ADDIS ABABA UNIVERSITY
SCHOOL OF GRADUATE STUDIES
INSTITUTE OF BIOTECHNOLOGY

THE EFFECT OF URINE-DERIVED FERTILIZERS ON SOIL MICROBIOTA AND NUTRITIONAL CONTENTS OF SELECTED VEGETABLES

MSc Thesis

Tseganesh Darsema

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Advisor- Adey Feleke (PhD)

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DECLARATION

I declare that the thesis hereby submitted for the degree of Masters in Biotechnology to Addis Ababa University, institute of Biotechnology is my own work and has not been submitted by me or anybody else at another university. The material obtained from other sources have been acknowledged in the thesis.

Examinee	Name	____________________
	Signature	____________________
	Date	____________________
Advisor	Name	____________________
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TABLE OF CONTENTS

List of Tables

List of Figures

Abbreviations

ANOVA	Analysis of Variance
DNA	Deoxyribonucleic Acid
K	Potassium
N	Nitrogen
NGS	Next Generation Sequencing
P	Phosphorus
PCR	Polymerase Chain Reaction
PPM.	Parts per million
WHO	World Health Organization
SDS	Sodium dodecyl sulphr

ABSTRACT

There is now a growing trend to explore environmentally friendly, low cost and effective soil fertility techniques in substitute of chemicals that applied to the soil. Application of human urine is being used as organic fertilizer because of its nutrient rich and pathogen free nature. The aim of this study is to explore the effect of human urine fertilizers on soil microbial community and nutritional values of the plant by comparing it with widely used chemical fertilizer. On this specific study four different vegetables: Cabbage, Ethiopian kale, Carrot and Tomato were used as experimental subjects in green house condition. Five different treatments (Stored urine, struvite urine, chemical fertilizer and unfertilized and control) with twice replication of each were used for this experiment. The plants grown by these different treatments in soil were measured for their nutritional content values and physiological characters. Soil sample obtained from each of experimental pots were also used to elaborate the effects of chemical and organic fertilizers (urine) on the microbial community of soil. Shotgun metagenomic analysis was used to assess the distribution of soil microbial community at phylum and genus level upon the application of various fertilizers. In all selected plants, the distribution of the identified bacterial groups was relatively better in struvite urine fertilized soil than unfertilized ones. This was the same both in Phylum and genus levels. In carrot, struvite urine fertilized soil showed better composition of identified bacteria compared to chemical treated soil both at phylum and genus levels. In Ethiopian kale, the soil bacterial community distributed better in stored urine fertilized soil than both struvite urine treated soil and unfertilized soil. The nutritional analysis section of this study concluded that there was no significant difference in nutritional values of the plants treated with different fertilizers. However, the phosphorous, nitrogen and protein contents were slightly higher in plants fertilized with organic fertilizers while moisture contents were slightly higher on those plants treated with chemical fertilizers.

Key Word: Fertilizer, Shotgun metagenomics, Human Urine, Organic, Soil, Vegetable.

1. INTRODUCTION

1.1. Background

Soil is fundamental component of plant growth by providing anchorage, nutrients, water storage, aeration and suitable environment for organisms living underground (Angima and Terry, 2011). The scale of soil fertility is measured by its capacity to support population of plants above the ground & flora and fauna below the ground (Hamady and Knight 2009). Although the interest of maintaining the soil originated with the development of agriculture, almost half of the world still uses the same practices of soil management as 2000 years ago (Hamma and Ibrahim, 2013).

Fertilization is among the most important soil improvement operations introduced in modern agriculture (Mandic *et al.,* 2011). Fertilizer stands for any organic or synthetic material that is added to the soil to provide one or more essential nutrients important for plant growth (Rajani *et al.,* 2015). Fertilizers in general include bulky organic fertilizers, such as cow manure, organic compost, bone meal, and green manure crops (Singh *et al.,* 2014), and the chemical/ inorganic fertilizers which is made up of different composition of nutrients (like N,P,K….) that are suitable for specified uses (Silva *et al.,* 2000). Nitrogen is the most important facilitator of nutrient availability and any changes in the availability of soil N through fertilization or other causes can affect the soil microbial community (Carreiro *et al.,* 2000).

Although plants commonly prepare their food through photosynthesis, major nutrients like nitrogen cannot be prepared or absorbed from the air. This is the reason to apply different fertilizers to the soil. Even though plant can grow almost everywhere, Inorganic fertilizer exerts strong influence on plant growth, development, and yield (Stefano *et al.*, 2004). Organic fertilizers preferred over the synthetic ones for plant nutritional capacity mainly because they are environmental friendly and slow-release types which leave appropriate time for accumulation and proper use of plant nutrient (Mofunanya *et al*., 2015).

Different chemical products like chemical fertilizers, pesticides, and herbicides have been widely applied to maintain soil fertility and global agricultural production since the first Green Revolution (Leita *et al*., 1999). Especially chemical fertilizers have been widely popular around because they are less expensive and easy to obtain relative to the high returns often achieved (http://www.utextension.utk.edu/).

It is estimated that Africa holds 52% of the world's remaining arable land that can be utilized for agricultural production (Deininger *et al.,* 2011). Ethiopia has wide range ecological diversity, ranging from altitude of -126 in Danakil depression to 4620 in the Ras Dashen Mountains (Fantaye, 2016). The gross domestic product (GDP) from agriculture sector in Ethiopia is estimated to be about 41.2% (Central Statistics Agency, 2021). Ethiopian farmers have been using different soil management techniques like manure, crop rotation, mixed cropping, relay cropping and fallows since the ancient times (Spielman *et al.,* 2011). With growing global trend, the Ethiopian government has strongly been promoting the use of artificial fertilizer for few decades. According to Ministry of Agriculture and Rural Development, use of Fertilizer has increased from 250,000 t/yr in 1995 to 850,000 t/yr in year 2014 (Fantaye, 2016).

Despite their known merits, it has become difficult to use these artificial fertilizers because of their irreversible environmental issue. Availability of different materials that use to make chemical fertilizers is an emerging obstacle. The nitrogen used to make artificial fertilizer is made from natural gas and is subjected to price changes (Vaccari, 2009). Beside the issue of availability, the misuse and overuse of these chemical fertilizer eventually led to degradation of the soil, loss of crop genetic diversity, reduction in soil microbial diversity, contamination of ground-water resources and the pollution of the atmosphere (Chaudhry *et al.,* 2009).

Building connection between sanitation and agriculture is one of the most efficient approaches towards better ecosystem. The global population is now trapped in circle with discharging of human excreta to freshwater while we replace missing nutrients to soils artificially, which leads to the exploitation of soils in the long run (Richert *et al.,* 2010). It is estimated 2.5 billion individuals in the world have no access to adequate sanitation facilities. This number is more prominent in Sub-Saharan Africa (WHO, 2012). Sanitation in Ethiopia is among the lowest in the world with 31% for clean water and 18% total sanitation coverage (Kassa *et al.,* 2010). Nowadays the so called Closed-loop sanitation (Figure 1) is receiving a great deal of attention to tackle environmental problems around the world. This system is very essential as a way to reduce health risks while recovering useful nutrients and return them back to food systems quickly (WHO, 2006).

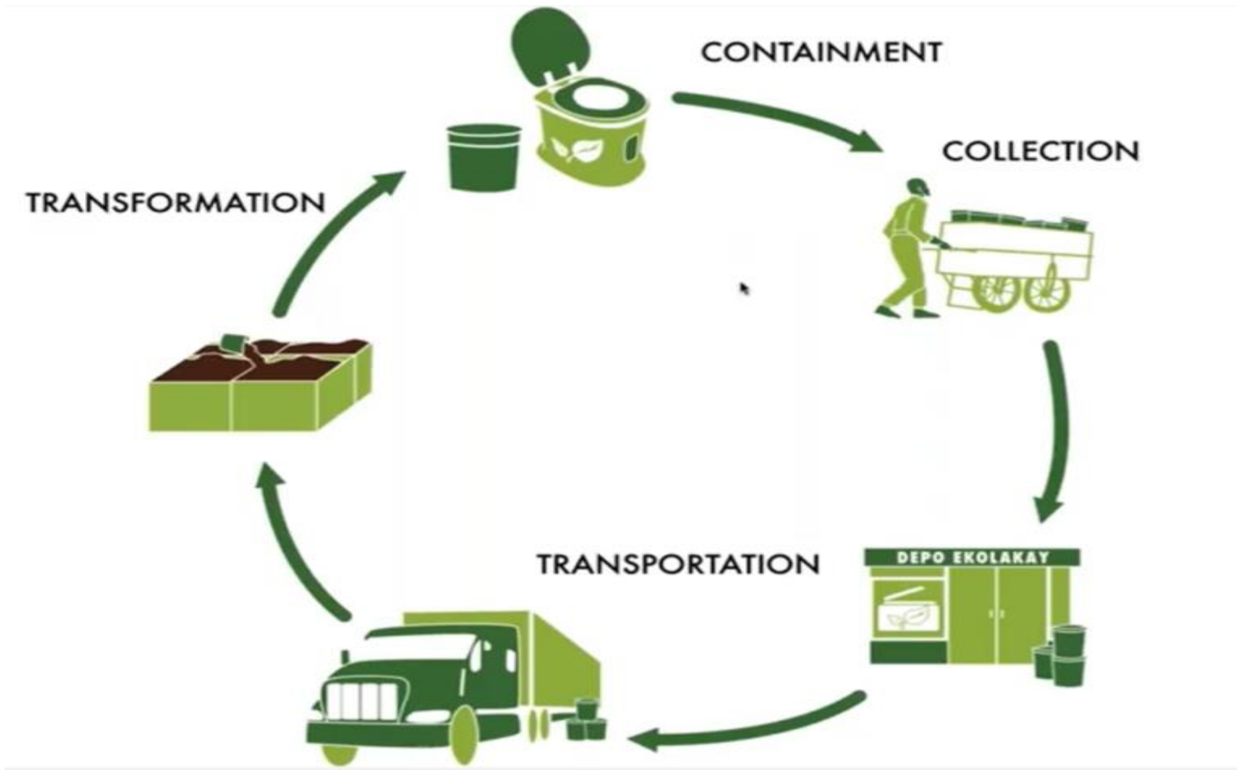

Figure 1: The closed loop sanitation method (Wikiwand.com).

Application of human wastes like urine as organic fertilizers is part of this new established system used to reduce risk and sustain the environment. High level of nutrients together with low levels of pathogens makes human urine a potential candidate as a liquid fertilizer to the soil (Ranasinghe *et al.*, 2016). Urine is an aqueous solution produced by kidney and made up of more than 95% water. The remaining constituents of urine include urea, creatinine, dissolved ions, inorganic and organic compounds and salts (Richert *et al.*, 2010). Depending on the amount of liquid a person drink, urine produced by each individual could range between 1.0 and 1.5 liters per day (WHO, 2006). Urine contains the major nutrients required for plant growth with approximately 80% of N, 55% of P and 60 % of the K (Sene, 2013).

1.2. Statement of the Problem

Ethiopia, like many other developing nations, has rapidly growing population and overcoming various environmental obstacles and securing enough food production has become a very essential national issue. Change in weather patterns and increase in food price have provided reason for introduction of new and advance measures toward ecosystem. Application of different kinds of fertilizers for soil fertility is common practice around the world. Ethiopia, as one of the countries with agriculture led economies has been using different kinds of fertilizers to increase food production. Although using animal wastes for soil fertility is common throughout the country, the use of human excreta for soil improvement is still taken as taboo. Therefore, studying the effects of human wastes like urine on detailed manner could come handy in order to further elaborate the use of organic fertilizers and convince people to use it as a replacement to chemical fertilizers.

1.3 Objective

General objective

-To investigate the impact of application of urine-derived fertilizers on nutritional contents and soil properties of selected vegetables.

Specific objective

-To determine the effect of application of stored urine and struvite urine on the soil microbial communities on the following vegetables: Carrot, Ethiopian kale, Cabbage and Tomato.

-To assess the effect of urine-derived fertilizers on productivity and yield of the mentioned vegetables.

-To determine the nutritional values of the vegetables grown with urine-derived fertilizers in comparison to the synthetic ones.

2. LITERATURE REVIEW

2.1. The soil and its contents

Soil is a center to sustainable development of any kind and has been crucial to global issues such as food, water security and climate regulation (McBratney *et al.,* 2014). Soil requires macronutrients (needed in higher quantity) and micronutrients (required in lower amount) for proper plant growth. The three main nutrients nitrogen (N), phosphorus (P) and potassium (K) together with calcium, magnesium and sulfur are required in higher amount while trace elements like iron, manganese, zinc, copper, boron and molybdenum are needed small quantities (Gensch *et al.*, 2011). Nitrogen the main nutrient in soil is key element for plant growth (Sanchez, 2002). Most nitrogen in soil is obtained from the atmosphere although few produce it in their roots. The role of phosphorous in soil is transferring energy from sunlight to plants and stimulates early root and plant nutrient (Kassa *et al.,* 2010). The other major nutrient, potassium provides the growing plant disease resistance and help move starches, sugars and oils in plants (Erisman *et al.*, 2008).

