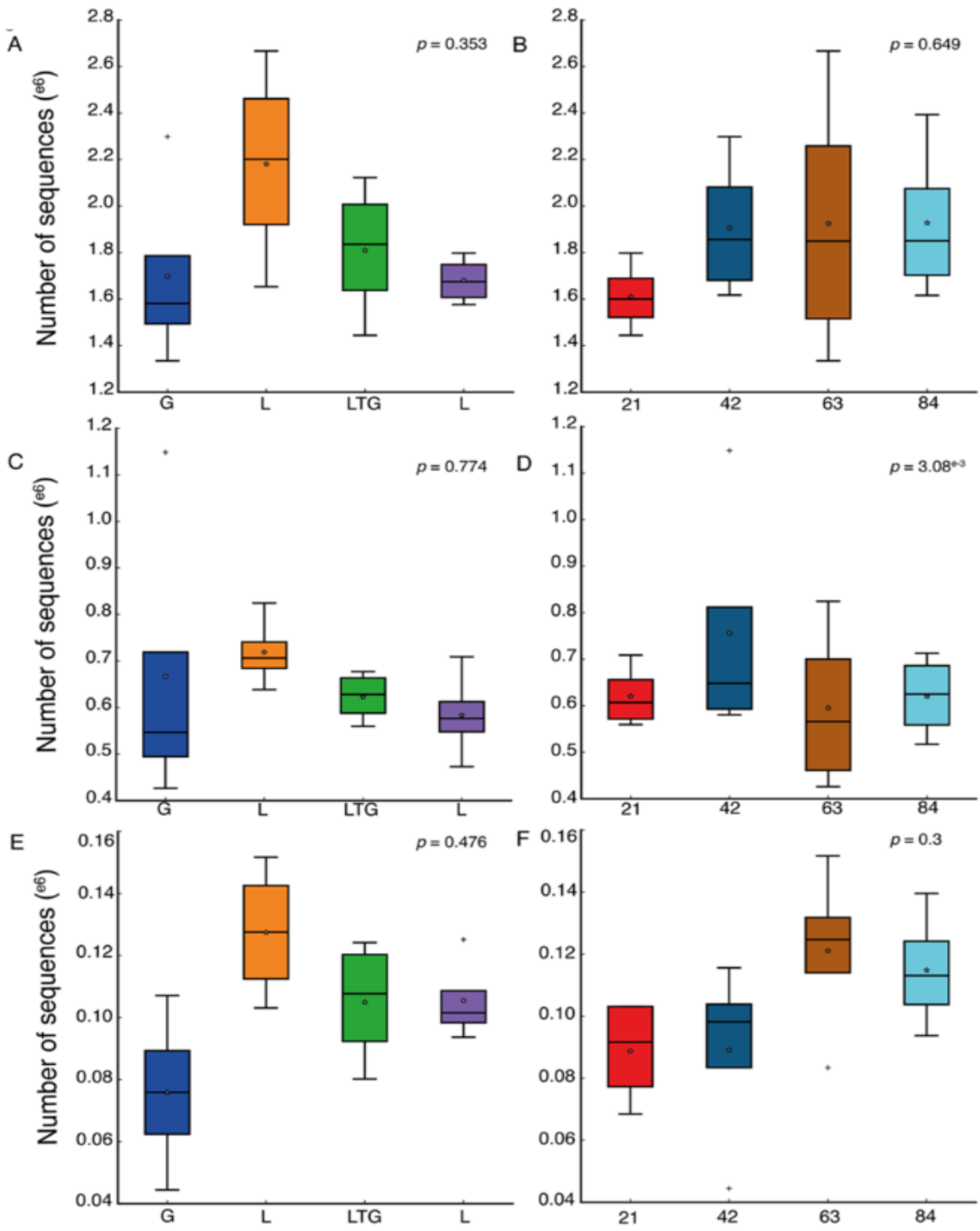


Figure 7. 4: **The abundance of prokaryotic sequences responsible for assorted metabolic pathways.**
Carbohydrate biosynthesis as influenced by composting material (A) and composting days (B); carbohydrate degradation as influenced by composting material (C) and composting days (D); Nitrogen biosynthesis as influenced by composting material (E) and composting days (F).

7.3.5 Influence of green feedstock materials on compost pathways associated enzymes and proteins

The study deduced differences in abundances and diversities of assorted enzymatic classes as influenced by the composting materials. Grass-based compost had the least total abundance of proteins (1.6e + 0.5), while Tithonia-based compost had the most abundance of these proteins (10.6e + 0.5). There were also notable differences in the abundance of individual proteins among the assorted compost treatments. For example, Grass-based compost had the most abundance of ribosomal and cytoskeletal proteins among all treatments. On the other hand, Lantana-based compost had the most abundance of transporter proteins among all the composting treatments, with mixed compost accounting for most of the hydrolase enzymes among all the treatments (Figure 7.5).

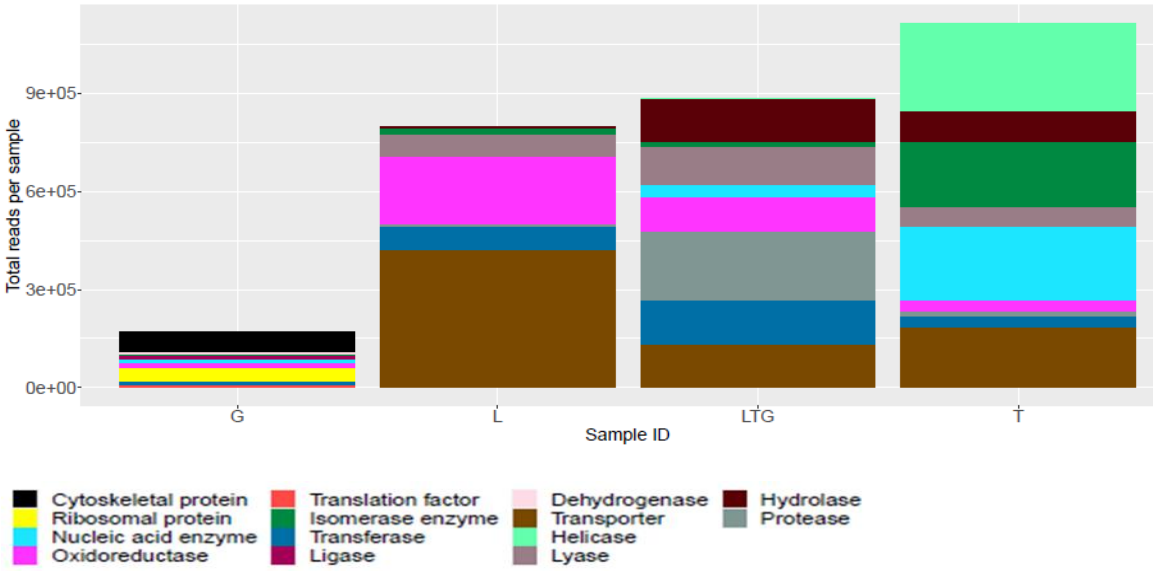


Figure 7. 5: **The relative abundance of compost assorted enzymatic classes and allied proteins**
G is Grass-based compost, L- Lantana-based compost, LTG is mixed compost, and T is Tithonia-based compost.

The intra-treatment activity of assorted enzymes and allied proteins showed the transporter proteins prominently featured in Lantana, mixed, and Tithonia-based composts, which was

not the case in Grass-based compost. The relative activity of oxidoreductases was most prominent in Lantana-based compost compared to other treatments. On the other hand, cytoskeletal proteins accounted for most of the enzymatic activity in Grass-based compost (Figure 7.6).

