BACTERIOLOGICAL EVALUATION AND PLANT GROWTH PROMOTING PROPERTIES OF ANIMAL MANURE

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CERTIFICATION

We certify that this work was carried out by Amenze Edokpolor in the Department of Microbiology, Faculty of Life Sciences, University of Benin, Benin City, Nigeria.

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CERTIFICATION OF THESIS

We the undersigned attest and declare that the thesis of Amenze Edokpolor titled: "Bacteriological Evaluation and Plant Growth Promoting Properties of Animal Manure" has successfully passed the anti-plagiarism test and does not violate any copyright regulations.

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DEDICATION

This work is dedicated to God Almighty.

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ABSTRACT

Animal manure, a sustainable alternative to chemical fertilizers, is rich in beneficial microorganisms capable of improving soil structure, nutrient availability, and crop yield. Understanding the bacteriological characteristics of these manures is crucial for optimizing their agricultural application while minimizing potential pathogenic risks. The study was conducted to investigate the microbial composition and plant growth-promoting potential of ruminant and non-ruminant animal manures to enhance soil fertility management. The research aimed to isolate and identify bacteria present in animal manures using phenotypic and molecular methods, evaluate their PGP traits, and assess their effects on the germination and growth performance of *Telfairia occidentalis* (fluted pumpkin).

A total of twenty-four manure samples from ruminant and non-ruminant animals were collected from different farms in Benin City, Edo State, Nigeria. Standard microbiological procedures were used for isolation, enumeration, and biochemical identification of bacterial isolates. Phenotypic characterization involved Gram staining and biochemical assays such as oxidase, indole, catalase, urease, citrate utilization, and triple sugar iron tests. Molecular identification was performed using 16S rRNA gene sequence analysis. Pathogenicity tests, including gelatin liquefaction, DNase, lipase, and hemolysin assays, were conducted to assess the virulence potentials of isolates. The isolates were also screened for plant growth-promoting traits such as indole-3-acetic acid (IAA) and ammonia production. In addition, the impact of different manure treatments on *Telfairia occidentalis* was evaluated through germination rate, vine length, leaf area, chlorophyll content and biomass yield.

The results revealed that the heterotrophic bacterial count ranged from 13.30×10^5 cfu/g in non-ruminant manure to 27.80×10^5 cfu/g in mixed manure, while the coliform count varied between 6.40×10^4 cfu/g and 13.69×10^4 cfu/g, indicating a higher microbial load in the mixed manure samples. Six major bacterial species were identified *Enterobacter cloacae*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella enterica* and *Bacillus velezensis* with *E. coli* showing the highest frequency of occurrence (25%). Molecular characterization further revealed *Pseudomonas aeruginosa* strain PA3 (88.5% identity), *Escherichia coli* strain NCCP 15734 (92.1%), *Bacillus velezensis* strain UA0297 (97.3%) and *Klebsiella pneumoniae* strain BUH3 (96.8%). Most isolates demonstrated positive results for plant growth-promoting

traits such as nitrogen fixation, Indole Acetic Acid (IAA) and ammonia production. Growth trials on *Telfairia occidentalis* showed that plants treated with mixed manure had the best performance, with an average vine length of 76.05 cm, leaf number of 33, and leaf length of 25.13 cm by week eight, compared to control plants with 55.13 cm vine length and 20 leaves. These findings confirm that animal manures, particularly mixed manure, significantly enhanced plant growth and soil microbial quality and therefore can serve as biofertilizers for sustainable agriculture and improved crop productivity.

CHAPTER ONE

INTRODUCTION

1.0 Background of Study

Organic farming and livestock production have expanded worldwide in recent years. These systems emphasize simple production methods and environmental balance while maintaining productivity and supporting farmer income (Moura and Jesus, 2015). Organic farming and bioformulations are gaining popularity among farmers and people across the globe for various health benefits and eco-friendly approaches, also to fight against the contrary effects of chemical fertilizers and pesticides. Hence, there is urgent need for the scientific validation of microflora resident in organic manure sourced from indigenous and exotic breeds (Shweta *et al.,* 2025).

Conventional farming depends heavily on non-renewable resources and industrial inputs, often employing them in an extractive manner. This causes a considerable increase in production costs, as well as harms the environment (Scheffer-Basso *et al.,* 2008). It is therefore important to evaluate alternatives which do not degrade the environment and maintain the current levels of productivity. Among the available alternatives, the use of animal manure has aroused interest in the scientific community due to the diverse properties of its composition and its availability in areas of high animal density (Schroder and Stevens, 2000).

Nutrients are key ingredients required for plant growth and development (Rashmi *et al*., 2020). The major source of these nutrients is the soil. However, the soil often cannot supply these nutrients at the required amount because of deficiency or other factors that may hinder their supply. To supplements the soil nutrient supply, various sources of nutrients such as inorganic fertilizer and organic fertilizer are added. Inorganic fertilizer provides easily accessible nutrients essential for plant growth (Rashmi *et al*., 2020). However, excessive use of it can result in

nutrient leaching leading to surface and underground water pollution and accumulation of heavy metals in soil (Dharma-Wardana, 2018). It is also expensive, particularly in developing countries like Nigeria. The use of organic fertilizer such as animal manure as an alternative means of supplying nutrients to plants in a sustainable way has attracted a lot of attention because it is available, affordable, and environmentally friendly (Zafar *et al.,* 2011; Chemura, 2014).

Animal manures serve as critical soil amendments in agricultural systems by supplying essential macro- and micro-nutrients, enhancing organic matter content, and improving physical soil properties. For example, the integration of livestock and cropping systems allows for nutrient recycling: one tonne of cow dung may contain approximately 8 kg N, 4 kg P_2O_5 and 16 kg K_2O, though the exact composition depends on species and diet (FAO, 1992). Incorporation of manure increases soil organic carbon, which in turn fosters a beneficial soil microbial community, enhances cation exchange capacity (CEC), and improves water-holding capacity and infiltration rates (Gross and Glaser, 2021) as described in the context of Sub-Saharan Africa.

Furthermore, long-term applications of animal manure have been demonstrated to change chemical, physical and biological soil attributes in favour of productivity: a review noted that repeated manure use led to increases in soil nutrient content and crop yields, with improvements in both fertility and food quality for horticultural crops (Loss *et al.,* 2019). In the context of degraded tropical soils, such as Ultisols, application of animal manure (cow or chicken manure) at higher doses significantly was observe by Palupi *et al.* (2023) to increase soil nitrogen and phosphorus, raised CEC, enhanced plant height and yield of a medicinal ginger species (*Zingiber montanum*) and improved the concentration of secondary metabolites in the rhizomes. Given these benefits, the use of animal manure is increasingly advocated as a sustainable strategy to

restore and maintain soil fertility, particularly in environments where mineral fertiliser is cost-prohibitive or environmentally challenging.

Manures derived from ruminants (such as cattle, goats and sheep) differ from those of non-ruminants (pigs, poultry) in terms of nutrient composition, decomposition dynamics, and microbial load. For instance, manures from poultry are often richer in readily available nutrients (particularly nitrogen and phosphorus) compared to many cattle manures, because of higher feed nutrient density and excreta characteristics. In the African smallholder context, cattle manure remains one of the most prominent manures used, but the quantity available per unit cropped area is often low; ruminant manures also tend to have higher fibrous bedding material addition, affecting C:N ratio and microbial decomposition rates (Soul and Dorine, 2023).