Soil is also a home to most populous community in the earth, the soil microorganisms. Soil contains more living organisms in a handful than there are people on planet Earth. Soil organisms found in most soil include Nematodes (5 million), Protozoa (10^9), Fungi (10^{10}) and Bacteria (10^{12}) (Stark *et al.,* 2007). Bacteria and fungi found in soil are a useful in phytoremediation under heavy metal stress as they can be able to absorb significant amounts of heavy metals (Garbeva *et al.*, 2004). They are also be able to produce a wide range of secondary metabolites with biological properties like being used as plant growth promoters, and enzymes resulting in the enhancement of plant growth under different conditions (Dokić *et al.,* 2010).

2.2. Soil management and fertilizers

Soil is losing its most important nutrients at an alarming rate around the globe due to growing urban setting and rapid climate change (Connor, 2006). Major problem that comes with modernization include degradation of soil quality by contamination of soil with excess salt, acidity and heavy metals; compacting them under heavy machinery. Climate change and related extreme weather can also contribute to low fertility of soil by reducing moisture and depleting the layers of nutrient-rich top soil (McBratney *et al.,* 2014).

The majority of the world's soil resources are now only in fair, poor or very poor condition as 33 percent of land is moderately to highly degrade due to erosion, salinization, compaction, acidification, and chemical pollution of soils (Connor, 2006). Land degradation in Sub-Saharan Africa (SSA) is believed to be expanding at an alarming rate, accompanied by the lowest agriculture and livestock yields of any region in the world (IFAD, 2009). Smallholder farmers in sub-Saharan Africa are continuously struggling to maintain the productive capacity of their land while trying to maintain their soil (Sanchez, 2002).

Soil amendments and fertilizer are the central mechanism to maintain fertility and decrease loss of soil nutrients. Soil amendment could be done by variety of mechanisms like introducing virgin raw material, composts and wastes, such as sewage sludge to the degraded soil (Andersson, 2014). Soil fertilization is also major agricultural practice that is very common around the world. The idea of fertilization is simply based on replacement of soil nutrient for plant production by natural or synthetic means (Erisman *et al.,* 2008). China, the US, and India together accounted for over 50% of fertilizer consumption globally (FAOSTAT, 2015). According to FAO Ethiopia alongside with South Africa, Nigeria, and Kenya is the major user of fertilizers in Sub-Saharan Africa. Ethiopia had only been using Urea and DAP fertilizers until 2013. But the three blends of NP (NPS, NPSB, NPSZn) have been imported for use as substitute of DAP since 2014 (Richert *et al.*, 2010).

Increased production and application of fertilizers has contributed to raising agricultural productivity and reducing hunger worldwide (Erisman *et al.,* 2008). On the other hand, excessive fertilizer use is proven to cause a number of environmental and ecological problems such as air pollution, soil acidification, water eutrophication, crop yield reduction, (Bouwman *et al.*, 2005).

2.3. Types of fertilizers

Inorganic fertilizers

Inorganic fertilizers were first introduced at the end of 19^{th} century and have been center for modern agriculture (Jansen *et al.,* 2010). These fertilizers are manufactured mixtures of chemical products that might contain N, P, K and other necessary nutrients. As nitrogen is the most important nutrient for healthy plant growth, nitrogen fertilizers are the main artificial fertilizer used in agriculture. The total nitrogen in fertilizers can be supplied as nitrate compounds, ammonium compounds, or urea (Flesa *et al.*, 2002). When applied to soil, the nitrogen is converted to mineral form, nitrate, so that plants can take it up (Goldstein, 2012)

Organic fertilizers

Organic fertilizer refers to materials used as fertilizer that occur regularly in nature, usually as a byproduct or end product of a naturally occurring process (Pradhan *et al.,* 2007). These organic fertilizers can be **plant-based** like Cottonseed meal, Molasses, Green manure, Kelp seaweed and Compost tea or **mineral-based** like Calcium or Epsom salt (Robert and Stewart, 2002). However the most important organic fertilizers would be the **animal-based** fertilizers such as manure, bone meal or blood meal, which add lots of nitrogen to the soil. These also include fish emulsion, manure tea milk, Urea (urine) (Goldstein, 2012).

2.4. Human Urine as organic fertilizer

2.4.1. What is human urine?

Urine is a liquid product of the human body that is secreted by the kidneys which contains large amounts of soluble nutrients- macro and micro nutrients (Gensch *et al.,* 2011). Urine contains four of most important macronutrients: nitrogen (N), phosphorus (P), potassium (K) and sulphur (S). Beside these urine contain 200 compounds including electrolytes, vitamins, hormones, and other organic and inorganic compounds (Sanchez, 2002). Human urine produced by human body is differed by its nutrient concentrations, as well as the amount depending on factors, such as diet, climate, gender, water intake, physical activity and body size (Eawag, *et al.,* 2014). In fully-grown human, the consumed nutrients do not stay in the body (for the growth of muscles, bones and nerves) but leave the body with the excreta almost in the same amount as consumed. Because of this on average, one person produces between 0.8 and 1.5 L of urine per day, which equals around 550 L of urine per person a year (Jönsson *et al.,* 2004).

2.4.2. Why to use human urine as fertilizer?

Urine is a valuable, yet underestimated resource for plant fertilization (Goldstein, 2012). The nutrient contents present in human urine may mean it can be a good fertilizer for plants. Studies showed that annual amount of urine and feces of one person consist of equal amount of nutrients than what is needed to grow grain for one person’s annual food requirements. Physiological measurements indicate that the amount of plant macronutrients excreted via urine per person and year has been measured at 2.5-4.3 Kg-N, 0.7-1.0 Kg-P, and 0.9-1.0 Kg- K (Sene, 2013). Urine has a fertilizer value of N/P/K 18:2:5 (Pradhan *et al.,* 2007). The major merits of using urine fertilizer include mainly recovering and recycling nutrients controlling micro pollutants and pathogens and reducing wastewater flow. An advantage of urine in comparison with other organic fertilizers is that the phosphorus in urine exists in forms that are plant-available. This means that urine is also quite efficient as a phosphorus fertilizer, which has implications for the future with regard to the fact that phosphorus is a finite resource (Richert *et al.,* 2010). Urea found in human urine is essential for use of urine as organic fertilizer by rapidly degrading into ammonium and water, and elevates the PH value. This rise in pH negatively affects the survival of most harmful bacteria making urine safer for use as fertilizer (Stintiing *et al.,* 2001).

The effect of human urine on agricultural practices has been studied in different part of the world such as Ethiopia (Kassa *et al*, 2010), India (Andersson, 2014), Philippines and developed countries like Germany and Sweden (Andersson 2014). There are researches that also compared urine with commercial fertilizer using diverse type of vegetables and cereals including wheat (Tidaker *et al.,* 2007), maize (Guzha *et al.,* 2005), spinach (Mnkeni *et al.,* 2005), cucumber (Heinonen-Tanski et *al.,* 2007), carrot, beetroot (Mnkeni *et al.,* 2008), pumpkin, tomato (Pradhan *et al.,* 2009), red beet, cauliflower, potato and radish (Pradhan *et al.,* 2010). Human urine as a crop fertilizer is studied for the first time in Finland on a large scale (Andersson 2014). Despite good starts on experimental level, the use of human as a fertilizer in agriculture in Ethiopia is generally faced with general cultural objection (Kassa *et al.,* 2010).

2.4.3. How to treat human urine for use?

Challenges faced on process of urine utilization in agriculture include separation techniques, storage time, urine amount to be applied, odor prevention and transport. Normally Urine from human body considered sterile until contamination from the environment. Pathogens transmitted through urine may not constitute a significant public health problem (Höglund, 2001). The possible positive and negative effects of urine are described in Table 1.

Table 1: **Compounds in urine, their potential and possible negative effects (Udert *et al,* 2015).**

Compound	**Beneficial reuses**	**Negative impacts**
Water	-Recycling, for example for irrigation	-Water volume requires large storage tanks -Water weight makes transport expensive
Nitrogen	-Fertilizer -Raw product for chemical industry	-Smell and toxicity of gaseous ammonia -Environmental pollution: nitrate in groundwater, eutrophication of receiving waters and soils, fish toxicity, atmospheric particles.
Phosphorus	-Fertilizer -Raw product for chemical industry	-Environmental pollution (eutrophication) -Precipitates block pipes and valves
Bicarbonate	-None	-Precipitates block pipes and valves
Sulfur	-Fertilizer -Raw product for chemical industry	-Environmental pollution -Smell and toxicity of hydrogen sulfide
Potassium	-Fertilizer -Raw product for chemical industry	-Salinization of agricultural soils and groundwater
Bulk organic substances	-None	-Pungent smell -Treatment problems such as foaming -Organic reduction of sulfate and nitrate: sulfide production or NO and N_2O production (pollutants, climate gases)
Trace organic compounds	-None	-Human health concerns -Environmental concerns
Pathogens	-None	-Human health concerns

Urine contains micro pollutants such as salts, heavy metals, hormones, pharmaceutical residues and pathogens. Among these, salts found in urine might cause permanent damage to the soil by causing water contamination and accumulation of several ions in soil. Ions, and excess of sodium in soil inhibits plant growth; while excess of N can built up in plant tissue and affect negatively amount of sugar and vitamin in vegetables (Andersson, 2014). Pathogens like Salmonella typhi, Salmonella paratyphi, and Shistosoma haematobium can also get discharged as urine from human body. However, they die off during simple storage or soon in the soil after application (Sene, 2013). The urine can be treated depending on the location of the collection site and the application target (Goldstein, 2012). There are different options to eliminate concerns. Two of the possible treatments are: urine storage and struvite precipitation.

Urine storage

Urine storage is hygiene treatment which is mostly sufficient on a small scale. This increases the pH and the ammonia content, improving the die-off rate of pathogens (Jönsson *et al,* 2004). According to the WHO guidelines for safe use of excreta in agriculture, the optimal storage period recommended is 1 month if it is collected from single household is six months at 20°C or higher if it is collected from wide range of household (WHO, 2006). At excretion, the pH of urine is normally around 6.0 but can vary between 4.5 and 8.2. During storage, the pH of urine increases from 9.0 to 9.3 and it has a high ammonium concentration. An advantage of this technique is its simplicity and low-cost implementation and maintenance (Jönsson *et al.*, 2004).

Struvite Precipitation

Struvite Precipitation is other technique used for treating urine. It is a simple and fast concentration technique which is mostly used for the recovery of phosphorous (Jönsson *et al.*, 2004). Struvite precipitation happens naturally, when magnesium ions react with phosphate and ammonium ions contained in urine (Udert *et al.*, 2015). The mineral struvite ($MgNH_4PO_46H_2O$) can be precipitated from stored urine because of suited requirements like high pH, high ammonia and phosphate concentration (Wilsenach *et al.*, 2007). In struvite precipitation the concentration of phosphorous becomes twice higher than liquid urine and can be used for crops with a high phosphorus demand (Behrendt *et al.*, 2002).

Beside this most common hygienic treatment of urine, there are techniques like Ammonia stripping (Winkler *et al.*, 2013) and combination of electrodialysis, microfiltration and ozonation (Pronk *et al.*, 2007) that are used to treat urine for agricultural use.

Table 2: Advantage/Disadvantage of chemical and urine fertilizers

	Advantage	**Disadvantage**
Urine fertilizer	-Free/no cost -Complete fertilizer (contain all nutrients) -Improved water capacity and soil structure -No build- up salt -Rich live of soil microorganism	-Concentration of nutrients can't be predicted - Not concentrated -Quality depend on quality of raw product -Easily washed out of soil
Artificial fertilizer	-Easy to handle -Small amount required -Composition adapted to needs of different crops	-Costly -Salt build-up -Not balanced, not complete -Destroy soil structure -Negative impact on soil microorganism

2.5. Methods to study soil microbial community

Different Organisms that live in the soil are among the most important components that considered as most sensitive indicators of ecosystem function (Stark *et al.*, 2007). The microbial community in the soil has beneficial, neutral or pathogenic effects. They are key players in environmental processes such as soil structure formation, decomposition of organic matter and recycling of essential elements and nutrients (Garbeva *et al.*, 2004). There are two major techniques developed to study the soil microbial community. These techniques are the biochemical and molecular techniques (Fakruddin and Mannan, 2013).

2.5.1. Biochemical methods

The biochemical method of soil microorganism analysis was the first technique to be introduced. Early microbiologists studied metabolic properties such as utilization of different carbon, nitrogen and energy sources in order to distinguish between different types of soil microbes (Kirk *et al.*, 2004). This technique includes Plate Counts (Tabacchioni *et al.*, 2000), Community level physiological profiling (CLPP) and Fatty acid methyl ester analysis (FAME) (Kirk *et al.*, 2004). These methods have been widely used due to their fast and cost effective characters however these techniques use only to study the culturable microorganism, which only count 1% of the soil microbial community (Fakruddin and Mannan, 2013).