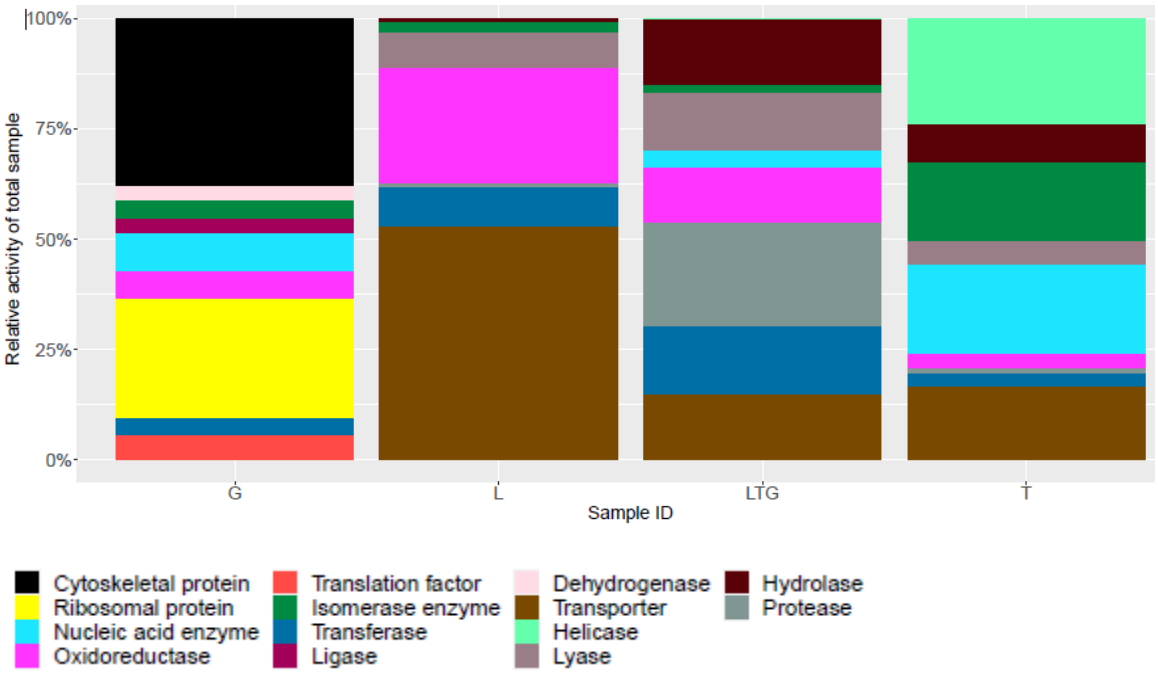


Figure 7. 6: **The relative activity of compost assorted enzymatic classes and allied proteins**
G is Grass-based compost, L- Lantana-based compost, LTG is mixed compost, and T is Tithonia-based compost.

7.4 Discussion

Many *Pseudomonadota* classes are known to degrade recalcitrant material accounting for most bacteria categories in compost since composts are dominated by recalcitrant material such as lignocellulose which is in maize stalks. Environmental *Spirochaetes* have been reported in anoxic environments where they scavenge biomass, therefore, enabling the recycling of necromass (Lopez et al., 2021). This, therefore, points out their dominance in an environment with antagonistic microbial communities with each community structure

being responsive towards the constituent green material source (LTG). This antagonism is also attributable to the constituent material inhibiting non-persistent microbial communities. Their dominance in the metabolism of wood was reported in the hindgut biome of termites (Nishamura et al., 2020).

Pathways for biosynthesis of nucleic acid components (nucleosides and nucleotides), vitamins, amino acids, cofactors, carrier molecules, fatty acids, and lipids were shown as critical contributors to nutrient cycling and microbial proliferation in compost. Nucleosides and Nucleotides are required for cell growth and replication pathways, while amino acids, fatty acids, and lipids are building blocks for microbial cell structure (Berg et al. 2002; Moore 2021). Cofactors, carriers, and vitamins play a critical role in microbial enzymatic functions, ultimately directly influencing nutrient cycling through catabolic and anabolic processes within the available pool of nutrients in the compost environment (Xavier et al., 2017). The high abundance of energy metabolism pathways explains the rise in compost temperatures, generally higher than ambient temperatures until the compost is mature. Energy metabolism is a critical pathway in central carbon metabolism, hydrogen oxidation, and Nitrogen cycling through pathways such as ammonia oxidation (Tang et al. 2011; Koch et al. 2014).

The uniqueness of G2 (Grass-based compost at 42 days of composting) in the heatmap is attributable to the higher abundance of pathways responsible for aromatic compound degradation and the lower Nucleoside and nucleotide biosynthetic pathways compared to other samples. The pathways for aromatic compound degradation are associated with *Gammaproteobacteria,* the most abundant phylum in G2. *Gammaproteobacteria* is

responsible for aromatic compound degradation through anaerobic peripheral pathways (Hassanshahian et al., 2015).

The study observed that Lantana-based compost had the highest abundance of Carbohydrate biosynthesis pathways. Such pathways include the Calvin-Benson-Bassham cycle and reductive acetyl-coenzyme A pathway (Serrato et al., 2009). These pathways predominantly utilize diverse carbohydrate metabolites in compost for microbial cellular energy needs or for building stable materials such as carboxylates. Therefore, Lantana-based compost could have had diverse, complex materials producing the metabolites necessary for the biosynthesis of stable organic forms. Furthermore, this points to the treatment as superior in the sequestration of volatile carbohydrate compounds like CO2 to produce readily available substrates such as acetate (Miltner et al. 2005; Abdullahi et al. 2018). The higher number of carbohydrate biosynthesis sequences points to that period of composting as having the best biogeochemical conditions for the assimilation of carbohydrate metabolites to produce stable organic substrates like acetate.

A similar scenario in carbohydrate degradation is a complementary indication of the presence of complex carbon polymers in Lantana-based compost compared to other treatments. Therefore, more pathways are necessary to convert complex materials such as lignin into simple carbohydrates (Jurak et al. 2015; Sinha et al. 2021). The significantly higher abundance of Carbohydrate degradation pathways at 42 days compared to other composting days indicates colonization of the compost heap by prokaryotes with high degradative capabilities of recalcitrant carbohydrates such as lignin. Such prokaryotes include *Pseudomonas* (Lee et al., 2021).

The study did not observe significant differences in nitrogen biosynthesis pathways among the compost treatments and composting days. Nonetheless, Lantana-based Compost (L) had the most abundant pathways of nitrogen biosynthesis, pointing to the complexity of the nitrogenous content of Lantana compared to other composting materials, therefore requiring more metabolic routes before Nitrogen is in available form.

The high abundance of nitrogen biosynthesis pathways in Lantana-based Compost (L) explains the higher total Nitrogen than other compost treatments. The study observed a higher number of nitrogen biosynthetic pathways at 63 days related to other composting days. This pathway abundance is related to the higher abundance of class *Polyangia* at 63 days in all composting treatments than on other composting days. *Polyangia* is a Myxobacteria that utilizes complex inorganic Nitrogen, biodegrading cellulolytic material (Dawid, 2000; Bhat et al. 2021). This period also coincides with the period when bacteria have fully utilized all available forms of carbohydrates, leaving complex materials like lignin and cellulose that are degradable by categories like *Polyangia* with the metabolic capability to break down these materials.

Through enzymatic activity, complex organic matter is cycled in the ecosystems into soil nutrients that are necessary for the promotion of plant growth and overall soil fertility. The differences in abundance and diversity of enzymes and allied proteins in the compost ecosystems as influenced by assorted green feedstock material indicate their contribution to elicit a complete ecological response. The differences in enzymatic response are due to the phytochemical composition and properties of the various materials, thus recruiting enzymes differently to break them down. For example, hydrolases, which include cellulases that hydrolyze complex carbon polymers such as cellulose in ecosystems to

simpler sugars imply the presence of these materials in the compost environment (Houfani et al., 2022).

CHAPTER EIGHT

GENERAL DISCUSSION, CONCLUSIONS, AND RECOMMENDATIONS

8.1 General discussion

Composting is a complex biological process that involves assorted interactions between eukaryotes, prokaryotes, and physical-chemical parameters as well as diverse functional capabilities (Lekasi et al. 2003; Meena et al. 2021). Eukaryotes, such as fungi and protozoa, are important in ecosystems due to their capacity to break down complex organic compounds, while prokaryotes, such as bacteria and archaea, utilize the products of this decomposition hence cycling nutrients (Matheri et al., 2023a).