Conversely, poultry manure tends to decompose more rapidly, releasing nutrients faster, but may also present higher risks of nutrient leaching or ammonia volatilisation if mismanaged (Kofi *et al.,* 2024). The physical form (e.g., litter vs slurry) and handling (fresh vs composted) also influence nutrient release patterns and microbial populations. It is therefore essential to consider the breed/source (ruminant vs non-ruminant) of the animal manure, their intrinsic nutrient and microflora profiles, and how those differences may affect bacterial communities and plant growth-promoting capacities

Alternatively, organic or biofertilizers can be used to provide plant nutrients and also increase the long-term sustainability of agricultural ecosystems (Mącik *et al*., 2020; Shaji *et al*., 2021). Organic fertilizer is a natural source of essential plant nutrients. Both natural organic fertilizers, such as manure and processed ones like compost and humic acid, have several benefits. These include mitigating the risks of groundwater pollution and excessive fertilization, improving soil properties and quality, maintaining soil fertility as they help to compensate for the loss of organic

matter in the short and long term, reducing environmental damage without reducing crop productivity and achieving sustainability of agriculture production (Shaji *et al*., 2021). Biological fertilizer (biofertilizer) is a substance containing microorganisms that when applied to plants, can enhance nutrient uptake and improve soil fertility and crop yield through several mechanisms (Mącik *et al*., 2020; Shaji *et al*., 2021). These mechanisms include N fixation, P and K solubilization, production of plant growth-promoting molecules, detoxification of soil contaminants, and protection of plants against pathogens, biotic and abiotic stresses (Mącik *et al*., 2020; Viji *et al*., 2021). Research into the widespread use of microbial inoculants (biofertilization) and organic additives (organic fertilization) in soil is one of the dominant areas of applied scientific research for sustainable agriculture development.

1.1 Statement of Problem

Animal manures harbour a diversity of microbial populations, including zoonotic pathogens such as *Salmonella* spp., *Escherichia coli* O157:H7, *Campylobacter jejuni, Listeria monocytogenes*, and *Clostridium perfringens*, which are frequently detected in livestock waste products and pose a risk to food safety and human health (Venglovsky *et al.,* 2006). Further, manures have been shown to serve as reservoirs for antibiotic-resistant bacteria, which can be disseminated into soil, crops and ultimately humans through the food chain. For example, untreated manure in Sub-Saharan Africa was found to contain high loads of antimicrobial-resistant bacteria, facilitating horizontal gene transfer in soils (Amon *et al.,* 2025).

The problem arises when manure is applied to soils (especially for food crops) without adequate treatment or monitoring of microbial safety; this creates a dual challenge of harnessing the beneficial aspects of manure while mitigating the health risks. While the nutrient value of animal manure has been long recognised, less is known about the beneficial microbial flora residing in

manures (particularly from different breeds or species) that may actively promote plant growth via PGP mechanisms. Recent studies such as that of Sagar *et al.* (2025) have isolated bacterial strains from cow dung of indigenous and exotic breeds and demonstrated their effects on pea growth. Thus, there exists a need to systematically evaluate manure as both a nutrient carrier and a microbial bio-resource for PGP traits, especially in local contexts such as Nigeria. By doing so, it becomes possible to optimise manure utilisation not just as organic fertiliser, but as a component of bio-fertiliser or bio-inoculant systems, thereby supporting sustainable agriculture.

1.2 Justification of Study

The use of animal manure aligns with principles of circular agriculture and sustainable soil management. It reduces reliance on inorganic fertilisers (which are expensive and environmentally intensive), helps build soil health (organic matter, structure, microbial diversity), and supports resilient agro-ecosystems. Manure enhances soil fertility, improves nutrient cycling, and contributes to carbon sequestration and water retention in soils. By evaluating the PGP microbial component of manure, this study may identify biologically-active microbial inoculants that further enhance productivity and reduce external input costs (Loss *et al.,* 2019).

Given the potential health risks associated with manure (pathogens, antibiotic-resistance dissemination), a scientific evaluation of microbial safety alongside agronomic benefits is essential. The findings from this research can inform guidelines for manure handling, processing (e.g., composting, ageing, pathogen reduction), and application rates for different manure types (ruminant vs non-ruminant). This will help farmers, extension services and policymakers adopt manure use in a manner that maximises benefits while minimising risks. Furthermore, by elucidating the PGP potential of manure-derived microbial communities, the study contributes to

the development of biofertiliser tools tailored to local agricultural systems in Nigeria and comparable settings.

1.3 Aim of Study

The study was aimed at evaluating the bacteriological and plant growth promoting properties of animal manure.

The following were the objectives of the study:

1. isolate and identity the bacteria present in different types of manure (cow dung and poultry droppings) using phenotypic and molecular techniques.
2. determine the plant growth-promoting properties present associated with in the bacterial isolates from the animal wastes.
3. assess the effect of the different manure (cow dung and poultry droppings) on the germination, growth and yield of *Telfairia occidentalis* (fluted pumpkin).
4. carry out comparative analysis on the effect of the different manure (cow dung and poultry droppings) and combined manure on the germination, growth and yield of *Telfairia occidentalis* (fluted pumpkin).

CHAPTER TWO

LITERATURE REVIEW

2.1 Organic manures and Historical Perspective

Organic manure refers to natural materials of plant or animal origin applied to soil to supply nutrients and improve soil quality. Common forms include animal dung, poultry litter, composted crop residues, green manure, and organic wastes from farms and households. Unlike synthetic fertilizers, organic manure releases nutrients gradually as soil organisms break it down. This slow release supports steady plant growth, improves soil structure, increases water holding capacity, and enhances microbial activity. Studies have shown that organic manure contributes significant amounts of nitrogen, phosphorus, potassium, and micronutrients while supporting long term soil fertility and biological balance (Adekiya *et al*., 2018).

The use of organic manure predates modern agriculture and forms one of the earliest soil fertility management practices. Ancient farming systems in Asia, Africa, and Europe relied heavily on animal dung, crop residues, and household waste to maintain soil productivity. Historical records from China and India describe the systematic recycling of animal and human wastes on farmland over thousands of years to sustain crop yields (Lal, 2006). In traditional African agriculture, farmers applied cattle manure, composted plant materials, and ash to improve soil fertility, especially in nutrient poor tropical soils (Bationo *et al*., 2007). These practices were based on observation and experience rather than chemical inputs, yet they supported food production for generations.

With the introduction of synthetic fertilizers in the twentieth century, reliance on organic manure declined in many regions due to faster nutrient availability and ease of application. Over time, concerns related to soil degradation, declining organic matter, rising fertilizer costs, and

environmental pollution renewed interest in organic manure use. Research from long term field experiments shows that continuous application of organic manure improves soil carbon content, nutrient retention, and crop resilience when compared with sole dependence on inorganic fertilizers (Bhattacharyya *et al*., 2015). These findings have reinforced the role of organic manure as a core component of sustainable agriculture.

Today, organic manure remains central to organic farming systems and integrated soil fertility management. Its historical use provides strong evidence of its value in maintaining soil health, supporting crop productivity, and reducing dependence on external inputs. Modern research continues to refine application methods and rates to align traditional knowledge with current environmental and food security needs (Palm *et al*., 2014).