2.5.2. Molecular methods

The inability to culture most microorganisms from environmental samples is a fundamental obstacle to understanding microbial ecology and diversity. The use of DNA-based techniques can overcome this limitation by allowing the fate of particular genes or organisms to be monitored directly in environmental samples (Yeates, 1997). Molecular tools in soil microbiology have been applied extensively in last decades because of the limitations in culture-dependent methods (Fakruddin and Mannan, 2013). Studies revealed that bacterial diversity is approximately one hundred times greater than first indicated by direct culture methods (Schneegurt *et al.*, 2003). These molecular techniques include nucleic acid re-association and hybridization, DNA microarrays, PCR-based methods & sequencing. (Dokić *et al.*, 2010). The research area of metagenomics can be categorized in two ways. The first one is the **single gene survey** in which the focus is in a particular gene which will be amplified, sequenced and analyzed (Kim *et al.*, 2016). The second category is the **random shotgun method** in which all the genes are involved in sequencing of the DNA isolated (Hamady and Knight, 2009).

2.5.2.1. Shotgun metagenomics methods for soil microbial community analysis

Shotgun metagenomics is untargeted sequencing of microbial genomes present in a sample. It is an unconventional approach to study uncultured microbiota which can then been used to profile functional potential and taxonomic composition of microbial communities (Sharpton, 2014). This sequencing method has an ability to combine many samples in single sequencing run and obtain high sequence coverage per sample. The shotgun sequencing strategy provides a technical guarantee for large-scale sequencing like environmental and human genome studies (Bentley, 2006). The procedures of shotgun sequencing varies depending on different methods it applies however all of them contain the following major steps (He *et al.*, 2020).

(i) Pre-processing of the sequencing reads
(ii) The sequence analysis
(iii) Post-processing statistical and biological analysis
(iv) Validation

The shotgun method can be categorized to several sequencing strategies based on its library construction and advances in the technology.

The hierarchical shotgun sequencing (clone-by-clone or top- down sequencing) is characterized by two major steps-the genome amplification and genome fragmentation (Green, 2002). In this sequencing method the genome is fragmented to large pieces and cloned to vectors in which it will then be arranged to recombinant vector library. The recombinant vector clones then proceed undergo shotgun sequencing individually followed by restriction digestion to reduce the size of the genome (Waterston and Robert, 2002). This method is time-consuming and costly. **The whole genome shotgun sequencing** in other hand has only one advanced step. The shearing of the whole genome to small fragments takes place first followed by each fragments undergoing sequencing randomly (Kuczynski *et al.,* 2012). It is rapid and less costly compared to the hierarchal process however it is less efficient, unordered and it is difficult to use in repetitive sequence like that of eukaryotes (Waterston and Robert, 2002).

The next-generation shotgun sequencing is modern high throughput sequencing processes which depends on capillary electrophoresis instead of overlapping sequences like hierarchal and whole genome sequencing methods (Mardis, 2008). It is sequencing that use post-Sanger technologies to sequence large numbers of DNA fragments in parallel (Glenn, 2011). It utilize a dramatically simplified and accelerated method of library construction from the whole genome shotgun sequencing. It is highly sensitive and accurate sequencing compared to above mentioned methods. However the read length (the number of nucleotides sequenced off one fragment) has seen a large decrease compared to the other two methods (Lozupone, 2013).

There are several platforms for this sequencing technology including Roche ('454' sequencing) (Margulies *et al.,* 2005), Illumina's platform (Hiseq 2000 sequencing) (Glenn, 2011), Life technologies (SOLID and ion torrent sequencing) (Shendure and Ji, 2008) and Pacific bioscience (PacBIO RS sequencing) (Metzker, 2010). Illumina's platform is the most widely used for projects as it provides a good balance between read length and cost. It is also widely available, has very high outputs (up to 1.5 Tb per run) and high accuracy (with a typical error rate of between 0.1-1%) (Liu *et al.,* 2012).

Procedures of shotgun sequencing by using Illumina platform include the following steps

A. Library preparation

The sequencing library is prepared by fragmenting random DNA by using sonication or nebulization and ligating it to 5′ and 3′ specific double-stranded adapters at both ends generating a shotgun library (Knierim *et al.,* 2011).This process is known as Tagmenation (Adey *et al.,* 2010).

B. Cluster Generation

It is a process where each fragment is isothermally amplified. It takes place on the surface of flow cell, a thick glass slide with channels or lanes which are coated with a lawn of oligos that are complementary to library adapters. Each fragment is then amplified into distinct clusters (bright spots with each cluster represents thousands of copies of the same DNA strand) through bridge amplification (Chen *et al.,* 2016). Bridge amplification is a process in which a single-stranded molecule flips over and forms a bridge by hybridizing to adjacent complementary primer. It is repeated on the surface of the flow cell until many bridges are created (Krishna *et al.,* 2019).

C. Sequencing

It uses branded reversible terminator–based method that detect single bases as they are incorporated into DNA template strands (Bentley, 2006).Each of the four fluorescently labeled DNA bases emit an intensity of a unique wavelength (Krishna *et al.,* 2019).

D. Alignment and Data Analysis

The newly sequenced strands are aligned with the genome a reference. After the alignment different methods of analysis could be conducted depending on the result required like single nucleotide polymorphism or insertion-deletion identification (Su *et al,* 2011).

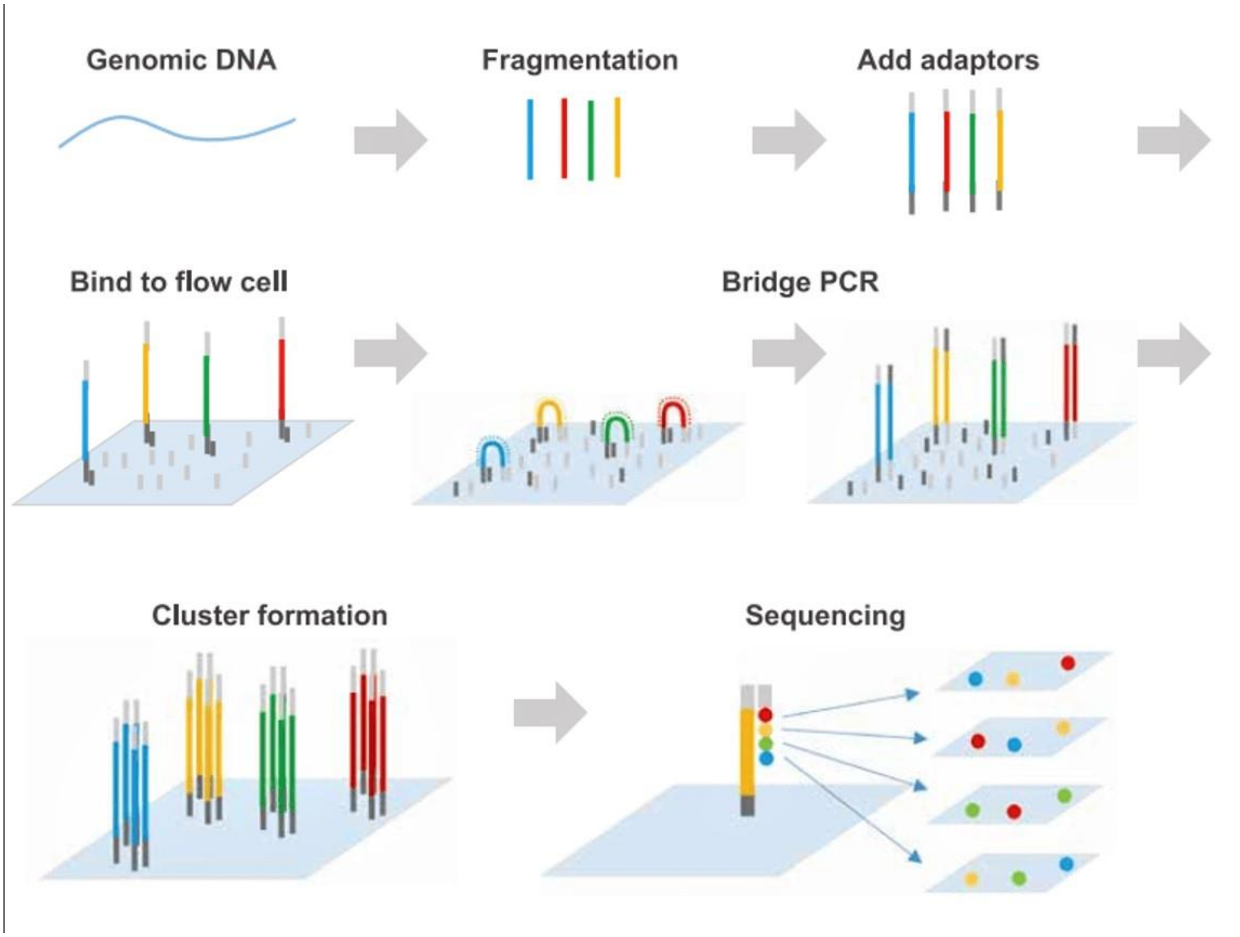

Figure 2: The new generation advanced shotgun sequencing procedure (Yuan *et al.*, 2016)

The application of new generation shotgun method in the environment used to quantify the abundance of species to provide bio monitoring which is used for determination of functionality and dynamics of microbes in a certain environment (Sharma *et al.*, 2021).

The main benefit of the new generation shotgun system in the environmental studies is that it can help to detect very low abundance members of the microbial community in a given complex environmental sample (Delmont *et al.*, 2012). It also allows researchers to sequence thousands of organisms in parallel and has the ability to combine many samples in a single sequencing run to obtain high sequence coverage per sample. It helps to characterize the soil microbial community by quantification of its taxonomic diversity (Krishna *et al.*, 2019). This includes determining which microbes are present in a community and its abundance. It also doesn't require any restriction enzyme as it only involves random breaking (Liu *et al.*, 2012).

The short coming of this random process is that some nucleotide could be cleaved out or lost so there might be a missing data in the process. Other limitations include to potential experimental biases as it can be biased toward easily cultivable bacteria and pathogens based on its availability (Kulski, 2016). It also has high complexity of computational analysis and interpretation. It is still expensive to sequence and analyze large numbers of metagenomes without access to sequencing and computational facilities (Mardis, 2008).

Shotgun sequencing is widely being used in different environmental research areas. The soil microbial community is especially require high end technologies to study as it is too complicated to study in the conventional method (Krishna *et al.*, 2019). The microbial community is studied both through the plant or directly the soil by using shotgun sequencing method. The new generation shotgun sequencing is being used to study the shift in microbial community caused by different organic fertilizers in sectors like in maize production (Ji *et al.*, 2021), sugarcane fields (Li *et al.*, 2020), Irish Grassland (Karpinska, 2021), Tea production (Sarkar *et al.*, 2020) and others .There are also some studies that use the shotgun sequencing to show the effects of both organic and inorganic fertilizers in soil microbial communities in longer time interval (Li *et al*,2017) like in maize rhizosphere (Enebe and Babaloa, 2020), in Paddy Fields (Kim *et al*, 2020) and the 20 years observational study in Czech Republic (Kracmarova *et al*, 2022).

3. MATERIAL AND METHODS

3.1. Experimental Site description and duration

The experimental trial was carried out under greenhouse condition at main campus of Addis Ababa University, Addis Ababa located at 9˚1’48’’N 38˚44’24”E with elevation of 2200 m. The planting and growing had been taken place from February to May 2019 with average 25 ^{0}C temperature and 87 mm precipitation under controlled condition. The greenhouse had a temperature ranging from 27 to 29 ^{0}C and well ventilated to minimize humidity during the day time when the temperature gets high.

3.2. Experimental Design and Plant Materials

A total of 40 pots each filled with 3Kg of clay soil type were prepared and randomly assigned for four types of vegetable seeds (Figure 3). Commercial vegetable seeds of Cabbage *(Brassica oleracea),* Tomato (*Solanum lucopersicum)*, Carrot (*Daucus carota*), and Ethiopian kale (*Brassica oleracea*) were placed separately on each pot. Five of each identical vegetable seeds were planted separately on each pot. The experimental pots were placed horizontally on wire setting at the green house.

Figure 3: The experimental pots used to grow selected vegetables.

3.3. Application of treatments

Three treatments (Chemical fertilizer, stored urine, struvite urine) with unfertilized control and unplanted control were used in this experiment. The chemical fertilizer was applied 12 days after seeding. It is applied twice, three days apart as indicated in Singh *et al* (2000). The application of stored urine and struvite urine fertilizers were also carried out twice, three weeks after seedling. Both fertilizers were applied 10 days apart based on the recommended application guideline by Etter B. (2011) and other related documents at AAU center for food science and nutrition.

For this experiment, a mixture of Di-ammonium Phosphate and Ammonium sulphate ($(NH_4)_2HPO_4 + (NH_4)_2SO_4$) was used as chemical fertilizer. The application of this fertilizer had been arranged based on the fertilizer nutrient ratio and the plant nutrient demand as shown in Table 3.