Physical-chemical metrics like moisture, pH, temperature, and nutrient availability influence the functional capability of compost eukaryotes and prokaryotes (Adamtey, 2005; Aguilar-Paredes et al. 2023). Moisture content is also an essential factor that influences composting, as it affects microbial activity and oxygen availability. pH is another critical parameter that influences composting, as it affects the growth and metabolic activity of microorganisms. Nutrient availability is also crucial, as it influences the metabolic activity and colonization capacity of microorganisms (Rastogi et al., 2020).

The nature of the composting feedstock has a crucial influence on the nutrient content and microbial composition of compost (Rynk et al., 2022). The nutrient content of the composting material is directly proportional to the nutrient content of the final compost. Materials such as food waste, yard waste, and animal manure are rich in essential nutrients such as nitrogen, phosphorus, and potassium. The addition of such materials to the compost pile results in the production of compost that is rich in nutrients, making it an ideal soil amendment for growing plants.

Moreover, microbial diversities and abundance also influence the composting process since assorted microbial categories utilize assorted forms of organic materials. In principle, bacteria and fungi are the main microbial contributors to nutrient cycling in compost, by breaking down the organic matter during composting (Antunes et al 2016; Matheri et al. 2023a; Matheri et al. 2023b). The composition of the composting material determines the type of microorganisms present in the compost pile. For instance, food waste contains a high concentration of nitrogen, which favors the growth of bacteria that thrive in nitrogen-rich environments. On the other hand, woody materials, such as sawdust and wood chips, are high in carbon and encourage the growth of fungi (Noll and Jirji 2012; Clocchiatti et al. 2020).

The microbial structure of the composting material affects the rate and efficiency of the composting process. A diverse microbial community in the compost pile results in a faster and more efficient composting process. The presence of a wide array of organisms ensures that all the organic matter is broken down into compost, resulting in a nutrient-rich product.

Metagenomics and metatranscriptomics are emerging molecular techniques that offer several advantages in studying the microbial community structure in compost. Metagenomics involves the direct sequencing of the entire microbial community DNA, whereas metatranscriptomics involves the sequencing of the RNA transcripts produced by the microbial community. The combination of these techniques provides a more thorough understanding of the community structure and function of assorted compost microbes (Boparai and Sharma, 2021).

Another advantage of metagenomics and metatranscriptomics is their ability to provide a quantitative assessment of the microbial community structure. Traditional methods of studying microbial communities in compost, such as 16S rRNA gene sequencing, only provide a qualitative assessment of the microbial community structure. In contrast, metagenomics and metatranscriptomics can provide a quantitative assessment of the relative abundance of different microbial taxa and their gene expression levels. This information can be used to identify the key microbial players in the compost ecosystem and their contribution to the composting process.

Metagenomics and metatranscriptomics also have the potential to provide insight into the functional relationships between different microbial taxa in the compost ecosystem (Antunes et al. 2016; Matheri et al. 2023b). For example, metagenomics can identify the presence of genes involved in the digestion of complex (cellulose proteins and lignin) materials in the ecosystem. Metatranscriptomics can reveal the expression of these genes and the microbial taxa responsible for their expression. This information can be used to identify potential synergistic relationships between different microbial taxa in the compost ecosystem and to optimize composting processes for maximum efficiency.

8.2 Conclusions

A polyphasic approach, integrating multiple methods, is recommended for the comprehensive determination of functional potential in compost ecosystems. This study evaluated the influence of different green composting feedstocks and composting durations on manure stability, microbial community dynamics, and metabolic profiles. The following conclusions were drawn:

i) Physical and Chemical Shifts in Manure: Contrary to expectations, the different green composting feedstocks did not significantly influence the physical and chemical properties of the compost. The composting duration played a more prominent role in determining manure stability and nutrient cycling, with longer periods enhancing compost maturity and stabilization, regardless of the specific feedstock used.

ii) Prokaryotic Community Evolution: The microbial community, particularly prokaryotic organisms, exhibited shifts that were more dependent on composting phases rather than the type of green feedstock. Classes such as *Firmicutes* and *Proteobacteria* dominated in early thermophilic phases, while *Actinobacteria* and others became prominent in later stages, reflecting typical microbial succession patterns during composting.

iii) Fungal and Non-Fungal Eukaryotic Communities: Similar to prokaryotic communities, fungal and non-fungal eukaryotic populations evolved according to composting phases rather than the type of feedstock used. While feedstock variations had little impact, the duration of composting enhanced fungal diversity, which is essential for breaking down complex organic matter in the later stages of composting.

iv) Metabolic Potential of Compost Microbes: The study found that metabolic enzyme activities, essential for nutrient cycling, were not significantly altered by the type of green feedstock. Instead, these activities were primarily influenced by composting duration, suggesting that microbial metabolic potential in compost

ecosystems is driven more by composting time than by the specific green materials added.

8.3 Recommendations

Based on the findings of this study, it is recommended that future composting practices prioritize composting duration over the specific type of green feedstock to enhance compost quality and stability. While variations in compost materials may have minimal influence on physical chemistry and microbial metabolic potential, extending the composting duration ensures optimal microbial activity and nutrient cycling. Additionally, adopting a polyphasic approach; combining microbiological, chemical, and enzymatic assessments will provide a more holistic understanding of compost dynamics and help refine compost management strategies for sustainable agriculture.