2.2 Importance of Organic Manure

Nitrogen is one of the major limiting nutrients for the increase, or maintenance of productivity of crops in tropical soils, due to its complex dynamic and costs, which leads the search for viable alternatives to minimize the need for application, and to extend its availability time for the plants (Souza and Melo, 2000). Therefore, the need to fertilize agricultural lands, in view of high costs of synthetic fertilizers, causes farmers to think about the maximization of existent resources naturally found on rural properties. Nitrogen fertilizers are more expensive and mainly arise from petrochemical sources, representing a major limitation to forage. Nitrogen is the most abundant nutrient in manure. The value of animal manure in fertilization is so great that in some countries, it is required by law to capture, store and transport in appropriate tanks, and to distribute in crop or pasture areas (Campos *et al*., 2002; Sutaryo *et al*., 2013).

In the “new” livestock scenario, through the increase of organic production importance, it is, therefore, necessary to study rational use of manure as an alternative to enhance crop production.

Despite the momentum generated by this demand in recent years, organic manure production is not still considered a current way to trigger ecologic groups, although it provides a tangible reality to offer healthy products to consumers and a profitable activity for producers (Almeida, 2000; Schroder and Stevens, 2004).

2.3 Forms of Organic Manure

Organic fertilizer has two main sources: animal manure (poultry, small ruminants, large ruminants) and agricultural waste (compost and crop residues). Animal manure is rich in essential nutrients and has the ability to improve soil's physical, chemical, and microbiological properties thereby improving plant growth and development (Li *et al*., 2016; Zhang *et al*., 2016). The nutrient content of animal manures varies depending on the type of animal and the nature of feeds the animals are fed with. Poultry manure is reported to be rich in Nitrogen (N) and phosphorus (P) and low in potassium (K) when compared to sheep, horse, and pig manure (Therios, 1996). Cow manure is a good source of Nitrogen (N), Organic carbon (C), Magnesium (Mg), and Calcium (Ca) (Adegunloye *et al*., 2007), while the amount of N and K in goat manure was reported to be twice the quantities in cow manure (Midranisiah *et al*., 2021). The beneficial effects of animal manure on plants growth and development have been well documented (Naishima *et al*., 2019; Majolagbe *et al*., 2020; Ibode *et al*., 2022).

2.4 Ruminant and Non-ruminant Animal Manure

Ruminant animals such as cattle, goats, and sheep possess a distinctive digestive system composed of several stomach chambers that facilitate the breakdown of fibrous plant materials through the process of rumination (Dewell *et al*., 2018a). This adaptation allows them to extract nutrients efficiently, resulting in manure that is rich in essential elements like nitrogen, phosphorus, and potassium, which are beneficial for soil fertility and crop growth. During

rumination, fermentation promotes the decomposition of organic matter within the manure, thereby improving nutrient bioavailability. Nevertheless, improper disposal or management of ruminant manure can lead to environmental challenges such as greenhouse gas emissions and nutrient leaching into water bodies (Dewell *et al*., 2018b).

Conversely, non-ruminant species, including pigs and poultry, have a simpler, single-chambered digestive tract. Their manure generally contains higher nitrogen concentrations but less fiber than that of ruminants. Due to its concentrated nutrient content, non-ruminant manure is easier to store and transport; however, it often harbors elevated levels of pathogens, posing potential risks of contamination if mishandled (Elder *et al*., 2020). To enhance its agricultural utility and reduce environmental hazards, non-ruminant manure is typically treated through processes such as composting or anaerobic digestion before application.

2.5 Wastes of Ruminant and Non-ruminant Animals

The chemical composition of ruminant manure mirrors the complex digestive processes occurring within these animals (Elder *et al*., 2020). In contrast, waste produced by non-ruminant animals particularly swine and poultry present notable health and safety challenges. The high concentration of nutrients in non-ruminant manure creates favorable conditions for the growth of pathogenic microorganisms, including *Salmonella* and *Clostridium perfringens*, which can pose serious risks to human health during handling and field application (Fedorka-Cray *et al*., 2018). Research emphasizes the necessity of implementing strict biosecurity measures, adopting effective waste management strategies, and applying manure under controlled conditions to reduce the likelihood of disease transmission and environmental contamination.

2.6 Agricultural Utilization of Ruminant and Non-Ruminant Animal Wastes

Animal manures from both ruminant (e.g., cattle, sheep, goats) and non- ruminant (e.g., poultry, swine) sources have long been recognised as organic soil amendments with the potential to support crop production, improve soil health, and contribute to circular nutrient use. However, their effective utilization requires appropriate management to maximise benefits and minimise environmental risks.

2.6.1 Ruminant Manure Utilisation

Ruminant manures typically arise from animals with multi compartment stomachs that digest fibrous plant material. These manures are rich in organic matter and macro nutrients (nitrogen, phosphorus, potassium), making them valuable for agricultural application. For example, vermicomposted cattle manure has been shown to improve forage quality and soil properties. Nasiru *et al*. (2014) reported on the nutritive value of cattle manure vermicast and its effect on in vitro ruminal gas production, pointing to the potential value of dairy or beef manures for recycling back into feed/fertiliser systems.

In field trials, application of animal manure has improved degraded soils; Palupi et al. (2023) found that cow and chicken manure applied at 60 t ha^{-1} increased soil Nitrogen and Phosphorus and improved yields of Zingiber montanum grown on Ultisol soils. Furthermore, in integrated crop livestock systems, assessing availability and utilisation of livestock manure helped evaluate nitrogen management in smallholder systems; for instance, in rural Eastern Cape, South Africa, manure use was evaluated for crop livestock integration and nitrogen budgets. These studies demonstrated that ruminant manures can enrich soils, enhance fertility, support crop growth, and contribute to nutrient cycling. Key mechanisms include increasing organic matter content,

enhancing soil microbial diversity, improving cation exchange capacity, and supplying slowly released nutrients (Zhang, 2016).

2.6.2 Non- Ruminant Manure Utilisation

Manures from non ruminants particularly poultry and pigs are typically more nitrogen dense, less fibrous, and often more concentrated than ruminant manures. This concentration makes them attractive as fertilisers, but also introduces management challenges (e.g., higher potential for ammonia volatilization, pathogen risk and odour). Research shows notable positive impacts of poultry manure; for example, a long term (1998–2017) poultry manure application study in the US under corn–soybean and continuous corn rotations found that poultry manure treatments maintained high crop yields, improved soil health, and had lower nitrate N concentrations in drainage compared to urea ammonium nitrate fertiliser (Suhel and Wahidu, 2005). Meta-analysis across Sub Saharan Africa concluded that manure application (including poultry and non ruminant) increased soil pH, soil organic carbon, total nitrogen, available phosphorus, potassium, crop yield and biomass under smallholder systems with poultry manure among the more effective types (Hamond *et al.,* 2025).

A field experiment comparing poultry manure with inorganic NPK fertiliser on maize in Ghana found that 100 % poultry manure treatment out performed NPK in grain yield as reported by Afriyie Debrah *et al.* (2019) in their study of the "Omankwa" maize variety. From a practical standpoint, non-ruminant manures serve both as nutrient sources and as components of integrated systems; when applied judiciously, they can improve soil fertility, enhance soil structure, increase microbial activity, and support crop production without sole reliance on synthetic fertilisers (Debrah *et al.,* 2019).

However, effective utilisation requires careful treatment (e.g., composting, anaerobic digestion) because of higher risks of nutrient losses (especially nitrogen and phosphorus), odours, and pathogen spread (Suhel and Wahidu, 2005). The concentrated nature of non-ruminant manure means that storage, timing of application, and rate must all be managed to avoid environmental pollution, such as nutrient runoff, greenhouse gas emissions, and eutrophication of water bodies (Suhel and Wahidu, 2005).