Human urine had been obtained from male volunteers in Addis Ababa and had been stored at room temperature for around 30 days ahead of use. The urine used here was diluted to tap water at 1:1 ratio. The amount of urine for application had been determined by taking consideration of the volume of the pot, the weight of the soil in the pot and recommended application rates of all plants from different guidelines as presented on Table 3. Struvite urine fertilizer, which was applied on experimental pots, were stored for a year. It had pH of 10 and contained 87.07% of nitrogen and 91.55% of phosphorous.

The application of urine was done on slightly wet soil to avoid volatilization. In order to do this the plants were watered a day ahead of application. Urine had been applied at least 15 cm away from each plant by using plastic pipette. This is used to avoid ‘burning’ of the plant. The holes were then covered with soil immediately after urine application to avoid ammonia losses.

Table 3: Requirement and applied amount of different treatments for experiment

	Plant	Nutrient requirement from urine (Kg/m^2) (Etter, 2011)		Nutrient requirement from chemical fertilizer (Kg/m^2) (Singh *et al.*, 2000).		Applied amount		
		N	P	N	P	NPS(g)	Stored urine (ml)	Struvite urine (g)
1	Cabbage	0.00022	0.00003	0.016	2.07	36	4.2	35.5
2	Tomato	0.00014	0.00003	0.0135	3.26	54	4.1	52
3	Carrot	0.00001	0.00003	0.00012	1.4	34	4	31
4	Ethiopian kale	0.00022	0.00003	0.016	2.07	36	4.2	35.5

3.4. Sample collection and analysis

Soil before any application of fertilizer was collected from the greenhouse on February 7, 2019. Initial N and P content of the soil was analyzed by using Kjeldhal (AOAC, 2000) and Olsen (Horta and Torrent, 2007) methods respectively. Soil samples were also collected and measured after the first and second application of fertilizers. Each sample was brought to AAU, Department of microbial, cellular and molecular biology (Bioinstrumentation laboratory) and kept in freezer until future use for molecular analysis.

3.5. Analysis of the plant nutrient composition

3.5.1. Determination of Moisture content

Moisture content of the vegetables was measured by first slicing the Tomato, Carrot and Cabbage and adding 5 g of each plant to moisture analyzer (A&D ML-50). Each sample measurement was done according to the manufacturer's manual and took approximately 35-45 minutes.

3.5.2. Determination of Nitrogen and protein content

Nitrogen and Protein contents were determined by using the Kjeldahl method (AOAC, 2000). This analysis contains steps of predigestion, digestion, distillation and titration.

The samples were first dried overnight at 56 ^{0}C in oven and grinded until it is powder. The dried powder at the amount of 0.5 g were added to clean conical flasks. Sulfuric acid (6 ml) was then added and left for other 24 hours for predigestion. Titration with 3.5 ml of hydrogen peroxide was done on the next day and 3g of catalytic mixture of potassium sulphate and copper sulphate was added and reacted for 15 minutes. The mixture was then digested in digestion stove at 37 ^{0}C for 4 hours until a clear digest was obtained. Precipitation of sulphate was then prevented by adding 25 ml of distilled water. Neutralization of sulfuric acid and complete release of ammonia was accomplished by adding 25% of 35% NaOH. The Erlenmeyer flask containing a mixture of 25ml of 4 % H_3BO_3, 25ml of dilute water and 3 drops of methyl red indicator was used as a receiver at the end of distillation unit. Finally the distillate was titrated with standardize 0.1N HCI until the appearance of reddish color which can be used to measure the amount of HCI consumed.

The amount of nitrogen and protein were calculated by using:

$$\textbf{Nitrogen (\%)} = \frac{\mathbf{V_{HCl} \text{ in L} \times N_{HCl} \text{ (ca 0.1)} \times 14.00 \times 100}}{\mathbf{W_0}}$$

$$\textbf{Protein (\%)} = \mathbf{6.25 \times \% \text{ Nitrogen (g)}}$$

Where:

V_{HCl} = Volume of HCI in L consumed to the end point of titration

N_{HCl} = the normality of HCI (normally used 0.1)

W_0 = weight of dried sample

14= equivalent weight of nitrogen

6.25= conversion factor for food

3.5.3. Determination of Phosphorous

Phosphorus was determined by the colorimetric method using ammonium molybdate (AOAC, 1990). Two separate solutions were prepared. The first solution was prepared by dissolving 20 g of ammonium molybidate in 200 ml of distilled water. The other mixture was prepared by dissolving ammonium vanadate (NH_4VO_3) in 300 ml of warm distilled water. These two solutions were mixed together in 1L volumetric flask and cooled in room temperature. After the mixture was prepared 250 ml nitric acid (HNO_3) were added to it and the mixture is filled up to 1L by using distilled water (Figure 4).

For the preparation of stock solution at 50 ppm concentration, 2.5 gm of Potassium di-hydrogen phosphate (KH_2PO_4) (2.5 g) were dried at 105 ^{0}C for 2 hours. An amount of 0.2197 g of KH_2PO_4 was dissolved in 1 L of distilled water to make 50 ppm stock of P. Series of standard solution were prepared by diluting 1,2,3,4 and 5ml of stock solution by up to 100 ml distilled water. Respectively 0.5,1,1.5,2,2.5 ppm standard solution was prepared (Figure 4).

Preparation of sample for phosphorous determination was done by using wet digestion method (Henryk *et al.*, 2003). Each sample (0.25g) was placed in a 100 ml digestion flask and 5ml of 70% nitric acid was added to the digestion flask 0.08 and the sample allowed standing and predigesting for 20 minute. After that 1 ml of 60% perchloric acid and 0.5 ml of 98% sulphuric acid were added and the digestion was continued. This procedure was repeated few times by increasing the nitric acid predigestion time.

After the sample was prepared 5 ml of 70% nitric acid, 1 ml of 60% perchloric and 0.5 ml of 98% sulphuric acid were added simultaneously to the digestion flask. The sample was then allowed to stand and predigest in the mixture of all three acids. This was followed by immediate digestion at moderate heat. The digest was cooled, transferred into 50 ml volumetric flask by filtration and diluted.

Sample digest (5 ml) was then added to 100 ml volumetric flask and 10 ml of vanadate- molybidate solution prepared earlier was mixed. Standard solution and distilled water then brought up to final volume.

After that it was left for 15 minute in darker area until color development. It was then read on the absorbance by spectrophotometer at 460 nm wavelength and phosphorous was calculates as:

$$\mathbf{P}_{(ppm)} = \frac{\mathbf{C \times V_1 \times V_2 \times mcf}}{\mathbf{S \times A}}$$

Where

C = P concentration in sample digest read from the curve, ppm

V_1 = Volume of the digest (100 ml)

V_2 = Volume of dilution

S = Weight of the plant material (g) (0.5 g)

A = Aliquot (5 ml)

mof = moisture correction factor

Figure 4: Sample prepared and kept in digestion flask to determine the amount of phosphorus.

3.6. Molecular analysis of the soil bacteria

3.6.1. Soil DNA extraction

DNA extraction from the soil samples was done according to protocol by Tsai and Olson (1991) with few modification. Soil sample (1 g) were measured and mixed with of 120 mM sodium phosphate buffer (2 ml) by shaking it at 200 rpm for 15 min. The mixture was then centrifuged at 5000 rpm for 15 minute. The pellet was washed again with Sodium phosphate buffer and 2 ml of lysis solution (0.15 M NaCl, 0.1 M Na2EDTA [pH=8.0]) was added. The sample was ultrasonicated at power of 20 KHZ for 45 seconds with interval in each 15 seconds to separate the DNA from the microbial cell. After the separation 2 ml of a mixture containing 0.1 M NaCl, 0.5 M Tris-HCl (pH 8.0) and 10% sodium dodecyl sulfate was added to the sample while it was on the ice. The sample was then mixed with Chloroform/phenol/Isoamyl alcohol (ratio, 24:23:1) and centrifuged at 5000 rpm for 10 minute. The upper aqueous layer (3ml) was then collected and added to new falcon tube and mixed with 2.5 ml of chloroform mixture (chloroform/isoamyl alcohol ratio, 24:1). It was centrifuged again at the same speed and time. The top aqueous sample (3ml) was then collected again with new falcon and mixed with equal amount of cold isopropanol at (-20°C). It was then centrifuged at 5000 RPM for 10 minute and the supernatant was removed. The sample was then dried overnight at room temperature in safety cabinet and TE buffer (20 mM Tris-HCl, 1 mM EDTA [pH 8.0]) was added to the pellet. The crude DNA was then purified by using Sodium acetate precipitation method. DNA was mixed with 1 volume of 3 M sodium acetate and 4 volumes of 100% ethanol. It was Mixed thoroughly and incubated at –20° C overnight. The mixture was then centrifuged at highest speed for 30 min at 4°C at room temperature. The supernatant was removed and the pellet was washed with 500 µl of 70% cold ethanol and centrifuged for 10 min at 4°C or room temperature. It was dried and suspended with TE buffer.

Extracted DNA was quantified by using nanodrop spectrometric device (Thermo Fisher Scientific U.S.A.) using TE buffer for the blank solution. Likewise, agarose gel electrophoresis (0.8%) was used to separate DNA fragments based on their weight by using Ethidium bromide and observed under UV transilluminator and Gel image captured using Molecular imager Gel Doc XR+ system (BIO-RAD).

3.6.2. Shotgun sequencing

DNA Samples that fulfilled the quality requirements for sequencing were selected and shipped to Zymobiomics (San Deigo, USA) for sequencing. As per the company's description on the specific service for the submitted samples, the library preparation, cluster generation and quality checking were performed. To prepare library, genomic DNA samples were profiled with shotgun metagenomic sequencing. Sequencing libraries were prepared with Nextera® DNA Flex Library Prep Kit (Illumina, San Diego, CA) with up to 100 ng DNA input following the manufacturer's protocol using internal dual-index 8 bp barcodes with Nextera® adapters (Illumina, San Diego, CA). All libraries were quantified with TapeStation® (Agilent Technologies, Santa Clara, CA) and then pooled in equal abundance. The final pool was quantified using qPCR.

Cluster generation and sequencing: The final library was sequenced on the NovaSeq® (Illumina, San Diego, CA) platform. Quality was checked by using ZymoBIOMICS® Microbial Community DNA Standard (Zymo Research, Irvine, CA) as a positive control for each targeted library preparation. Negative controls (i.e. blank extraction control, blank library preparation control) were included to assess the level of bioburden carried by the wet-lab process.

3.6.3. Statistical data analysis

Comparison of means for moisture content, Nitrogen, protein and phosphorus contents was analyzed using one-way analysis of variance (ANOVA) using SPSS 16.0 program. The significant differences in mean values of the quantitative variables were tested at 5% level of significance and reported as mean ± standard deviation (SD). For the bioinformatic analysis, the raw sequence reads were checked for their quality using fastqc and trimmed to remove low quality fractions and adapters with Trimmomatic-0.33 (Bolger et al., 2014): quality trimming by sliding window with 6 bp window size and a quality cutoff of 20, and reads with size lower than 70 bp were removed. Assembly was done using the tool velvet (Zerbino and Birney, 2008).

4. Result and discussion

4.1 Nutritional composition

4.1.1. Moisture

The moisture contents for the subjected plants (Carrot, Tomato and Cabbage) by using both organic and inorganic treatments is shown in Table 4. In tomato, the plant treated with stored urine fertilizer had shown relatively higher moisture content with an average of 93.1%. Chemical and struvite urine fertilized soil had shown similar moisture content in tomato with an average of 92.1 %. The moisture contents of tomato showed stability towards different fertilizers compared to other plants. In carrot, unfertilized and artificially fertilized plants showed relatively higher moisture content with 83.7% and 86.4% respectively. Carrot treated with stored and struvite urine showed the lowest moisture contents of all plants used in this study with 73.15% and 55.2% respectively as shown in Table 4. Chemically fertilized cabbage had shown the better moisture content compared to organically fertilized soil with an average of 87.6% moisture content. The P-value indicating the significant difference between the mean values of the moisture contents of plants was calculated to be approximately 0.48%. According to the present study, all subjected plants (Cabbage, carrot and tomato) resulted higher than normal moisture contents (Table 4). A reason for this could be the harvesting time of the plants, which was August. According to the annual report from National Meteorology Agency, August is the rainiest month with 400mm average annual rainfall. Vegetable production is usually advised on dry and cool season to ensure lower disease and pest infestation (Duncan *et al.*, 2012). Vegetables like cabbage and tomato also easily get spoiled on rainy season due to their high moisture compositions (Hilmi, 2019). This specific study was carried out at a greenhouse condition with relatively controlled environment. However it doesn’t necessary mean it isn’t prone to the weather changes. Hochmuth (2012) greenhouse experiments also indicated that the supply of nutrients like Phosphorous, Sulfur and Iron from the soil could vary depending on the weather condition. Weather patterns also have an impact on the activities of fertilizers and their efficiency in the soil. This is reported by different studies on vegetable crops like Barrett *et al.* (2018) and Reza *et al* (2016). The effect of organic and inorganic fertilizers could also vary depending on the weather pattern and water availability in the soil (Quansah, 2010).