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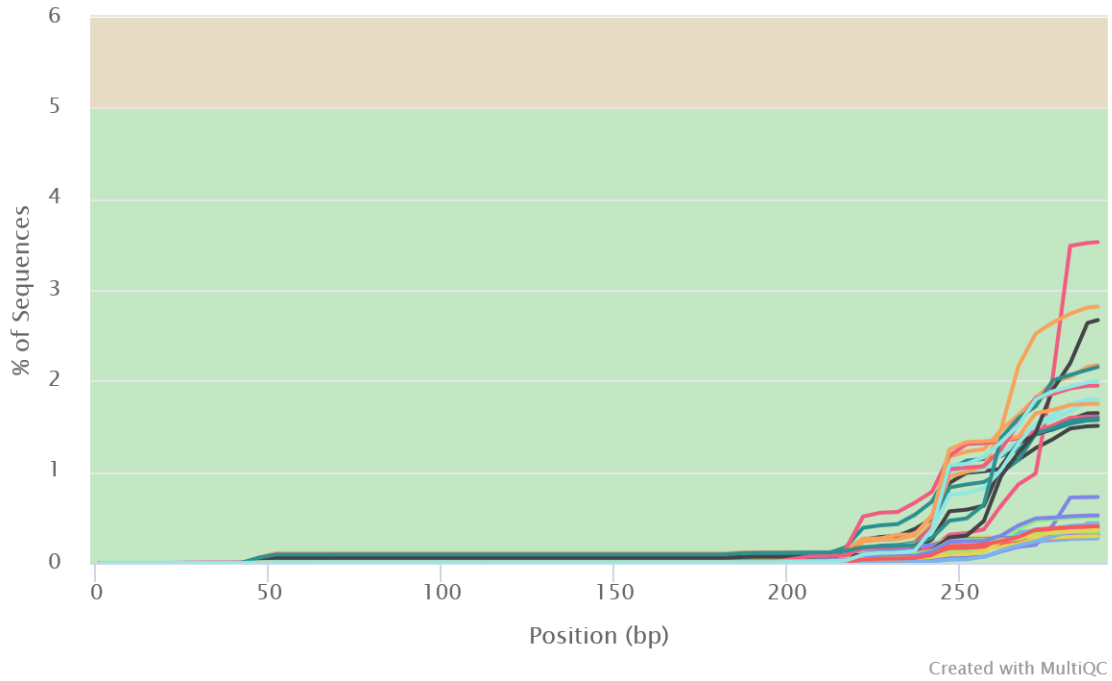
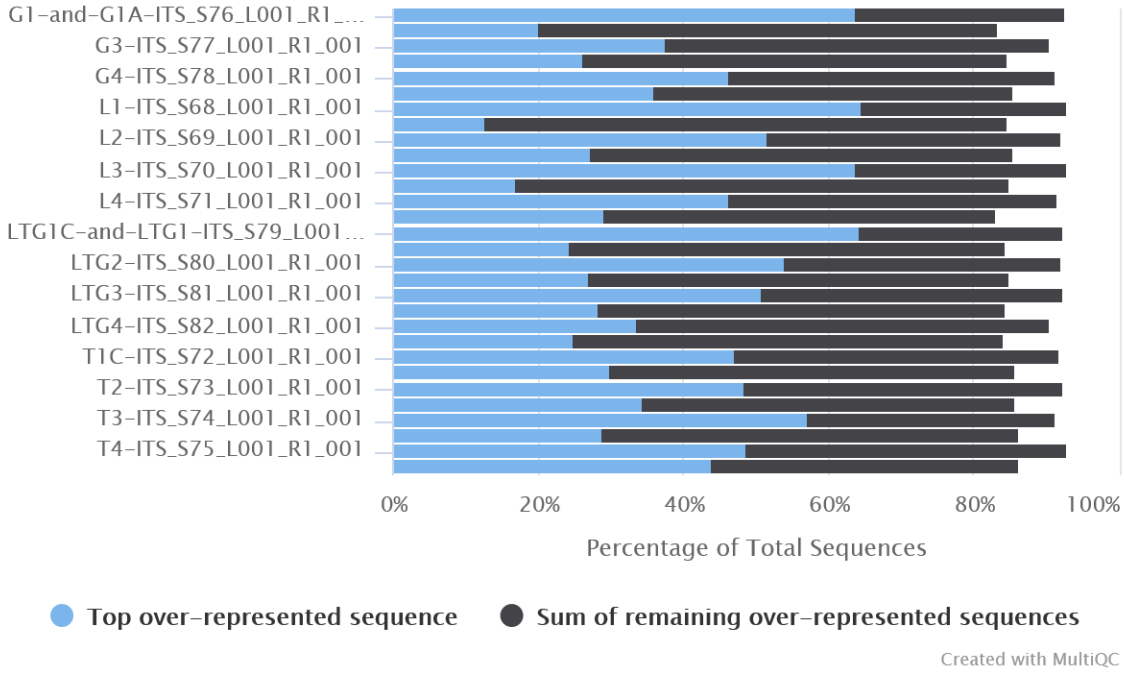
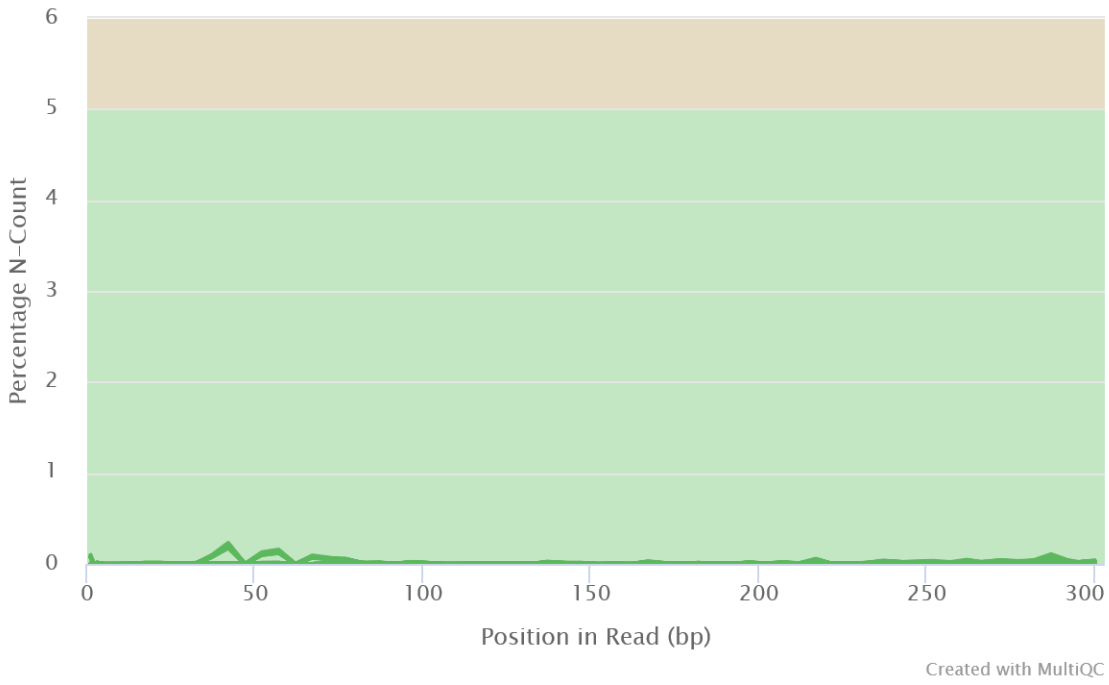
APPENDICES



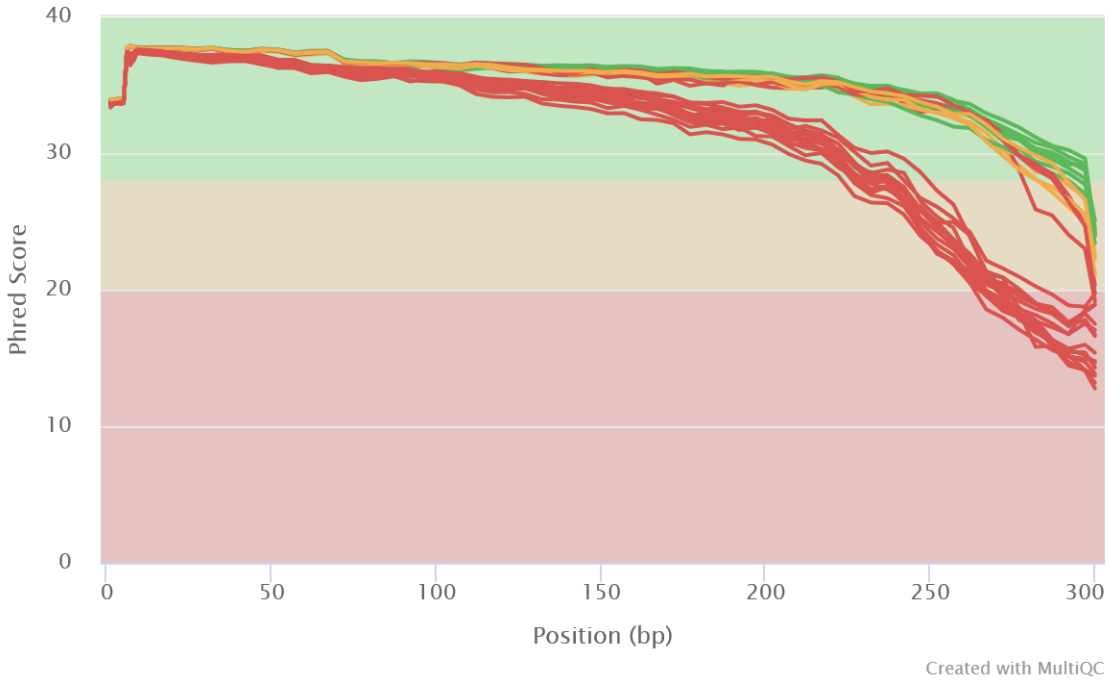
Appendix 1: **FASTQC generated plot of adapter content of DNA sequences**