2.7 Interactions Among Bacteria, Manure and Soil

Bacteria are capable of adapting to a wide range of environmental conditions, including variations in oxygen concentration, temperature, pH, and moisture levels. Research on maize cropping systems in China revealed a strong correlation between bacterial diversity, precipitation, and soil pH (Fernandez *et al*., 2020). Microorganisms that cannot tolerate fluctuating soil conditions are often outcompeted and eliminated during the struggle for nutrients (Dewell *et al*., 2018). Conversely, newly introduced microbes that encounter favorable conditions are more likely to survive and dominate. In addition, bacterial survival is influenced by biological interactions such as predation from protozoans, which play a regulatory role in maintaining microbial population balance (Awasthi *et al*., 2019).

The bacterial composition of animal manure is influenced by several factors, including the animal species, diet, and waste management practices. However, the use of manure in agriculture can inadvertently spread zoonotic diseases, posing risks to humans, livestock, and crops (Nielsen *et al*., 2022). These bacterial pathogens not only threaten health but also carry significant economic costs, as poor soil health and contamination can affect food quality, reduce yields, and hinder marketability (Dewell *et al*., 2018b).

Common manure-borne pathogens such as *Escherichia coli* and *Salmonella* have been frequently associated with outbreaks of human diseases. When manure containing viable pathogens is applied to agricultural fields, these organisms can spread through surface runoff, leaching, and contamination of water sources or harvested crops. Such transmission pathways establish a clear link between agricultural practices and the spread of pathogens across ecosystems. For instance, *E. coli* has been found to transfer from untreated poultry manure to fresh produce, highlighting the importance of safe waste handling (Elder *et al*., 2020). Safeguarding food and water resources from fecal contamination is essential to ensuring public health, animal well-being, and sustainable agricultural productivity.

The type and origin of manure influence its pathogen load, underscoring the need for effective livestock disease control to maintain high biosecurity standards (Nielsen *et al*., 2022). Reducing pathogen presence in manure enhances its environmental safety and sustainability (Fedorka-Cray *et al*., 2018). Moreover, regional differences such as climate, soil type, feeding practices, and precipitation patterns influence pathogen survival and microbial diversity (Awasthi *et al*., 2019), meaning that manure management strategies must be tailored to local conditions.

Manure application affects soil properties, often improving its physical and chemical characteristics, such as enhancing organic carbon content, nutrient availability, and water retention capacity (Awasthi *et al*., 2019). However, excessive manure use may increase soil salinity and promote particle dispersion, particularly in semi-arid and humid environments. Climatic factors also affect manure's impact on soil, as leaching in wetter regions can help remove excess sodium ions, mitigating adverse effects (Nielsen *et al*., 2022).

Changes in soil characteristics can alter biological properties and microbial community composition. When manure is applied, it introduces new microbial populations into the soil, and

variations in environmental factors like salinity and pH may favor certain microbial groups over others, reshaping the soil microbiome. For example, long-term use of synthetic fertilizers containing nitrogen, phosphorus, and potassium has been observed to lower soil pH, whereas composted cattle manure tends to increase pH, improve crop yield, and stimulate microbial activity, particularly in rice cultivation systems (Xiongwei *et al.,* 2025). Manure-enriched soils typically host more active microbial communities, with nutrient-loving bacteria such as *Proteobacteria* becoming dominant due to the abundance of organic matter (Bello *et al*., 2020). Conversely, soils lacking manure are often dominated by *Acidobacteria*, which thrive in nutrient-deficient environments (Éva-Boglárka *et al.,* 2024).

2.8 Bacterial Pathogens Associated with Animal Manure

The occurrence of pathogens in animal manure varies widely and is influenced by several factors, including the type of animal producing the waste, its diet, and the use of antimicrobial feed additives. Dietary modifications such as increasing the acidity of animal feed have been shown to effectively lower *Salmonella* levels in fattening pigs (Rasmussen *et al*., 2023). When manure is applied to agricultural soil, it can serve as a medium through which pathogens spread to other environments, either through direct contact with livestock, contamination of fresh produce, or runoff into nearby water sources (Bello *et al*., 2020).

Different pathogens exhibit varying levels of resilience under stressful or suboptimal environmental conditions. For instance, *Listeria* species are highly adaptable and can survive in environments characterized by low water availability, acidic pH, and cold temperatures (Awasthi *et al*., 2019). Similarly, the application of poultry and pig manure to croplands can facilitate the spread of *Escherichia coli* into soil ecosystems, while *Salmonella* has been detected in anaerobic

environments such as slurry pits, as well as under low-temperature conditions (Awasthi *et al*., 2019).

Modern agricultural practices particularly intensive farming, monoculture cropping systems, and the concentration of similar livestock species within limited areas create ideal conditions for pathogen persistence and reinfection cycles (Rasmussen *et al*., 2025). These homogeneous systems allow certain bacteria to adapt and specialize, enhancing their ability to survive in specific hosts or environmental niches. In contrast, diverse cropping systems and rotational practices tend to suppress pathogen establishment by promoting ecological balance and microbial diversity (Awasthi *et al*., 2019).

Common bacterial pathogens frequently detected in manure and linked to disease outbreaks include *Campylobacter*, *Salmonella*, and pathogenic strains of *E. coli* such as O157:H7 (Bello *et al*., 2020). Emerging pathogens, such as *Providencia* species, have also been identified in manure and soil environments, underscoring the importance of continuous monitoring. As agricultural practices continue to evolve, maintaining vigilant surveillance and adopting adaptive management strategies are essential to minimize the risks associated with manure-borne pathogens.

2.8.1 *Campylobacter* spp.

Campylobacter species are among the most significant bacterial agents responsible for foodborne illnesses in both the United States and Europe. Human infection, known as campylobacteriosis, can occur from a remarkably low infectious dose typically between 500 and 800 colony-forming units (CFU) with *Campylobacter jejuni* accounting for the majority of reported cases of campylobacteriosis (Chibisa *et al*., 2016). These bacteria are commonly detected in unpasteurized milk, untreated water, animal manure, and various food-producing animals such as

poultry, cattle, pigs, and sheep. Studies have shown that nearly all *Campylobacter* isolates from poultry and cattle feces are *C. jejuni*, while approximately 97% of isolates from pig feces are *C. coli* (Chibisa *et al*., 2016). Other research have identified cattle as important reservoirs for *C. jejuni* and *C. lari* (Hussein *et al*., 2017). Lopez-Gonzalez *et al*. (2015) reported *Campylobacter* prevalence rates of 76.0%, 63.8%, and 50.0% in cattle, pig, and poultry fecal samples, respectively, suggesting that manure from these animals can serve as a potential source of soil contamination when applied to agricultural lands.

Campylobacter jejuni is particularly resilient, capable of adapting to challenging environmental conditions such as elevated oxygen concentrations and limited nutrient availability (Neher *et al*., 2023). Interestingly, this bacterium can also contribute to increased microbial diversity by consuming oxygen and thereby reducing oxidative stress in mixed microbial communities. Under aerobic conditions, *C. jejuni* has been observed to form or integrate into mixed-species biofilms, particularly those containing *Pseudomonadaceae*, to which it can attach for enhanced protection and survival (Neher *et al*., 2023).