In the present study soil treated with chemical fertilizer showed slightly higher moisture content in Cabbage and Carrots (Table 4). One major reason for this could be the faster effect of chemical fertilizer in shorter time interval. Cabbage and carrot take relatively shorter time to grow as it takes only 60 to 90 days to grow the crops (Badgley *et al.*, 2007). This might result more efficiency of chemical fertilizers than organic ones. Commercial fertilizers have readily available N which can be consumed and processed faster in the soil unlike organic fertilizers which are the slow release types (Ojatayo *et al.*, 2011). Organic fertilizers do not release nutrients fast but through longer time as it decomposes in soil. Organic fertilizers also extra sensitive to water supply, heat and other elements than the commercial fertilizers (Shaji *et al.*, 2021). However the moisture content of tomato was high on the plants treated with stored urine fertilizers (Table 4). This might be due to the fact that tomato takes longer time to grow (80-110 days) resulting better reaction to organic treatment. Stored urine is also found to be more quick fertilizer that other organic fertilizers like manure or bone meal mainly because it is easily soluble and more evenly distributed in soil (Berkelaar, 2010). This is in agreement with reports made by Wu *et al.* (2020), Islam *et al.* (2017) and Biswas *et al.* (2022).

4.1.2. Protein and nitrogen content

Cabbage responded considerably well to stored urine fertilizer than other treatments with an average of 5.5% N and 34.74% protein contents (Table 4). The nitrogen contents of carrot showed a slight increase when treated with different fertilizers than unfertilized soil. Stored urine fertilizer had also shown better results in tomato with 2.282% N and 14.2625% protein contents in average recorded. In all three plants, vegetables taken from unfertilized soil showed the lowest content of Nitrogen and protein. The P-value indicating the significant difference between the mean values of the Nitrogen and protein contents of plants were calculated to be approximately 0.1% and 0.01% respectively. According to the present study, Carrot resulted slightly higher value of Nitrogen in inorganically fertilized soil. This report is supported by similar study by Lairon (2010). The study attributed the readily available Nitrogen in commercial fertilizers as the reason. It suggested that nitrogen available in organic fertilizer is less, thereby slows down its production and accumulation of Nitrogen in the plant. This report however, contradicts the outcome from cabbage and tomato as they resulted higher N value on those plants treated with organic fertilizers. This unsynchronized response from vegetable crops to fertilizers could be the result of varying nitrogen-releasing process in the soil and crops' nutrient demand (Kirchmann *et al.*, 2008).

Table 4: One way analysis of variance (ANOVA) on the effect of organic and inorganic fertilizer on the nutritional composition of selected plants.

Plant	Treatments	Average moisture (%)	Nitrogen (%)	Protein (%)	Phosphorous (ppm)
Cabbage	NPS fertilizer	87.6 ± 0.5	4.172 ± 0.1	26.075 ± 0.1	17.598
	Stored urine	83.2 ± 0.1	5.558 ± 0.75	34.7375 ± 0.75	19.578
	Struvite urine	84.15 ± 0.5	2.31 ± 0.25	14.4375 ± 0.25	26.106
	None	77.75 ± 1.65	1.932 ± 0.1	12.075 ± 0.1	18.084
Carrot	NPS fertilizer	86.4 ± 0.1	0.882 ± 0.35	5. 5125 ± 0.35	18.54
	Stored urine	73.15 ± 1.65	0.798 ± 0.05	4.9875 ± 0.05	18.84
	Struvite urine	55.2 ± 0.1	0.798 ± 0.05	4.9875 ± 0.05	22.296
	None	83.7 ± 0.85	0.644 ± 0.1	4.025 ± 0.1	16.386
Tomato	NPS fertilizer	92.1± 0.3	1.974 ± 0.1	12.3375 ± 0.1	8.298
	Stored urine	93.1 ± 0.8	2.282 ±0.15	14.2625 ± 0.15	20.532
	Struvite urine	92.1 ± 0.4	0.896	5.6	16.524
	None	92.35 ± 0.45	0.364 ± 0.9	2.275 ± 0.9	19.182
F		4.330407	3.292238	6.629953	1.077837
P-value		0.048144	0.10838	0.016992	0.380505
F-crit		10.10671	14.54411	10.10671	10.10671

4.1.3 Phosphorus

The phosphorous values of cabbage and carrot were higher on those treated with struvite urine with 26.1 ppm and 22.2 ppm respectively as shown in Table 4. In both Carrot and cabbage, unfertilized soil resulted the lowest phosphorus content with 18.084 ppm and 16.386 ppm respectively. Tomato treated with stored urine fertilizer showed the highest phosphorus content while samples treated with chemical fertilizer reacted the least with considerably lower phosphorus content of 8.298 ppm. In all three vegetable crops organic fertilizers had showed better phosphorus content compared to chemical fertilizers. The P-value indicating the significant difference between the mean values of the phosphorous content of plants was calculated to be approximately 0.38%. On this study, the phosphorous values of selected crops were higher on those vegetables treated with organic fertilizers (stored and struvite urine) (Table 4). This result is in agreement with reports made by Wang *et al* (2019) and Vieria *et al.,* (2023). Study by Wang *et al* (2019) suggested that, the reason for this could be the faster depilation of available P in the soil upon use of Chemical fertilizers. This will lead to poor root development and lower plant yield. Organic fertilizers in the other hand, takes enough time to distribute in soil and have significantly higher capacity to facilitate absorption of nutrients from the soil (Vieria *et al.*, 2023). On present study, Cabbage and carrot showed higher P value on plants fertilized by struvite urine than stored urine (Table 4). Struvite urine have low solubility and guarantees a slow but steady nutrient supply to the soil (Miso, 2008).

4.1.4 Yield

The graph depicting yield showed that tomato had given considerably higher yield when treated with chemical fertilizer than the other samples with an average of 6.5 tomato yield per pot (Figure 5). The rest of the treatments had shown similar results with similar amount of fruits. However, the yield from carrots when treated with struvite urine was the highest. Cabbage reacted well to chemical fertilizer as it produced 18 leaves per pot. Beside this, cabbage had given the same result to both organic treatments with similar numbers of leaves. The yields of these different plants are shown in Figure 5.

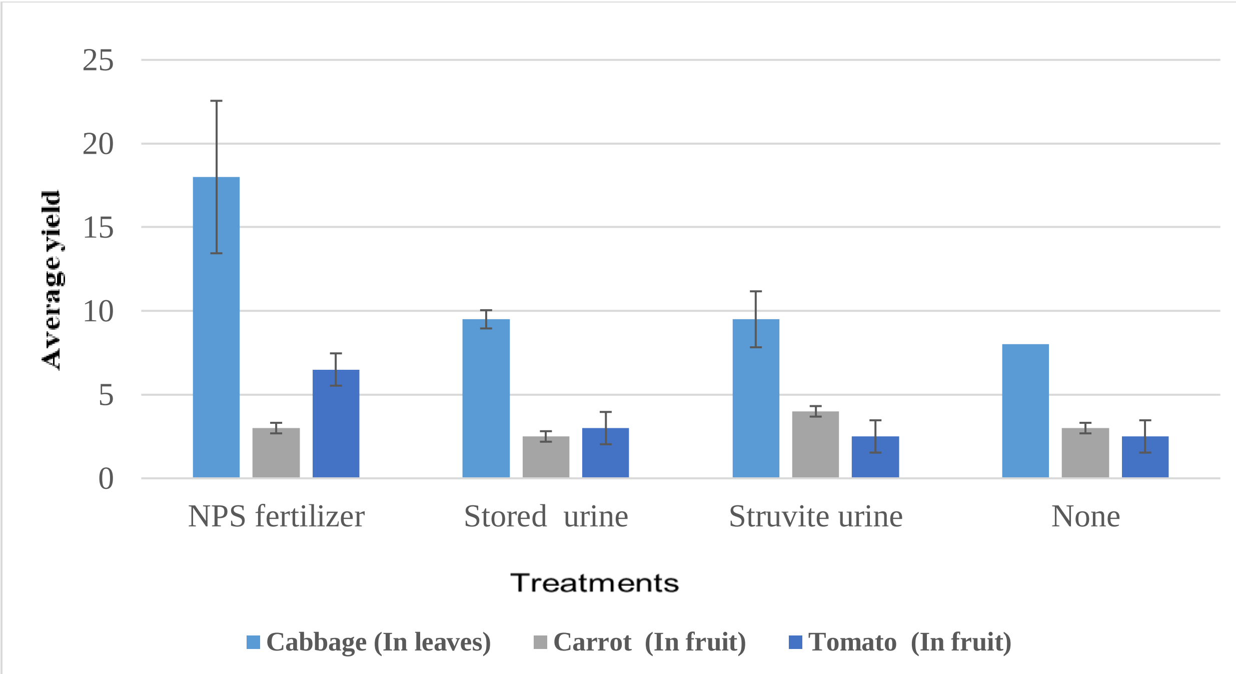

Figure 5: Mean comparison between yields of the selected treated with different fertilizers.

The physical characteristics of the soil used for vegetable growth also has an affect the nutrition composition of the plant. This it mainly because the pore space in the soil has a direct effect on the movement of air, water and nutrients in the soil and its supply to the plant (Huntley, 2023). This is indicated in several studies on vegetables like Pongrac *et al.*, (2019) study on cabbage and Zucco *et al.*, (2015) and Ahmed and Li (2021) study on tomato. On the present study, Clay soil was chosen to grow the vegetable crops mainly due to its availability and fertile nature. Soil with high content of clay is the best to grow vegetable because it has well-drained system making it hold the right amounts of water, nutrient and air for better plant growth and increased biological activity (Hong *et al.*, 2022). Clay soil also react differently to organic and inorganic fertilizers. Organic fertilizers can help improve clay soil by loosening it and improving its air pockets which will eventually lead to easier supply of vital nutrients to the crops (Sarkar *et al.*, 2018). However in clay soil, inorganic chemicals found to be more suitable fertilizers. This is because inorganic fertilizers are highly concentrated and often more effective on addressing nutrient deficiencies of clay soil (Si *et al.*, 2016). The yields from chemically fertilized crops (Cabbage and Tomato) were higher than organically fertilized plants (Table 4). Chemical constitute of fertilizers used is an important factor. Urine, as an organic fertilizer could contain growth- inhibitory salts like NaCI in higher concentration than other organic fertilizers like manure (Perveen *et al.*, 2012) and this is more problematic on the growth of salt intolerant crops like cabbage. However, carrot produced higher yield on those plants treated with organic fertilization as it is more tolerant to higher salt composition and takes enough time to benefit from organic fertilization. Studies like Auebach *et al.*, (2013) and Badgley *et al.*, (2007) also demonstrated the same results on studies conducted on different vegetables.

The statistical result obtained from the present study indicated that there was no significant difference ($P<0.05$) between the mean values of measured nutritional composition. Pradhan et al (2007) study on cabbage was also in agreement with this result. The study reported that the chemical and the biological composition of the plant resulted similar outcome for both organic and inorganic fertilization. However study by Alemayhew (2020), on urine fertilizer reported significant increase of production during organic fertilization. This report also emphasized that the dose of urine is very crucial and suggested 1:3 urine to tap water ratio as a preferable dose for better results. Similarly bigger and more extensive study by Katherine (2007) also reported a very considerable advantage of organic fertilization over the conventional one. This study conducted on crops like tomatoes, potatoes, cabbage and lettuce, reported 20% to 47% increase nutrient compositions on plants during organic fertilizers.

4.2. The distribution of identified bacterial groups in the soil

In this study, vegetable crops (Cabbage, Ethiopian kale, carrot and tomato) were used to show the distribution and abundance of bacterial groups in the soil during different fertilization practices. Soil from cabbage and tomato used to compare the distribution of bacteria in struvite urine treated soil with non-fertilized soil. Ethiopian kale was used to compare the bacterial distribution in organically fertilized soil with unfertilized soil. The soil from carrot was used to compare the bacterial distribution in organically and inorganically fertilized soil with unfertilized soil.

Alpha diversity index was used to measure the species richness and diversity of soil bacteria with different fertilization treatments (Figure 6). The number of bacterial species observed is reported in Figure 7. In cabbage and tomato, the number of observed bacteria were lower in struvite fertilized soil compared unfertilized soil, A total of 245 and 279 bacterial species were observed in unfertilized soil of cabbage and tomato respectively (Figure 7). In struvite treated soil however, the number of bacterial species decreased to 184 species and 211 species respectively as shown in Figure 7. Bacterial diversity was also lower in soil (cabbage, carrot and Ethiopian kale) treated with struvite urine fertilizer in comparison with unfertilized soil as shown in Figure 6. The result from present study is in agreement with study by Ritti *et al.*, (2019). The higher than normal heavy metal composition of struvite fertilizer can result lower bacterial diversity and composition (Huang *et al.*, 2019). Struvite urine is the most metal prone organic fertilizer, even compared with stored urine (Ronteltap *et al.*, 2007). Heavy metal from this struvite urine fertilization might lead to disturbed activity of carbon, nitrogen and other organic matters, resulting lack of soil microbial diversity (Huang *et al.*, 2019). The limited activity of struvite fertilizer on upper layer of soil could also be a reason for the outcome as most of the samples for the present study were collected from the upper soil layer (Rech *et al.*, 2019).