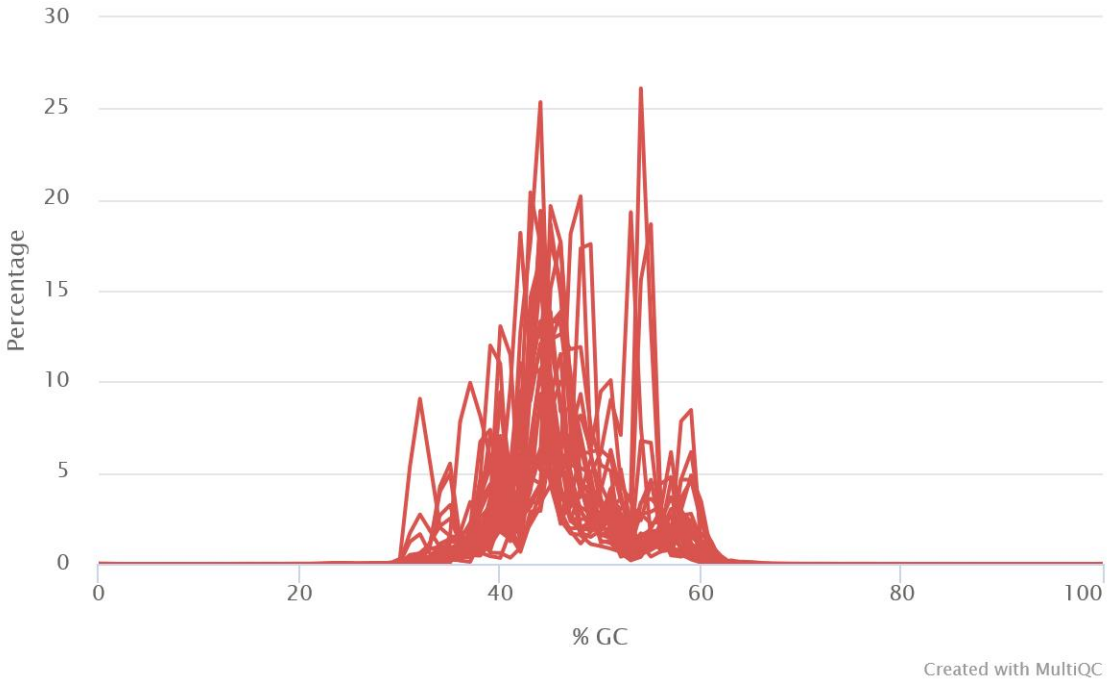
Appendix 2: **FASTQC generated a plot of overrepresented sequences**



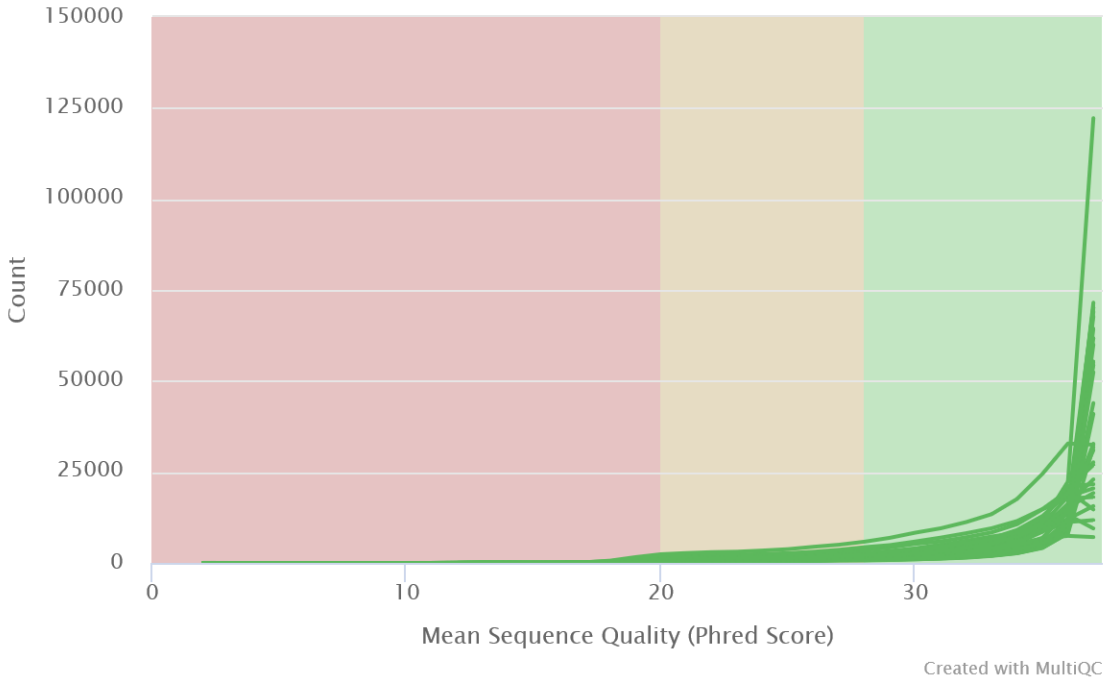
Appendix 3: **FASTQC generated plot of per base N content of compost DNA sequences**



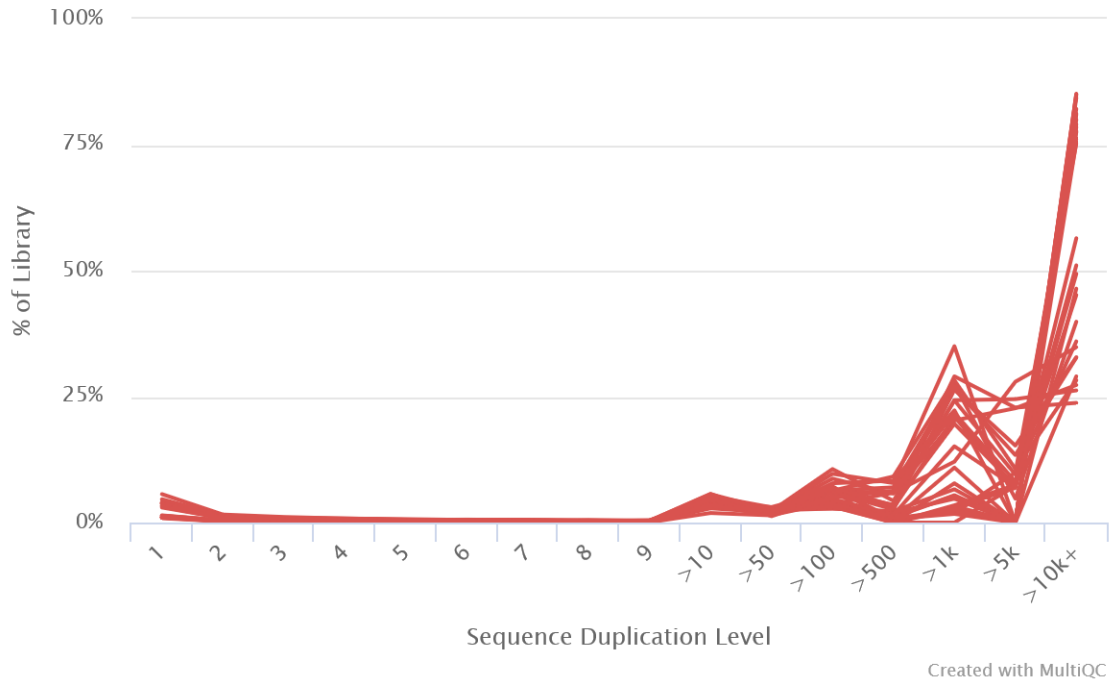
Appendix 4: **FASTQC generated mean per base quality scores of compost DNA sequences**