A notable adaptive mechanism of *Campylobacter* is its ability to transition into a viable but non-culturable (VBNC) state in response to environmental stresses, including high oxygen exposure (Oosteron *et al*., 2025). Although this state enhances persistence, the degree to which VBNC cells remain infectious remains unclear. Temperature also plays a crucial role in the survival of *Campylobacter* in external environments. Oosteron *et al*. (2025) found that *C. coli* was more vulnerable to aerobic stress at 15 °C and above compared to 4 °C, emphasizing temperature as a major determinant of environmental endurance.

The widespread occurrence of *Campylobacter* in various environments particularly within meat processing facilities following its release from animal hosts may explain the high frequency of

campylobacteriosis outbreaks globally (Awasthi *et al*., 2019). Despite its adaptability, *Campylobacter* is generally unable to multiply outside of host organisms due to environmental stressors and its intolerance to oxygen concentrations exceeding 5% (Awasthi *et al*., 2019).

2.8.2 *Escherichia coli*

Escherichia coli is a facultative anaerobic bacterium characterized by extensive genetic diversity, encompassing numerous pathotypes with varying degrees of virulence from harmless commensal strains to highly pathogenic variants capable of causing severe illness or death. Certain strains, such as Shiga toxin-producing *E. coli* (STEC), including *E. coli* O157, are responsible for gastrointestinal infections in humans (Bello *et al*., 2020). Food-producing animals serve as important reservoirs for pathogenic *E. coli* and may carry the bacteria either symptomatically or asymptomatically. Although the precise infectious dose remains under debate, it is widely accepted that very low doses can cause infection. Chibisa *et al*. (2016) reported that 50.8% of sheep and 35.9% of cattle herds harbored STEC strains of pathogenic *E. coli*, demonstrating their widespread occurrence in livestock populations.

Pathogenic *E. coli*, particularly STEC strains, exhibits remarkable environmental resilience, persisting in diverse habitats such as soil, water, and manure. Despite its overall hardiness, *E. coli* O157 is notably sensitive to desiccation. A study conducted in Cameroon revealed that *E. coli* persisted in 59.1% of chicken litter samples collected from urban poultry farms, underscoring its environmental stability (Djuikoue *et al.,* 2022). Hussein *et al*. (2017) observed that greater microbial diversity within soil ecosystems can suppress *E. coli* invasiveness, likely due to increased microbial competition. Additionally, temperature plays a significant role in its survival, with higher persistence observed in non-autoclaved soils maintained at 5 °C compared to 15 °C indicating an adaptive advantage under cooler conditions (Hussein *et al*., 2017).

Lopez-Gonzalez *et al*. (2015) investigated *E. coli* O157 survival in microbially active soils in southeast China, reporting a decline from 10^6 to 10^0 CFU g^{-1} in chicken manure-amended soils after approximately 2.6 ± 6.6 days at 25 °C, while a similar reduction in pig manure amendments required around 25.7 ± 7.1 days. Furthermore, *E. coli* survival was found to increase in soils with higher pH levels and lower microbial diversity. These findings suggest that both environmental factors such as temperature, pH, and microbial competition and anthropogenic influences, including manure management practices, collectively shape *E. coli* persistence in agricultural settings (Neher *et al*., 2023).

The frequent detection of *E. coli* in agricultural soils highlights the importance of implementing strict manure management strategies before field application. Proper treatment and controlled use of manure are essential to minimize pathogen survival, reduce environmental transmission, and protect public health and food safety.

2.8.3 *Salmonella* spp.

Salmonella species are facultative anaerobic bacteria comprising numerous serotypes, many of which are capable of causing gastroenteritis and other foodborne illnesses in humans. Certain serotypes, such as *Salmonella enteric*a serovar Typhimurium and *Salmonella enterica* serovar Choleraesuis, are pathogenic to livestock, especially pigs. They are associated with severe, sometimes fatal infections, as well as asymptomatic or subclinical cases, which make detection and control more difficult (Collins *et al*., 2019). Although the precise infectious dose varies among studies, it is generally accepted that *Salmonella* requires a higher infectious dose compared to *Campylobacter* or *Escherichia coli* (Collins *et al*., 2019).

In recent years, the emergence and spread of antimicrobial-resistant (AMR) *Salmonella* strains especially *S.* Typhimurium have become a growing public health concern. The persistence and

survival of *Salmonella* in soil depend on several environmental factors, including temperature, moisture, predation pressure, and the method by which it is introduced. Lei and Vandergheynst (2020) demonstrated that *Salmonella* can be transmitted from contaminated soil to tomato plants. In their study, tomatoes placed with cut stems in inoculated, moisture-saturated soil exhibited a 2.5 $\log_{10}$ CFU increase in *Salmonella* populations within four days, with levels remaining stable for up to ten days at 20 °C under humid conditions. Further research by Nakasaki *et al*. (2025) revealed that *Salmonella* persisted in soil with minimal reduction over a 45-day period, indicating that contaminated produce, such as tomatoes, may act both as a habitat for bacterial survival and as a vehicle for human transmission.

Compounding the risk, *Salmonella* spp. have demonstrated the ability to invade the internal tissues of fruits and vegetables including tomatoes, lettuce, and green onions through a process known as *internalization* (Nakasaki *et al*., 2025). This phenomenon renders surface washing insufficient for bacterial removal, as the pathogens reside within plant tissues. Bernal *et al*. (2019) reported that extreme weather events, such as heavy rainfall or flooding, can facilitate bacterial internalization, emphasizing the need for region-specific studies and regulations tailored to local climatic conditions.

Salmonella can persist for extended durations outside a host when environmental conditions such as adequate moisture, favorable temperature, and suitable soil type support survival (Alnahdi, 2022). The bacterium's capacity to rapidly adapt to environmental stressors also enhances its resilience. As Bhardwaj *et al*. (2024) explained, *Salmonella* can transition into a viable but non-culturable (VBNC) state, enabling dormancy under hostile or nutrient-limited conditions. Moreover, *S.* Typhimurium tends to survive longer in both untreated and manure-amended soils

at lower temperatures, though protozoan predation appears to reduce its survival under certain conditions.

2.8.4 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is an environmental, opportunistic Gram-negative bacterium commonly found in water, soil and decaying organic matter; because of this ecological versatility it can be introduced into agricultural environments and become associated with animal manures and organic amendments (for example composts and slurry). When present in manure it is not usually a dominant faecal indicator organism, but multiple studies have confirmed its occurrence in manures, immature/mature composts and soils amended with animal wastes especially where moisture and nutrient availability favour survival and where manure management is poor (e.g., incomplete composting or storage at ambient temperature). *P. aeruginosa* survives in these matrices by forming biofilms and exploiting diverse metabolic pathways, which helps it persist through environmental stressors and during manure handling and land application (Deredijan *et al.,* 2014).

From a public- and animal-health standpoint the concern about *P. aeruginosa* in manure lies in two linked properties: (1) its capacity to cause opportunistic infections in animals and humans (mastitis and wound infections in animals; opportunistic infections in immunocompromised humans), and (2) its frequent multidrug-resistant (MDR) phenotypes. Studies sampling dairy farms, farm environments and animal products have documented MDR *P. aeruginosa* strains recovered from cattle and from farm environments, including bedding and other materials closely related to manure handling (Kaszab *et al.,* 2011). These resistant strains often carry combinations of efflux pumps, beta-lactamases and other determinants that reduce the effectiveness of common antimicrobials; such resistance genes can be maintained in the farm

environment and potentially transferred horizontally to other bacteria present in manure or soil. Consequently, incomplete or improper treatment of manure (e.g., inadequate composting or uncontrolled land spreading) may act as a pathway for persistence and dissemination of resistant *P. aeruginosa* into soils, water and the broader farm-human interface (Kaszab *et al*., 2011).