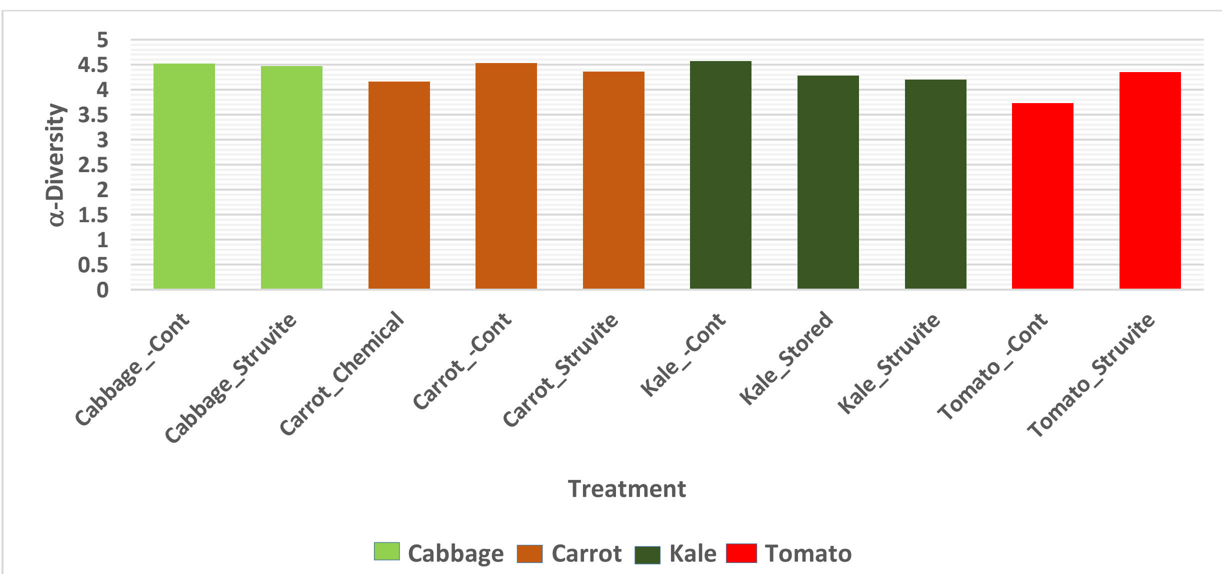

Figure 6: Diversity of soil bacteria planted with cabbage, carrot, Ethiopian kale and tomato with different fertilizer treatment.

In Ethiopian kale the bacterial richness was higher in stored urine fertilized soil than struvite (Figure 6). Struvite urine is phosphorous based fertilizer with limited N and almost zero potassium recovery which is not the case for stored urine (Ronteltap *et al*., 2007) and this might lead to lower composition of soil bacteria with high demand of Nitrogen and potassium. However the number of observed bacteria was higher in struvite treated soil than stored urine fertilized soil as shown in Figure 7. The reason for this could be the higher abundance of specific phosphate solubilizing bacteria like *Pseudomonas* and *Actinomycetes*. In carrot, soil fertilized with chemical showed the lowest microbial richness compared to organic fertilizers (Figure 6). Studies like Šarauskas *et al.*, (2021), Zebarth *et al.*, (2005), and Crusciol *et al.*, (2020) also reported increase in bacterial diversity with organic fertilization. However the number of bacterial species was considerably high in chemical fertilized soil with a total of 254 species observed (Figure 7). The reason for this could be the quick nutrient providing capacity of chemical fertilizers (Liu *et al.*, 2011). Some soil bacteria like *Gemmatimonadetes*, and *Chloroflexi* have a very short time span for growth and production hence requiring faster supply of nutrients which couldn't be provided by slow-functioning organic fertilizers (Lauber *et al.*, 2009). Administering the appropriate dosage of fertilizers is also very crucial for optimum activity of soil microbes as indicated by Callejaa *et al.*, (2015).

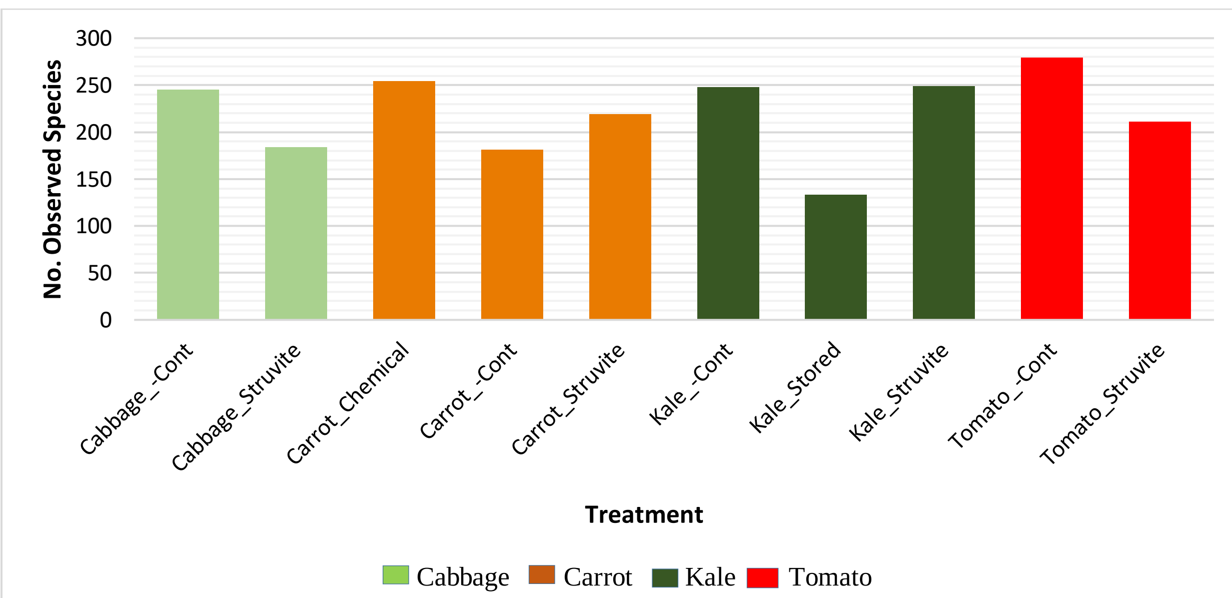

Figure 7: Observed species of soil bacteria planted with cabbage, carrot, kale and tomato with different fertilizer treatments.

4.2.1 Phylum-level distribution of identified bacterial groups in the soil upon different fertilizer application

Soil samples taken from pots planted with selected vegetables and fifteen bacterial groups were identified at the phylum level (Figure 8). The identified groups were *Thaumarchaeota*, *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Candidatus Rokubacteria*, *Chloroflexi*, *Cyanobacteria*, *Chlamydiae*, *Deinococcus-Thermus*, *Firmicutes*, *Gemmatimonadetes*, *Planctomycetes Proteobacteria* and Verrucomicrobia. In cabbage, *Chloroflexi*, *Cyanobacteria*, *Firmicutes*, *Gemmatimonadetes* and *Planctomycetes* were the most abundant groups in struvite treated soil while *Candidatus Rokubacteria* was mostly abundant in unfertilized soil (Figure 8). Similarly in carrot, *Candidatus Rokubacteria* was better distributed in unfertilized soil than organically or inorganically fertilized soil (Figure 9). The reason for this might be the extra sensitivity of *Candidatus* species to change in environment. *Candidatus Rokubacteria* have highly specific growth requirements including the need for a specific nutrient composition, specific pH conditions, temperatures, atmospheric pressure or levels of oxygen (Kopke *et al.*, 2005). Due to this, the bacteria might not adopt well to change in soil environment caused by any sort of fertilization.

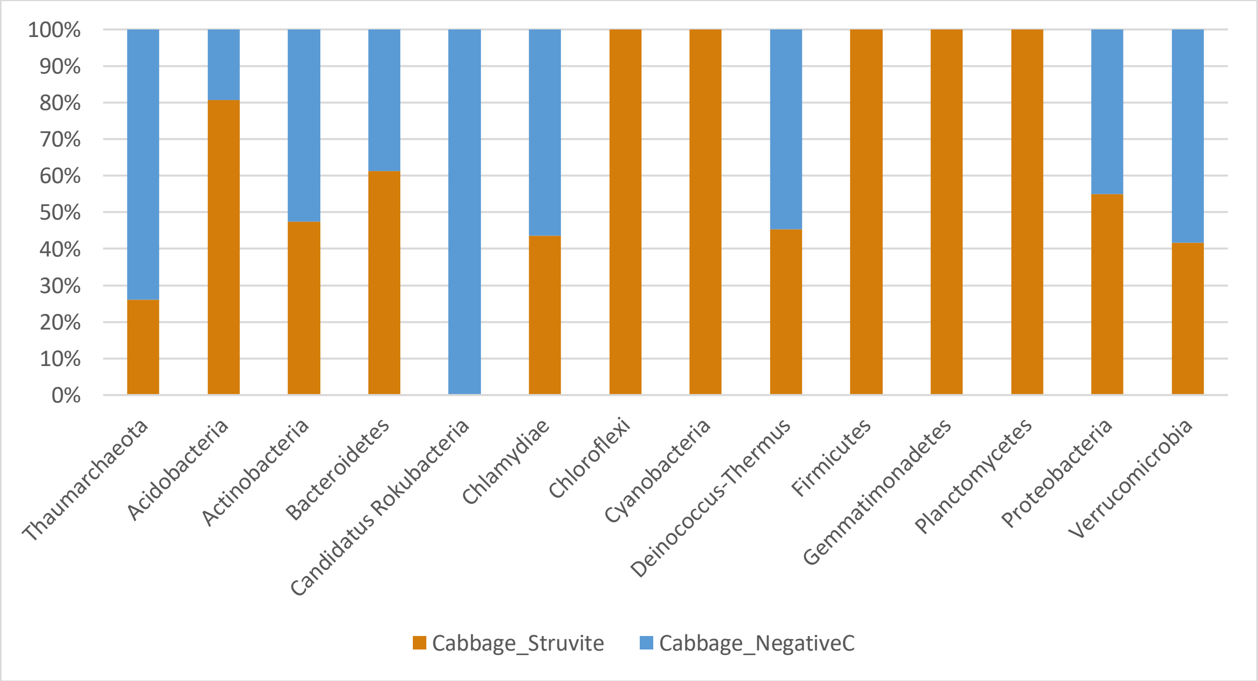

Figure 8: Phylum-level distribution of identified bacterial groups in the soil planted with cabbage.

Firmicutes is the most distributed bacterial group in struvite treated soil of cabbage and carrot as shown in Figure 8 and Figure 9. It is also abundant in soil fertilized with stored urine as shown in Ethiopian kale (Figure 11). This could be due to adaptability of the bacteria in different environment as *Firmicutes* known to thrive in such extreme environments, by producing spores that render them resistant to change in the soil (Yadav *et al.*, 2015).

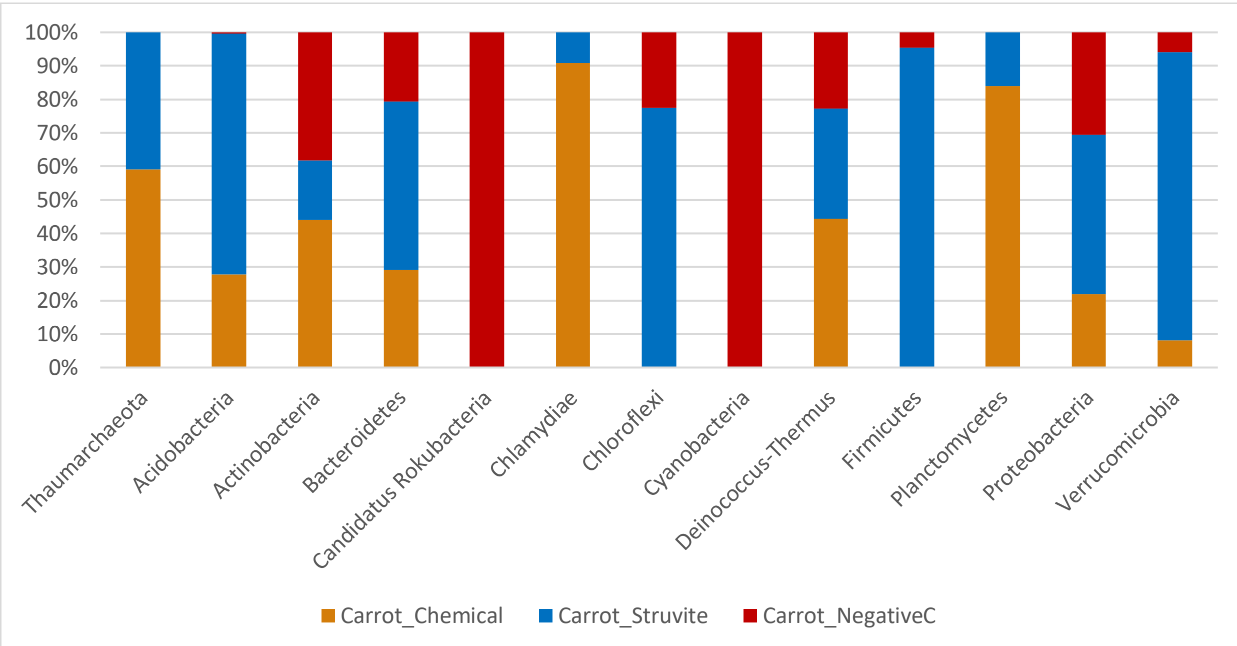

Figure 9: Phylum-level distribution of identified bacterial groups in the soil planted with Carrot.