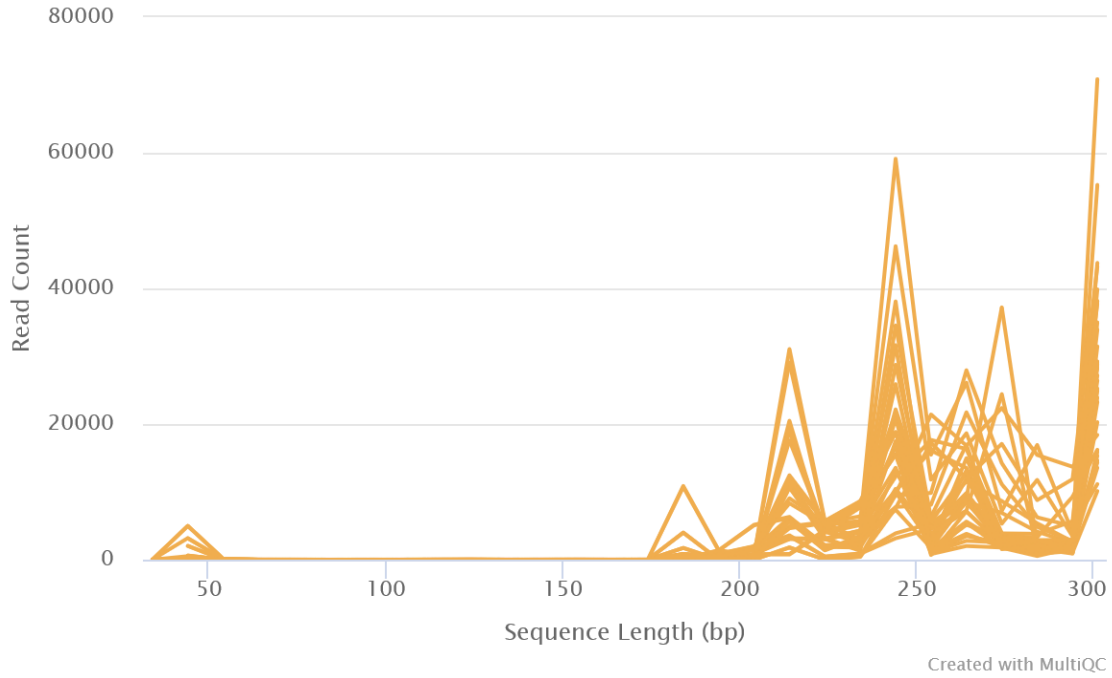
Appendix 5: **FASTQC generated mean per sequence GC content of compost DNA sequences**



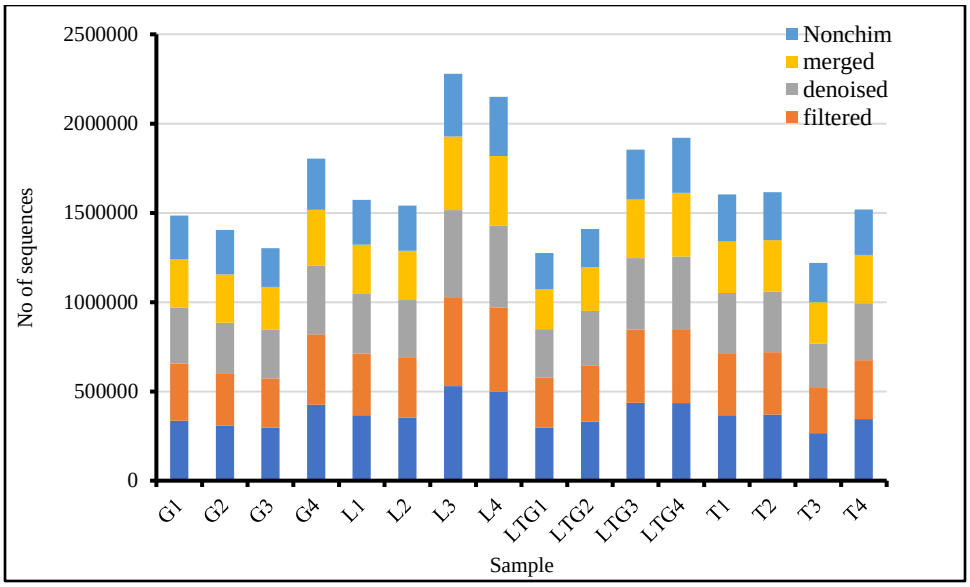
Appendix 6: **FASTQC generated mean per sequence quality scores of compost DNA sequences**



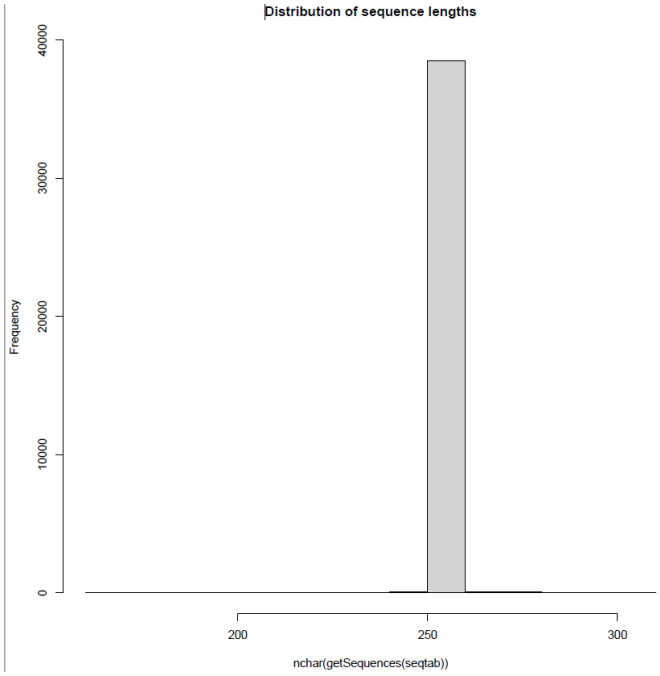
Appendix 7: **FASTQC generated sequence duplication of compost DNA sequences**



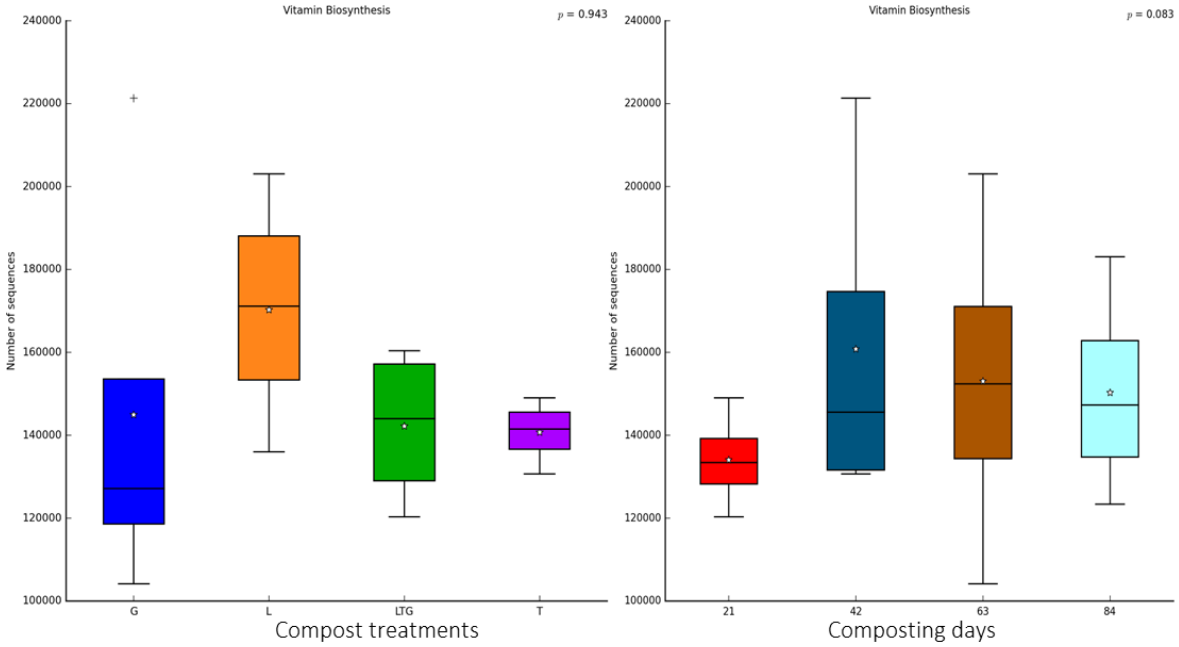
Appendix 8: **FASTQC generated sequence length distribution of compost DNA sequences**