Because *P. aeruginosa* is not strictly an enteric bacterium and its prevalence in fresh faeces can be variable, monitoring programs that target environmental amendments often combine culture, molecular identification and resistance profiling to detect and track *P. aeruginosa* populations and their resistance determinants in manure-derived materials. Effective manure management (proper composting to achieve thermophilic temperatures, controlled storage, minimizing runoff) and prudent antimicrobial stewardship on farms are therefore important control points to reduce persistence and spread (Obayiuwana *et al.,* 2025).

2.8.5 *Klebsiella* spp.

Klebsiella species most notably *Klebsiella pneumoniae* and *Klebsiella oxytoca* are common members of the Enterobacteriaceae and are frequently isolated from the gastrointestinal tracts of animals. Because of this, *Klebsiella* spp. are regularly found in fresh animal faeces and, as a consequence, in animal manures, bedding, farm soils and water linked to livestock operations. Several farm studies have shown high detection rates of *Klebsiella* in rumen contents, manure, water troughs and bedding, highlighting that animal husbandry and hygiene practices strongly influence the environmental distribution of these organisms on farms (Zadoks *et al.,* 2011).

The principal concern with *Klebsiella* in manure is their growing burden of antimicrobial resistance particularly extended-spectrum beta-lactamase (ESBL) producers and, in some settings, carbapenemase producers. Livestock and poultry have been identified as reservoirs for

ESBL-producing *Klebsiella*, and manure used as fertilizer can thus serve as a vehicle for dissemination of resistance genes (for example CTX-M family ESBLs) into soil microbiomes and potentially onto crops or into water systems (Zadoks *et al.,* 2011). Molecular and genomic surveillance studies of farm-associated *Klebsiella* have documented shared lineages and resistance determinants between animal, environmental and (in some cases) human isolates, indicating a porous interface at the animal–food–environment nexus. This means that manure management including composting regimes that reliably inactivate enteric bacteria, careful handling to limit environmental runoff, and measures to prevent cross-contamination between manure and food crops is critical to reduce the risk of spreading resistant *Klebsiella* strains (Zadoks *et al.,* 2011).

In practical terms, interventions to limit *Klebsiella* risks from manure combine on-farm hygiene (cleaning of housing, drinking water quality, limiting faecal contamination of feed), antimicrobial stewardship to lower selective pressure for resistant strains and post-excretion treatments (high-temperature composting, storage time/conditions that reduce viability) (Zadoks *et al.,* 2011). Surveillance that couples culture, antimicrobial susceptibility testing and genomic characterization remains important to detect emergent resistance and to inform targeted mitigation strategies at the farm and community levels (Zadoks *et al*., 2011).

2.9 Manure as a Reservoir and Vector of Antimicrobial Resistance

The presence of resistant and pathogenic bacteria in animal manure poses a substantial risk for the dissemination of antimicrobial resistance (AMR) in the environment, facilitating the transfer of antimicrobial resistance genes (ARGs) from livestock and animal-derived products to the human microbiome (Jiang *et al*., 2019). Evidence of ARG transmission from manured soils to humans has been demonstrated through the contamination of vegetables, such as lettuce,

highlighting the potential for plant-mediated resistome transfer into the human food chain. In southern China, fresh vegetables and fruits were found to harbor ARGs including *tet* and *ami* genes at high frequencies, with average abundances of 3.08 and 1.18 copies per 16S rRNA gene, respectively (Meng *et al*., 2019). Additional resistance determinants, such as *aadA*, *aph1*, *floR*, *sul1*, *intI1*, *qacE*, *sul2*, *tetB*, and *tetM*, were detected in over 90% of samples, while *cmlA* and *ermB* were present at slightly lower frequencies (Meng *et al*., 2019).

Various classes of antibiotics used in food animal production correspond to different ARGs: tetracyclines encode the *tet* gene family, sulfonamides correspond to *sul* genes, β-lactams are represented by *blaTEM* and *blaCTX-M*, macrolides carry *erm* and *mef* genes, colistins involve *mcr* genes, and quinolones include *qnr* genes (Davies *et al*., 2020). ARGs associated with manure also confer resistance to disinfectants and extend to aminoglycosides, tetracyclines, sulfonamides, and macrolide-lincosamide-streptogramin B (MLSB) antibiotics (Davies *et al*., 2020). Importantly, these resistance genes are often linked to mobile genetic elements (MGEs), including plasmids, transposons, and gene cassettes, which facilitate horizontal gene transfer between bacterial species across diverse environments (Davies *et al*., 2020).

Analyzing manure and soil resistomes is crucial to understanding the mechanisms of AMR and assessing public health risks posed by livestock effluents. Manure-soil microcosms can be simulated to study ARG transfer processes and identify strategies to limit environmental dissemination. A primary approach involves targeting ARGs with low natural decay potential, such as *sul1*, *sul2*, *intI1*, and *tetM*, before manure is applied to agricultural soils. Adjustments to manure management practices, such as extended windrow composting or prolonged stockpiling, have demonstrated reductions in ARG abundance by 0.5–3 log units compared to untreated manure (Okrend *et al*., 2020).

ARG concentrations in livestock manure are significantly higher than in human waste or unmanured soils, with some studies reporting levels up to 28,000 times greater (Stevenson, 2021). In a study of 12 large-scale Chinese farms, 109 ARGs associated with veterinary and human antibiotics were identified in poultry, swine, and cattle manure (Stevenson, 2021). Fresh manure application was shown to introduce more ARGs into soils compared to stockpiled manure, which releases fewer resistance genes over time. The persistence of ARGs also varies depending on composting and dietary practices; for example, genes such as *ermX*, *sul1*, *sul2*, and *tetB* remained detectable in cattle manure 175 days post-excretion, representing a potential environmental hazard (Beach *et al*., 2022). Tetracycline resistance genes (*tetM* and *tetW*) were among the most prevalent in cattle manure (Beach *et al*., 2022).

Thermophilic composting processes can reduce the abundance of erythromycin, sulfamethazine, and tetracycline resistance genes: - although some ARGs remained stable (Nikaido, 2024), emphasizing the need for prolonged composting to mitigate ARG dissemination upon land application. Monitoring ARG persistence in manure-soil microcosms is critical to understanding environmental AMR dynamics and bacterial community shifts (Nikaido, 2024). In a study using simulated manure-soil microcosms, 16S rDNA sequencing revealed differential ARG persistence over 90 days: ARGs from poultry manure decreased significantly, whereas soil microbial diversity increased (Barham *et al*., 2022). Genes such as *sul1*, *sul2*, *tetM*, and *intI1* exhibited slower decay, while *ermB* and *ermF* dissipated more rapidly. Cattle manure was enriched in macrolide resistance (*ermB*) and sulfonamide resistance (*sul1*) compared to poultry litter (Barham *et al*., 2022).