In soil planted with tomato, *Planctomycetes* and *Acidobacteria* were more distributed in unfertilized soil compared with struvite urine fertilized soil and *Cyanobacteria* was significantly more available in struvite treated soil like shown in Figure 10. *Cyanobacteria* was also well distributed in struvite treated soil of cabbage (Figure 8). *Cyanobacteria* is Nitrogen fixing bacteria which could benefit from Phosphorous based fertilizers like struvite urine (Nascimento *et al., 2019).* The bacteria is also heavy metal resistant and can grow and reproduce in metallic soil which might be created by struvite urine fertilization of soil. A study by *Al-Sherif et al*., (2015) suggested that heavy metal resistance nature of the *Cyanobacteria* makes it even suitable instrument for phytoremediation. The bacterial groups *Planctomycetes* and *Chlamydiae*, distributed well in chemically fertilized soil as shown in Figure 9. They are also well distributed in struvite soil as shown in cabbage (Figure 8) and carrot (Figure 10). This is in agreement with a study by Buckley *et al.*, (2006), which also reported the increased distribution of *Planctomycetes* with both organic and inorganic fertilization of soil. Most *Planctomycetes* and *Chlamydiae* groups are also slow – growing bacteria (Chodak *et al.*, 2015) which might make them benefit from both inorganic and urine fertilizations.

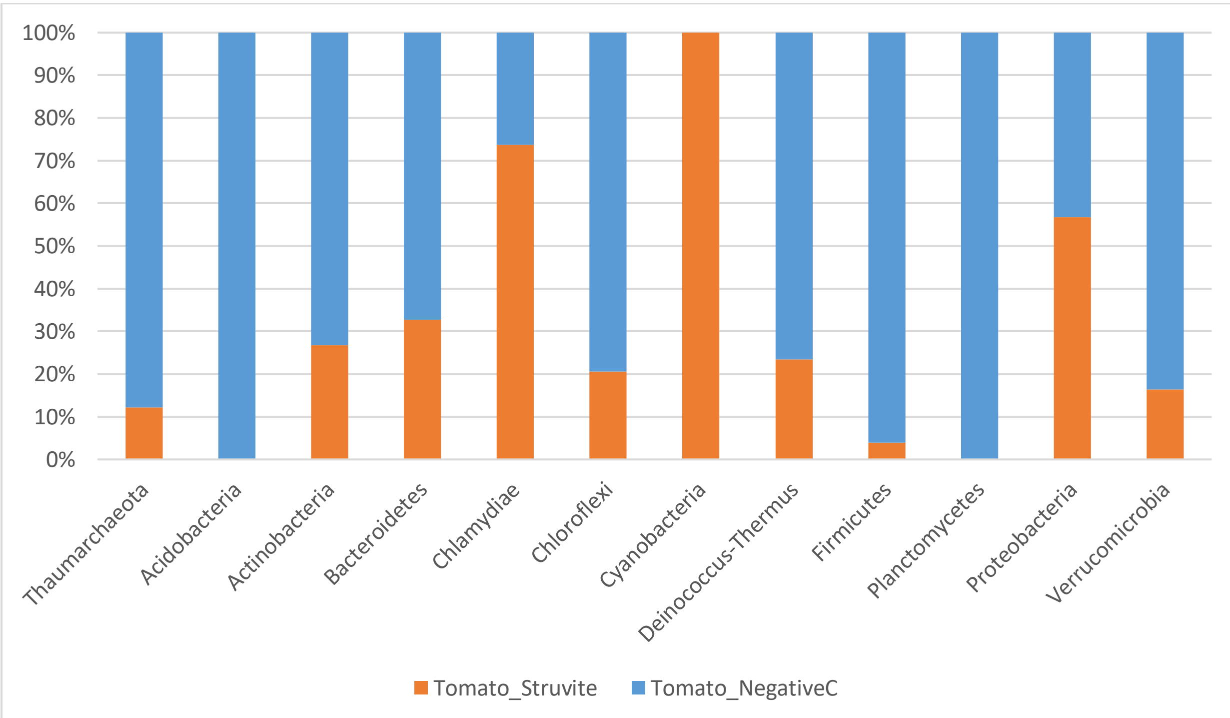

Figure 10: Phylum-level distribution of identified bacterial groups in the soil planted with tomato.

In the soil from Ethiopian kale, the composition of most of the identified groups were higher in stored urine fertilized soil than struvite treated soil (Figure 11). *Acidobacteria, Bacteroidetes, Candidatus Rokubacteria, Chloroflexi, Firmicutes, Gemmatimonadetes* and *Verrucomicrobia* were mostly abundant in stored urine fertilized soil (Figure 11). This is in agreement with study by Ren *et al.*, (2018). According to this study, the sufficient nitrogen and carbon provided by organic fertilizer promotes the growth and reproduction of soil microbes however, the excess available phosphorus provided by struvite urine fertilization might lead to reduced diversity of soil microbial communities. At the phylum level bacterial groups such as *Bacteroidetes*, *Deinococcus*, *Proteobacteria* had shown relative stability to different fertilization treatment among four vegetables planted (Cabbage, Carrot, Ethiopian kale and Tomato). Similar Study conducted by Enebe and Babaloa (2020) also reported that, regardless of the fertilization regimes, *Proteobacteria* and *Bacteroidetes* were well distributed in the soil, but in varying population sizes. According to the present study, *Chloroflexi* showed the most sensitivity to change in fertilization. This outcome disagrees with studies by Hallin *et al.*, (2018) and Li jin *et al.*, (2022) which reported the abundance of *chloroflexi* to be significantly higher during chemical fertilization.

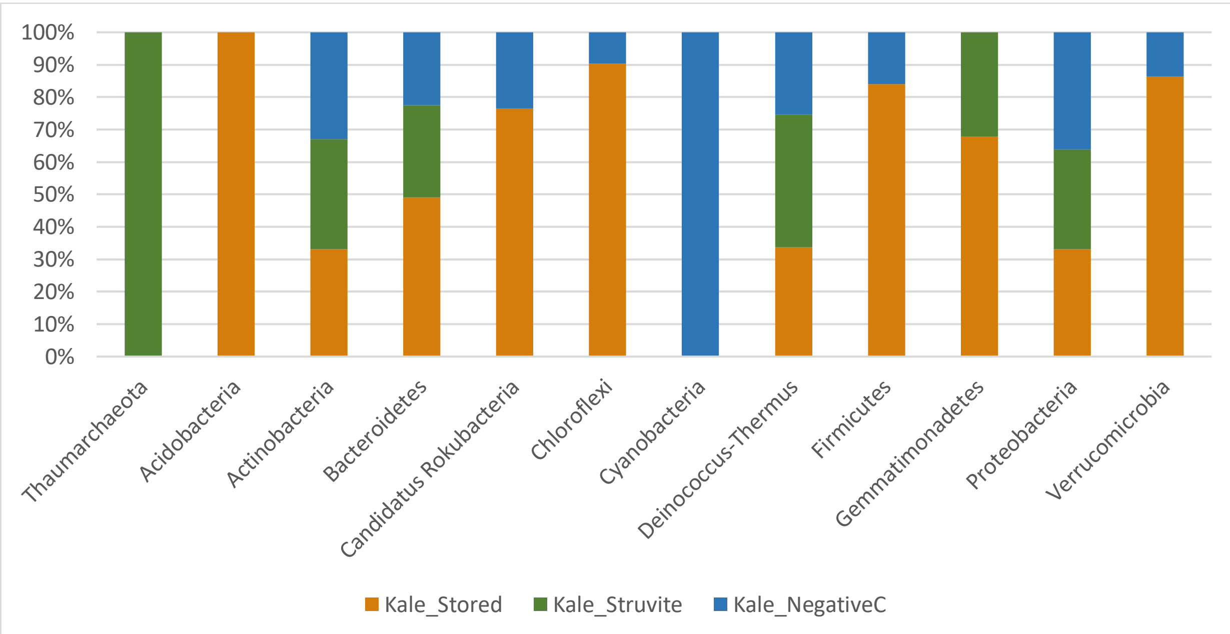

Figure 11: Phylum-level distribution of identified bacterial groups in the soil planted with Ethiopian kale.

4.2.2. Genus-level distribution of soil microbiota upon different fertilizer application

At the Genus level, the identified groups were *Nocardioides, Pseudomonas, Nitrosospira, Acinetobacter, Arthrobacter, Lysobacter, Solirubrobacter, Pseudonocardia, Aeromonas, Sphingmonas, Bradyrhizobium, Altererythrobacter, Rhodococcus, Brevundimonas, Stenotrophomonas, Sporichthya, Comamonas, Mesorhizobium, Mycolicibacterium* and*Acanthamoeba.* Genus *Aeromonas* was mostly distributed well in unfertilized soil as shown in cabbage (Figure 12) and tomato (Figure 14). Its composition is also relatively good in chemical treated soil as shown in soil planted with carrot (Figure 13). However the composition of *Aeromonas* is drastically lower in soil treated with stored urine. Stored urine is highly characterized by its significantly high alkaline pH. (Vinneral *et al.*, 2008). This might lead to lower distribution of bacteria like *Aeromonas,* which are well known to thrive better in acidic environment

(Vivekanandhan *et al.*, 2003). *Aeromonas* alongside with other bacteria like *Enterococci* and *Pseudomonas* are also the fastest pathogens to die-off during urine storage due to this same reason (Hoglund *et al.*, 2001).

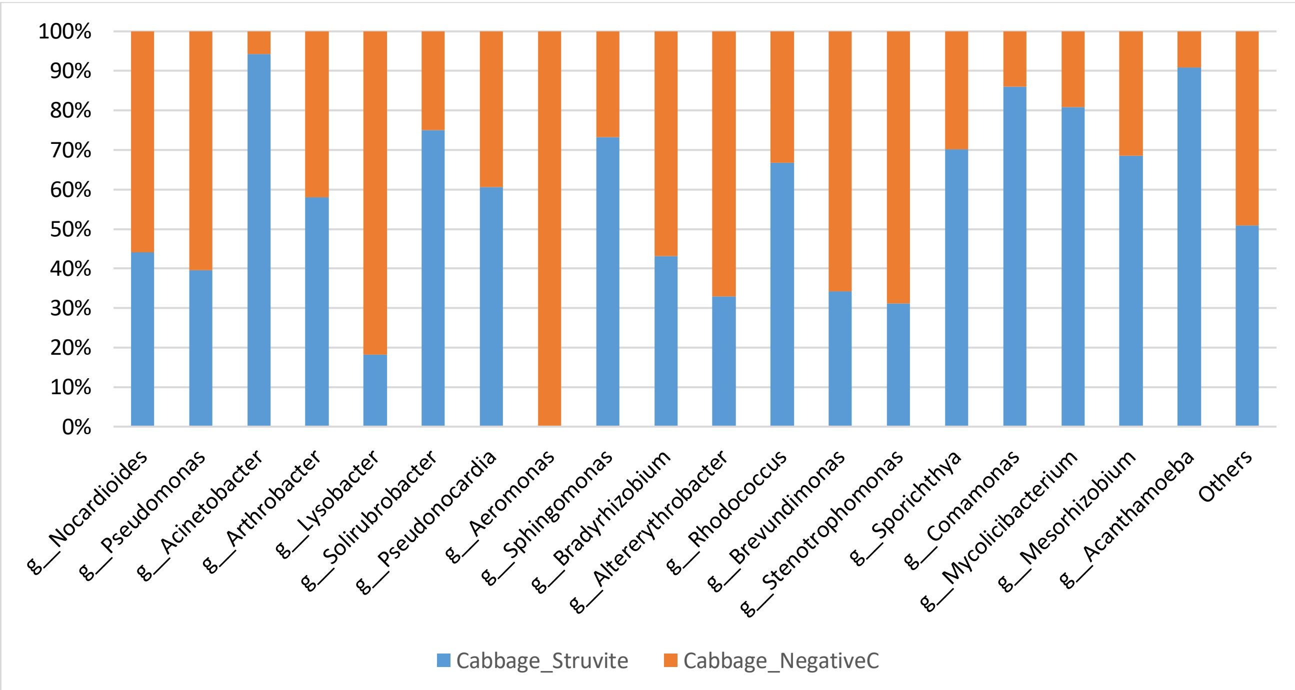

Figure 12: Genus-level distribution of identified bacterial groups in the soil planted with cabbage.