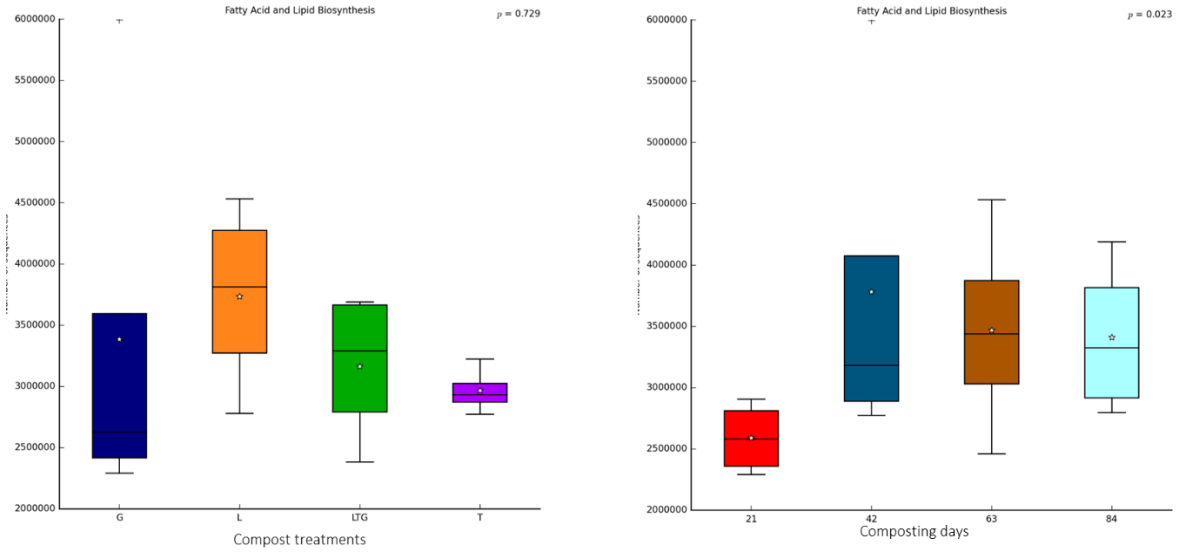
Appendix 9: **High-quality reads obtained from raw 16S rDNA reads after demultiplexing, quality filtering, denoising, and chimera removal.**



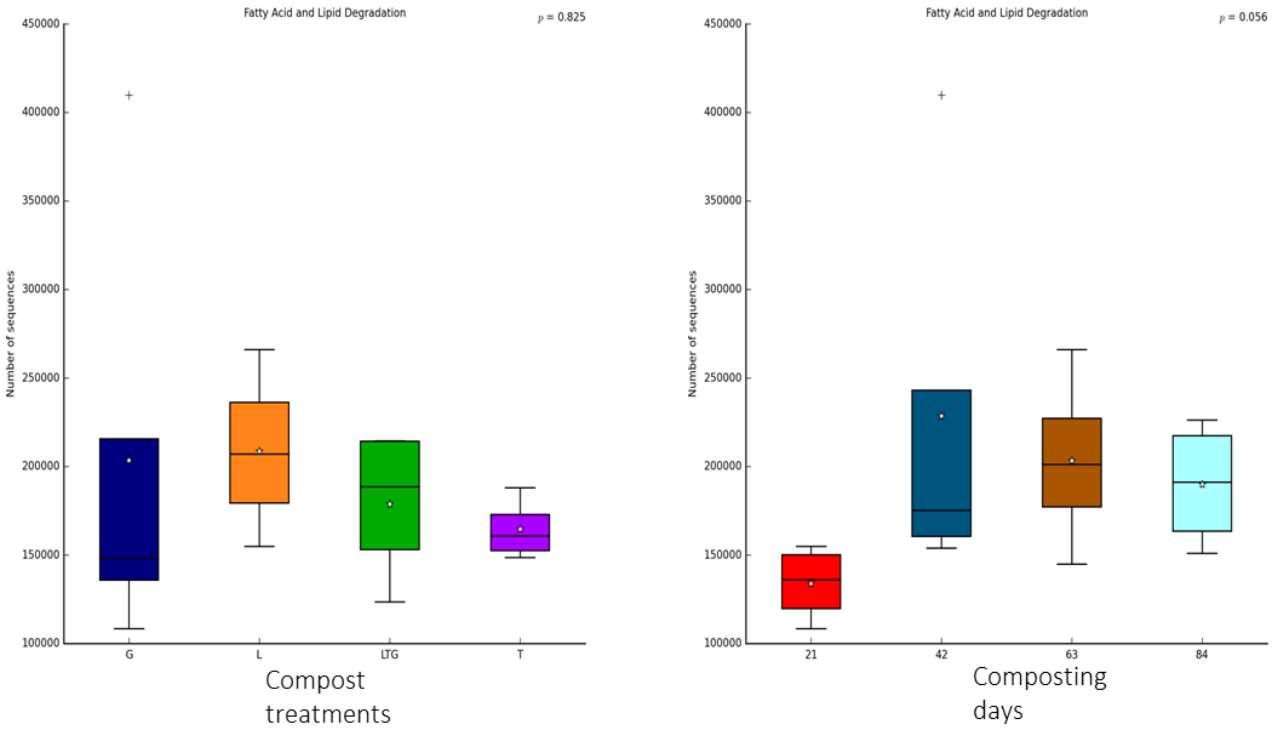
Appendix 10: **Sequence length of resultant ASVs from the four compost regimens. 99% of the ASV had an amplicon length of about 250 bp.**



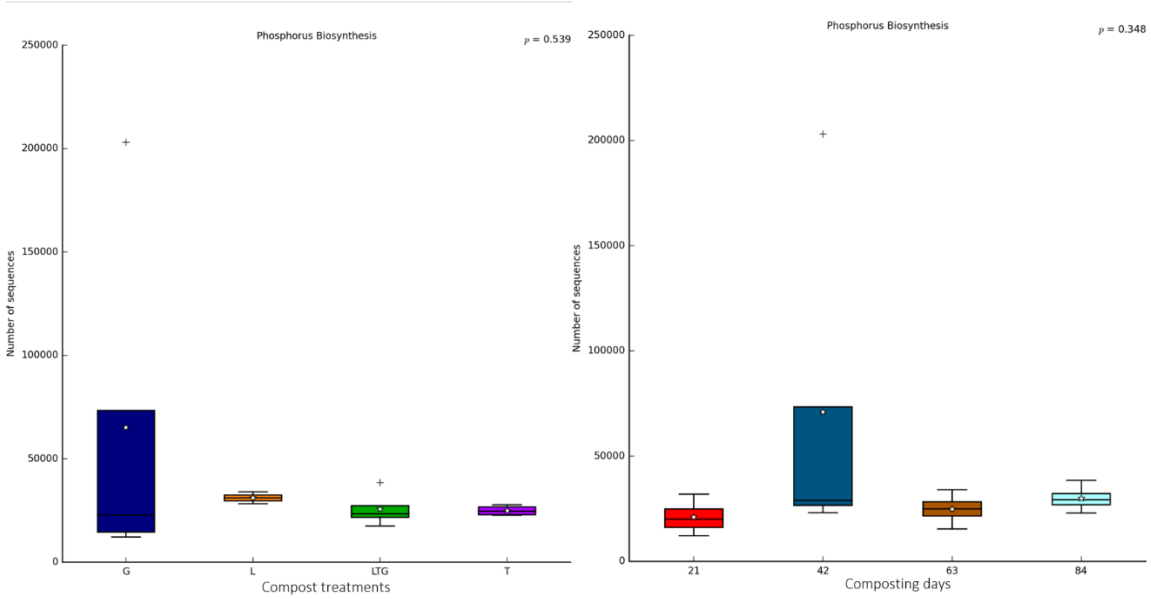
Appendix 11: **The abundance of prokaryotic sequences responsible for assorted vitamin biosynthesis pathways.**
A- influence of composting treatments; B- influence of composting days.