The widespread occurrence of *erm* genes corresponds to the function of Erm proteins, which demethylate a single adenine in 23S rRNA, conferring resistance to lincosamides, macrolides,

and streptogramin B (Chapman *et al*., 2017). The *cfr* gene, commonly plasmid-borne in *Staphylococcus* isolates, is co-expressed with *erm* genes but was detected at lower abundance. Similarly, *tetB* abundance, usually associated with Gram-negative bacteria, was lower than *tetM*, which is broadly distributed across bacterial taxa. Recent investigations identified 67 multidrug-resistant methicillin-resistant *Staphylococcus sciuri* isolates from 400 environmental samples, with 40 from manure and 360 from soil (Chapman *et al*., 2017). Notably, over 90% of these isolates were resistant to ampicillin, clindamycin, penicillin, cefoxitin, and ceftiofur, while 86.56% resisted tetracycline and 50.74% erythromycin (Chapman *et al*., 2017).

2.10 Environmental Impact of Non-Ruminant Animal Wastes

Improper handling of non-ruminant manure, such as that from poultry and swine, can result in nutrient runoff, leading to water pollution and adverse effects on aquatic ecosystems. The high nitrogen content in particular can drive eutrophication, disrupting ecological balance and reducing water quality (Chase-Topping *et al*., 2017). Additionally, non-ruminant manure often emits strong odorous compounds, which can create challenges in areas with high animal densities. Effective waste management strategies are therefore critical to mitigate odor issues and enhance the welfare of both livestock and nearby communities (Chase-Topping *et al*., 2017). Adequate storage infrastructure is essential to minimize nutrient losses and reduce the environmental footprint of manure application (Cízek *et al*., 2019). Common management techniques include anaerobic digestion, composting, and lagoon systems, all designed to treat and stabilize non-ruminant waste efficiently.

Beyond its environmental considerations, non-ruminant manure is a valuable agricultural resource. When applied appropriately, it supplies essential nutrients that improve soil fertility and structure, supporting sustainable farming practices. Anaerobic digestion of non-ruminant

manure also produces biogas a renewable energy source predominantly composed of methane which can be harnessed for heating, electricity generation, or other energy needs (Cízek *et al*., 2019). This approach simultaneously addresses waste management challenges while providing an alternative energy solution.

Compliance with environmental regulations is vital to ensure that manure management practices balance agricultural productivity with ecological sustainability. Research and innovation in advanced treatment technologies and precision application methods are essential for improving both efficiency and environmental performance. Integrated farming systems that combine non-ruminant manure management with crop production and other agricultural activities can enhance sustainability and resilience in agricultural landscapes (Cízek *et al*., 2019).

Equally important is increasing awareness among farmers, policymakers, and the public regarding the environmental impacts of non-ruminant manure. Education and training programs can encourage adoption of best practices, promote sustainable management strategies, and foster a broader understanding of the role of manure management in maintaining soil health, environmental quality, and agricultural productivity (Cízek *et al*., 2019).

2.10 Plant Growth-Promoting Bacteria (PGPB) and their Roles in Sustainable Agriculture

The indiscriminate use of agrochemicals, combined with physical soil degradation, pollution, wildfires, and droughts, exerts direct pressure on soil microbial communities, significantly reducing their population density and diversity (Figure 2.1A). Additionally, pesticides and herbicides disrupt microbial ecosystems by selectively promoting the proliferation of certain taxa at the expense of native species, ultimately compromising soil functionality (Baweja *et al.,* 2020).

The widespread adoption of intensive monocultures further exacerbates this issue by creating selective environments that favor a narrow range of microbial species, thereby reducing overall diversity and weakening soil resilience to pathogens and environmental stressors (Bourke *et al.,* 2021).

These disturbances degrade natural habitats and disrupt critical biogeochemical cycles, limiting microbial activity, reducing soil porosity, and hindering the movement of air and water within the soil matrix (Wei *et al.,* 2022). To address these challenges, the adoption of sustainable agricultural practices is essential to restore soil structure, alleviate compaction, and promote microbial activity. Moreover, the application of PGPB plays a pivotal role in conserving and restoring soil microbial diversity. These bacteria constitute a diverse group of free-living or endophytic microorganisms that promote plant growth and development through a range of direct and indirect mechanisms. They contribute to soil health and resilience by facilitating phosphorus solubilization, nitrogen fixation, and the biosynthesis of plant growth regulators that regulate key physiological processes, including cell division, elongation, and differentiation. Furthermore, PGPB help mitigate abiotic stressors such as salinity and drought through the production of osmoprotectants and enzymes that alleviate ethylene-induced stress in plants. In the context of drought, one critical limitation to the success of PGPB inoculants is desiccation, which reduces bacterial viability during formulation, storage and post-application in dry soils. Therefore, the use of desiccation-tolerant strains is particularly advantageous, as they maintain cellular viability under conditions of low water availability and improve plant colonization efficiency. This characteristic makes them highly suitable for application in arid and semi-arid regions (Molina-Romero *et al.,* 2017).

This integrated approach fosters the sustainability of agricultural systems while preserving the long-term health of soils and the surrounding environment. By combining responsible agricultural practices with the targeted use of PGPB, soil fertility can be restored, crop productivity enhanced, and the reliance on synthetic chemical inputs reduced, ultimately contributing to a more sustainable and ecologically balanced agroecosystem (Trivedi *et al.,* 2020).

2.12 Direct and Indirect Mechanisms of Plant Growth-Promoting Bacteria

Plants have coevolved with a diverse microbiota that plays a pivotal role in supporting their growth, development, and health. These microorganisms establish symbiotic associations with plant tissues, receiving carbon-rich compounds and other metabolites in exchange for beneficial services provided to the host (Santos and Olivares, 2021). This symbiosis dates to the early stages of terrestrial plant evolution and has enabled plants to overcome key challenges, including nutrient acquisition, abiotic stress tolerance, and protection against pathogens (Abdelfattah *et al.,* 2023).

In recent years, there has been an increasing interest in the use of microbial consortia, rather than single-strain inoculants, for restoring degraded soils. Numerous studies have demonstrated that PGPB consortia outperform individual strains due to functional complementarity among their constituent microorganisms. A recent meta-analysis revealed that microbial consortia inoculated into living soils exhibited superior plant growth-promoting effects compared to single-strain inoculants, primarily due to synergistic interactions involving mechanisms such as IAA production, phosphate solubilization, and stress tolerance induction (Liu *et al.,* 2023). Similarly, consortia comprising PGPB, arbuscular mycorrhizal fungi, and mineral-solubilizing

microorganisms have been shown to significantly improve plant biomass and soil microbial activity in alluvially mined and degraded soils (Schütz *et al.,* 2018).

In saline and degraded environments such as Mediterranean Technosols, the application of bacterial consortia has yielded promising results. These consortia enhanced both germination and growth in lettuce (*Lactuca sativa*) while stabilizing the soil's physicochemical properties, a key factor in fertility restoration (Li *et al.,* 2024). Likewise, under drought conditions, the inoculation of maize (*Zea mays*) with bacterial consortia improved leaf water retention and facilitated a more efficient regulation of stress-related gene expression, compared with single-strain applications (Wang *et al.,* 2024).

Collectively, these findings suggest that microbial consortia offer a more integrated and resilient approach to overcoming the limitations of degraded soils. The synergistic interactions among microbes enhance ecological stability, functional redundancy, and metabolic versatility, making consortia more effective and sustainable than single-strain inoculants (Molina-Romero *et al.,* 2021).