Bacterial groups like *Nitrosospira*, *Acinetobacter*, *Rhodococcus* and *Arthrobacter* didn't react well to stored urine as shown in Ethiopian kale (Figure 15). The reason for this could be the high amount of free ammonia in the soil caused by application of stored urine. Stored urine contains high rate of easily available ammonia due to urea Hydrolysis and the oxidation of this Ammonia is an extra slow process (WHO, 2006). The oxidation of this free ammonia could also be further lagged by other factors like the structure of soil. Heavy soil like clay have a limited presence of interfacial interactions among soil particles which could lead to very slow oxidation process resulted high accumulation of Ammonia (Wu *et al.*, 2018). Bacteria, especially those which mainly active around a root are affected by this unusual abundance of free ammonia (Braissant *et al.*, 2015). Similar outcomes are reported by researches conducted by Alimova *et al* (2006), Mueller (2005) and Kleber *et al.*, (2021).

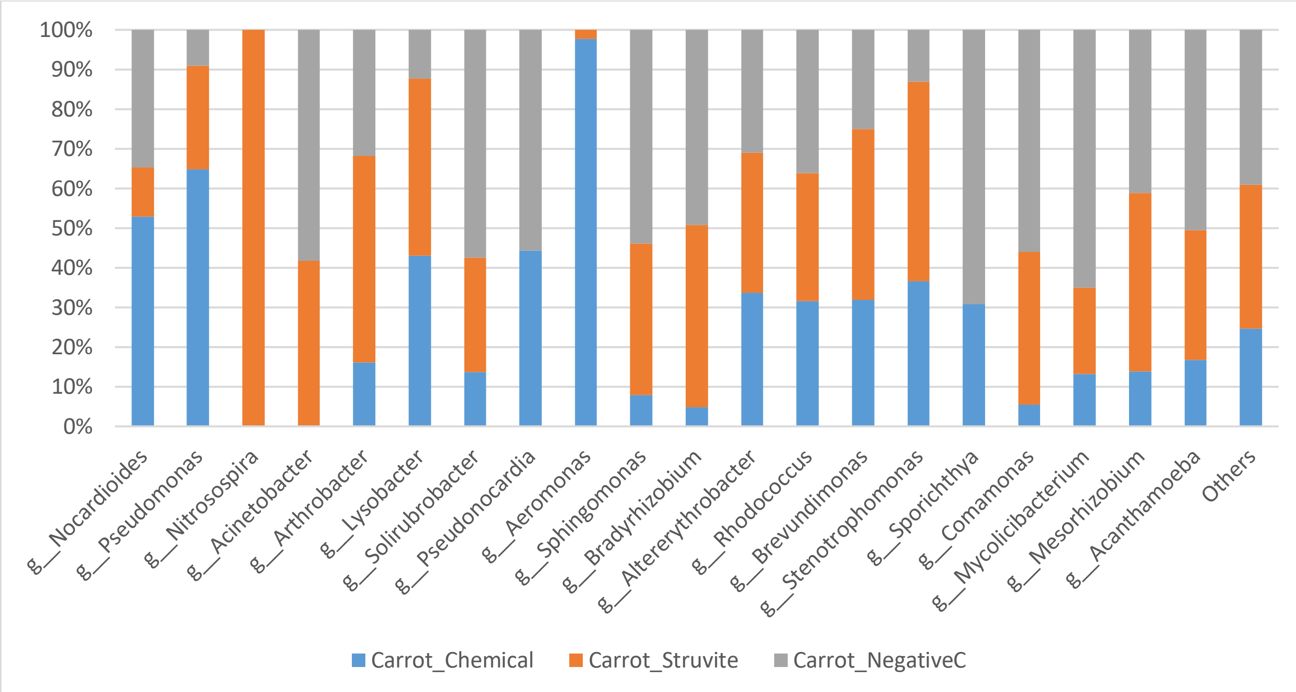

Figure 13: Genus-level distribution of identified bacterial groups in the soil planted with carrot

Genus *Nitrosospira* is the bacterial group that responded the most unfavorably to chemical fertilization as shown in Figure 13. Bacterial groups like *Arthrobacter* and *Stenotrophomonas* also showed better distribution in struvite treated urine than chemical fertilized soil (Figure 13). The main activity of *Nitrosospira* in soil is Nitrogen fixation (Joshi, 2020). This bacteria are associated with transforming atmospheric N to fixed nitrogen as they themselves become Bio fertilizers in soil. Due to this, they prefer natural soil environment over chemical fertilization of any sort to store fixed nitrogen permanently (Landry, 2011). Soil from cabbage (Figure 12), carrot (Figure 13) and tomato (Figure 14) all showed the distribution of most of identified bacterial groups were higher in struvite urine fertilized soil than unfertilized soil. Studies like Ganesapillai *et al.*, (2016) and Gerrewey *et al.*, (2021) also reported urine based fertilizers created better environment for most of soil bacteria.

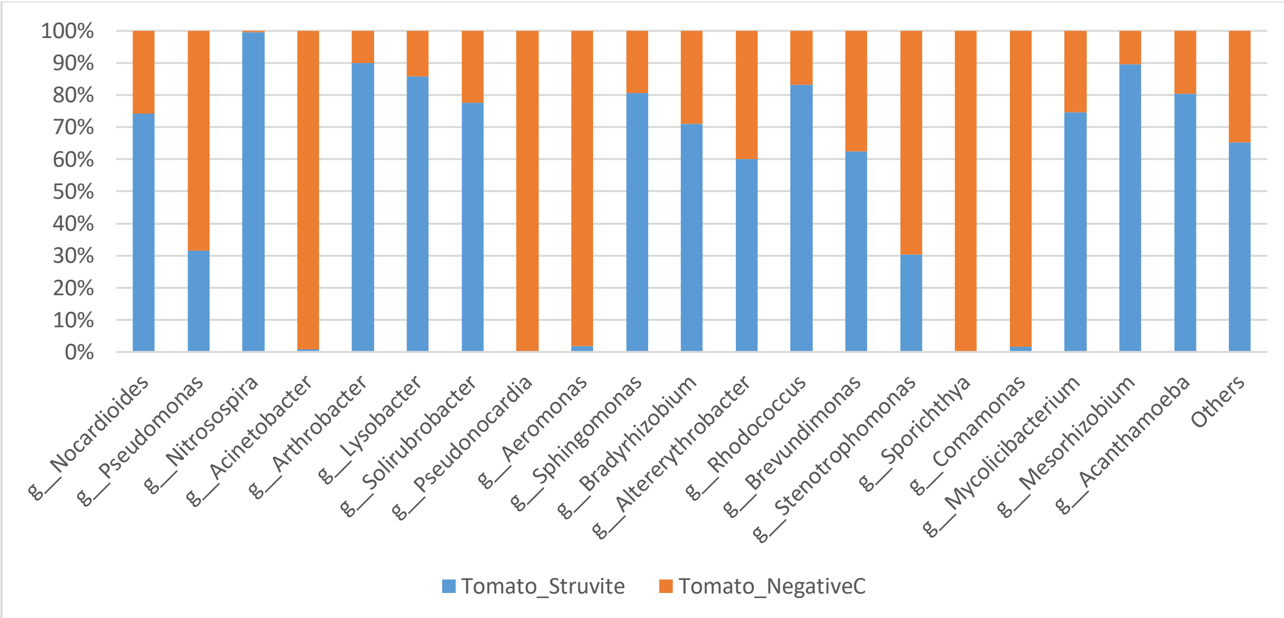

Figure 14: Genus-level distribution of identified bacterial groups in the soil planted with tomato

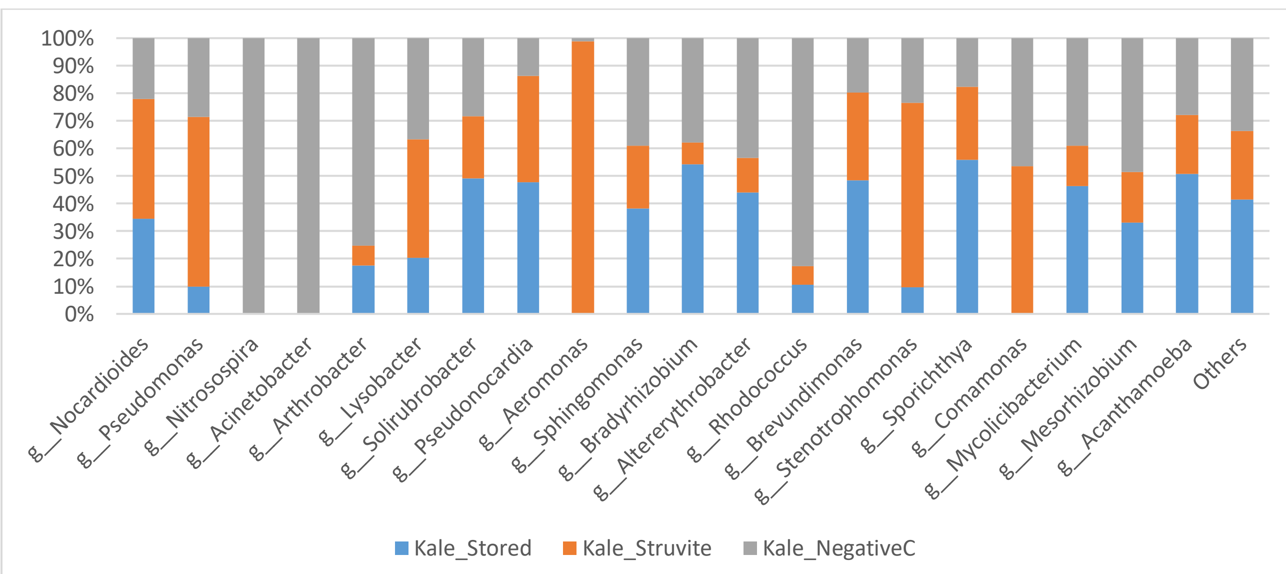

Figure 15: Genus-level distribution of identified bacterial groups in the soil planted with Ethiopian kale.

On the present study, groups like *Nocardioides*, *Pseudomonas* and *Aeromonas* distributed better in chemical fertilized soil as shown in Figure 13. This is in agreement with studies by Ling *et al.*, (2017) and Razanamalala *et al*, (2018) which reported that there are indeed few bacterial groups that responded better to chemical fertilizers than organic ones.

Other studies like Velimirov *et al.,* (2011), Pillai *et al.,* (2017) and Zhou *et al.,* (2020), further elaborated the impact of geographical and environmental conditions alongside fertilization practice on soil microbial community diversities and compositions.

5. CONCLUSION AND RECOMMENDATION

Conclusion

We examined the effect of organic (urine) and inorganic fertilizers on soil microbiota and nutritional contents of selected vegetables. Most common soil bacteria were identified and used to measure the shift in soil microbial community that could be caused by fertilization. A total of eleven bacterial groups identified at the phylum level and twenty at genera level. According to the present study, struvite urine fertilizer (organic fertilizer) resulted slightly better bacterial distribution both at phylum and genus level. Stored urine fertilization also resulted better reaction of some soil bacteria as shown in Ethiopian kale. The nutritional analysis performed on three vegetables (cabbage, carrot and tomato) also showed there is no significant difference in nutritional contents of plants treated with organic and inorganic fertilizers. In conclusion, the outcome of this study showed organic fertilization can indeed be considered as an alternative to chemical fertilization for vegetable growth. The potential of human urine as an organic fertilizer can also be maximized with better understanding of its correlation with other factors like climate, soil structure and soil chemical composition.

Recommendation

There are still several questions that remain unanswered on this topic area. Some recommended point for further study are;

- Studies conducted in longer time interval are recommended. The effect of both organic and inorganic fertilizers can be varied based on the climate condition so further study should be conducted to emphasize that.
- Detailed study based on functional analysis of the soil microbial community in response to different fertilization can also be useful.
- Further studies based on varied dosage of fertilizers (both organic and inorganic) and its respective effect on the soil microorganisms could also be useful.
- More studies should be conducted with an emphasis of the effect of soil fertilization on other soil microorganisms like Fungi, Protozoa and viruses.
- More Studies based on integrated use of organic and inorganic fertilizers and its effect on soil microbiota is also recommended.

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THESIS APPROVAL SHEET

We certify that Tseganesh Darsema Tadesse's M.Sc. Thesis entitled "**THE EFFECT OF URINE-DERIVED FERTILIZERS ON SOIL MICROBIOTA AND NUTRITIONAL CONTENTS OF SELECTED VEGETABLES** '' has been conducted under our direct supervision. Therefore, we kindly request the Institute of Biotechnology of Addis Ababa University to final approval and acceptance of the thesis.

	Date:	**Signature**
Examinee		
Tseganesh Darsema Tadesse	________________	______________
External examiner		
	_________________	_______________
Internal Examiner		
	__________________	_______________
Advisor's Name		
Adey Feleke (PhD)	__________________	________________

Dr. Addis Simachew (PhD), IoB, AAU Signature _____________Date_____________

Director, Institute of Biotechnology

Addis Ababa University