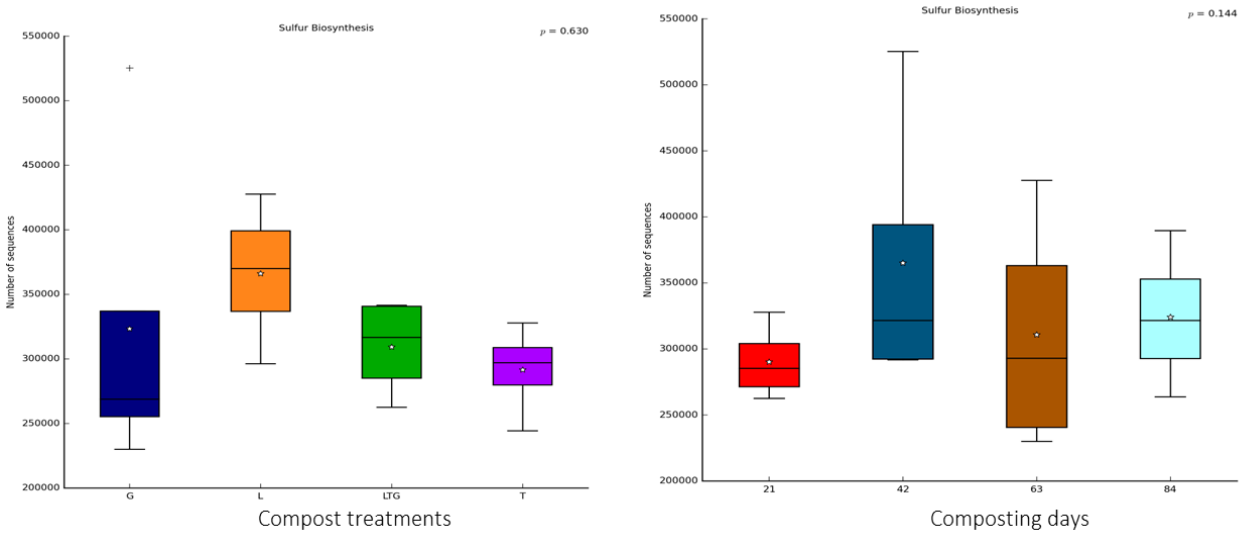
Appendix 12: **The abundance of prokaryotic sequences responsible for assorted lipids and fatty acid biosynthesis pathways.**
A- influence of composting treatments; B- influence of composting days.



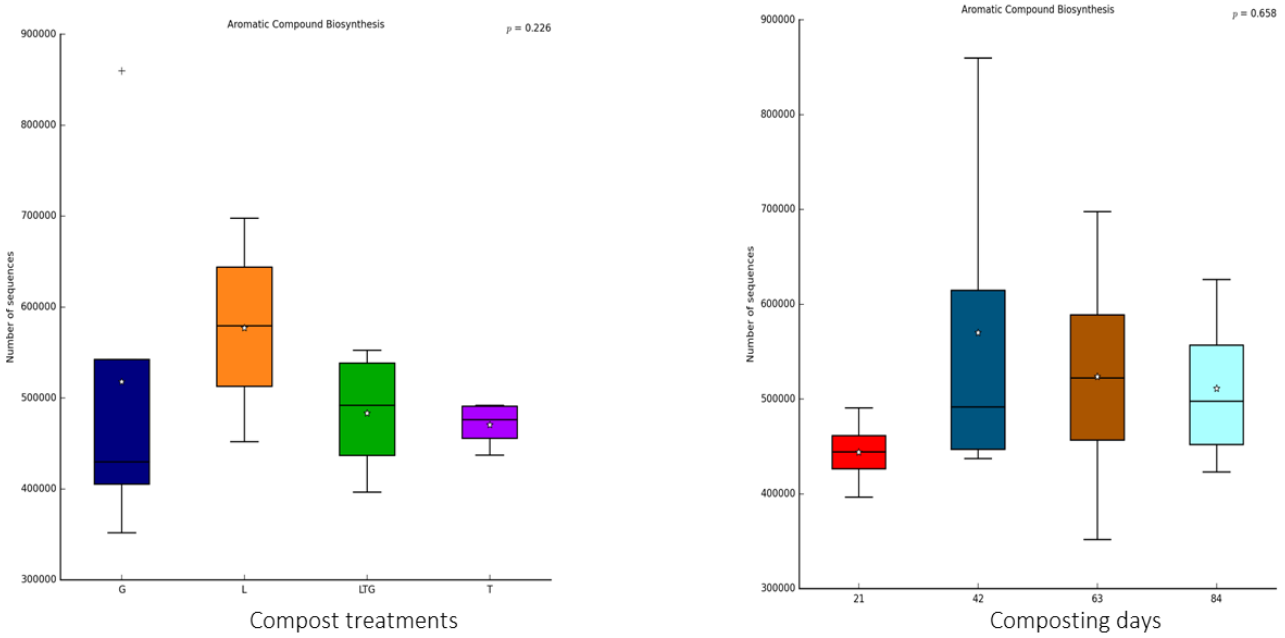
Appendix 13: **The abundance of prokaryotic sequences responsible for assorted lipids and fatty acid degradative pathways.**
A- influence of composting treatments; B- influence of composting days.



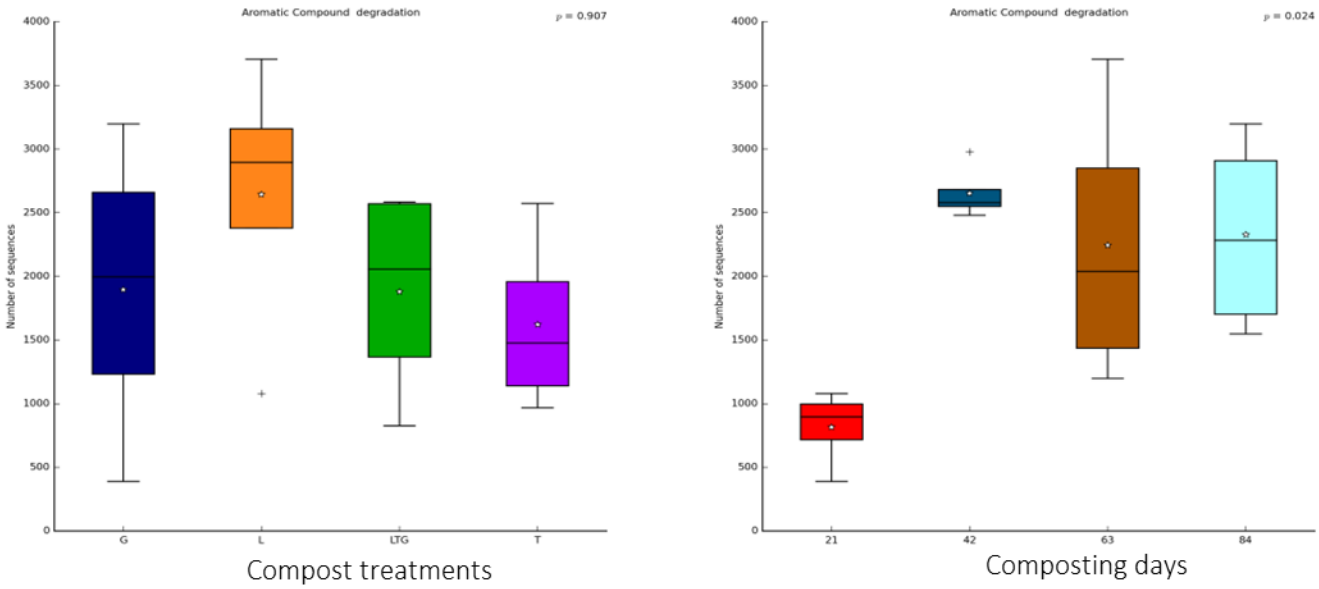
Appendix 14: **The abundance of prokaryotic sequences responsible for assorted phosphorous biosynthetic pathways.**
A- influence of composting treatments; B- influence of composting days.



Appendix 15: **The abundance of prokaryotic sequences responsible for assorted sulfur biosynthetic pathways.**
A- influence of composting treatments; B- influence of composting days



Appendix 16: **The abundance of prokaryotic sequences responsible for assorted aromatic compounds biosynthetic pathways.**
A- influence of composting treatments; B- influence of composting days



Appendix 17: **The abundance of prokaryotic sequences responsible for assorted aromatic compounds degradative pathways.**
A- influence of composting treatments; B- influence of composting days