Substantial progress has been made in characterizing the composition and dynamics of plant-associated microbial communities, as well as elucidating the functional attributes of key bacterial strains. PGPB have been isolated from a wide range of agriculturally and ecologically relevant plant species (Domínguez-Castillo *et al.,* 2021). Building on these insights, research has focused on the bioactive compounds secreted by PGPB including phytohormones, secondary metabolites, antibiotics, and signaling molecules, which function as biostimulants and mediate plant responses to abiotic and biotic stress. In addition to these benefits, PGPB also promote plant health by enhancing nutrient availability and uptake, improving soil aggregation and modulating rhizosphere signaling. These combined effects improve overall plant development, stress

resilience and productivity (Patel *et al.,* 2020). Nonetheless, while these microbial innovations show great promise, a deeper understanding of their molecular and ecological mechanisms remains essential for their effective, reproducible implementation in agricultural systems (Patel *et al.,* 2020).

Given the potential of PGPB and the urgent challenges facing modern crop production, integrating microbial innovations into agronomic practices represents a critical strategy for enhancing agroecosystem sustainability. Understanding the underlying molecular and physiological mechanisms of PGPB is essential for effectively translating microbial innovations into practical strategies for soil restoration and sustainable agriculture. The following sections explore the primary mechanisms by which PGPB enhance plant growth, including biological nitrogen fixation, phosphate solubilization, siderophore production, and the synthesis of plant growth regulators (Timofeeva *et al.,* 2023).

2.12.1 Indirect Mechanisms of Plant Growth-Promoting Bacteria

(i). Siderophores Production

Iron plays a fundamental role in plant photosynthesis, serving as an essential component of chlorophyll and participating in various biosynthetic pathways. However, the bioavailable iron fraction in soils is often insufficient to support optimal plant productivity (Molina-Romero *et al.,* 2017). To overcome this limitation, bacteria, fungi, and plants release specific low-molecular-weight compounds known as siderophores, which chelate iron from the surrounding environment (Saha *et al.,* 2013). Siderophores are primarily synthesized by bacteria to alleviate iron deficiency stress, thereby promoting plant growth. These microorganisms express surface receptors that regulate the uptake of ferric iron (Fe^{3+}), ensuring its availability for metabolic processes (Sayyed *et al.,* 2013). Notably, siderophores produced by PGPB exhibit an

exceptionally high affinity for Fe^{3+}. Once the iron–siderophore complex is formed, it is recognized by specific receptors on the bacterial or plant cell surface, internalized, and subsequently reduced to ferrous iron (Fe^{2+}) or released upon degradation of the siderophore, rendering iron bioavailable for cellular metabolism (Saha *et al.,* 2013). Importantly, siderophores produced by PGPB demonstrate significantly higher iron binding affinities compared to those synthesized by plants or fungi, enabling PGPB to sequester substantial amounts of this micronutrient (Saha *et al.,* 2013). This trait enhances iron acquisition under limiting conditions and contributes to plant protection. By releasing siderophores with superior chelating capabilities, PGPB effectively outcompete pathogenic fungi and bacteria for iron, thereby restricting their growth and serving as an indirect biocontrol mechanism (Perez *et al.,* 2019). The efficacy of this antagonistic strategy is attributed to the fact that PGPB siderophores can outcompete fungal siderophores in iron chelation (Wang *et al.,* 2022).

Structurally, microbial siderophores commonly possess functional groups such as hydroxamates and catecholates, in addition to carboxylates, citrates, or ethylenediamine moieties, which may coexist within the same molecule (Bilitewski *et al.,* 2017). Hydroxamate-type siderophores are predominant in fungi, whereas catecholate-type siderophores, which exhibit stronger iron-binding capacities, are more characteristic of bacterial species (Glick, 2015). In contrast, plant-derived siderophores, such as mugineic and avenic acids, belong to the amino carboxylic acid family and are distinguished by linear chains containing hydroxyl and amino functional groups that enhance metal chelation, including that of Fe^{3+} (Glick, 2015). These compounds exhibit high chelation efficiency under certain conditions. Additionally, bacterial siderophores can chelate other trivalent and divalent metal ions, although with significantly lower affinities than iron (Alori *et al.,* 2017).

(ii). Enzymatic Mechanisms in Indirect Plant Defense

Several microbial compounds contribute to indirect defense mechanisms, including hydrolytic enzymes produced by PGPB that exhibit antimicrobial activity and act as a barrier against pathogenic bacteria (ALKahtani *et al.,* 2020). Among these, proteases play a significant role in plant protection by degrading pathogen-derived proteins. These enzymes, secreted by species such as *Bacillus clausii* and *Bacillus lentus*, are categorized based on their optimal pH into alkaline, acidic, and neutral proteases, each contributing to defense under specific soil conditions (Razzaq *et al.,* 2019).

Another important mechanism involves catalase-positive bacteria that protect plant roots from oxidative damage caused by hydrogen peroxide (H_2O_2), thereby enhancing plant tolerance under oxidative stress. Notable catalase-producing strains include *Bacillus insolitus, Bacillus pasteurii, Bacillus laterosporus,* and *Staphylococcus aureus* (Atouei *et al.,* 2019). Hydrogen cyanide (HCN), a volatile and highly toxic secondary metabolite, is also synthesized by certain rhizospheric bacteria. HCN interferes with cellular respiration and inhibits the growth of pathogenic fungi, nematodes, insects and termites (Sehrawat *et al.,* 2022). Additionally, it acts as a natural herbicide by colonizing the rhizospheres of competing plant species and suppressing their growth without harming the host plant (Sehrawat *et al.,* 2022).

Amylases, another group of important enzymes, contribute to plant protection by degrading polysaccharides in the cell walls of phytopathogens (Ismail *et al.,* 2021). These enzymes are classified into α-amylases, β-amylases, and γ-amylases, with α-amylases being the most produced by endophytic bacteria associated with medicinal and crop plants (Ismail *et al.,* 2021). Bacterial species from the genus Bacillus, such as *Bacillus licheniformis, Bacillus stearothermophilus* and *Geobacillus* bacterium*,* are notable for their high amylolytic activity and

are widely studied for their biocontrol potential (Far *et al.,* 2020). Ureases also play a critical role in the rhizosphere by catalyzing the hydrolysis of urea into ammonium and carbon dioxide, producing ammonium (NH_4^+), a readily assimilable nitrogen source for plants (Witte, 2011). Moreover, ureolytic bacteria contribute to biomineralization through calcite precipitation, which occurs via an increase in pH and carbonate ion production. This process is relevant not only for soil nutrient enhancement but also for environmental applications such as biocementation and the repair of microcracks in concrete (Cui *et al.,* 2022).

2.12.2 Direct Mechanisms of Plant Growth-Promoting Bacteria

(i). Plant Growth Regulators and their Role in Plant Growth and Signaling

Plant growth regulators (PGRs) are essential chemical compounds that enable plants to adapt and respond to dynamic environmental conditions (Vejan *et al.,* 2016). Both plants and microorganisms synthesize PGRs, including cytokinins and auxins, which influence a wide range of physiological and developmental processes. However, research on cytokinin biosynthesis remains limited due to their structural diversity and typically low endogenous concentrations, which complicate detection and quantification (Mekureyaw *et al.,* 2022). Unlike animal hormones, PGRs are not synthesized in specialized organs but can be produced in nearly all plant tissues. Currently, five major groups of PGRs are recognized as key regulators of plant development: auxins (AUXs), gibberellins (GBRs), cytokinins (CTKs), ethylene (ETH), and abscisic acid (ABA) (Davies, 1995).

In addition to these canonical PGRs, other signaling molecules with hormone-like activities play crucial roles in plant defense against herbivores and pathogens. These include salicylic acid (SA), nitric oxide (NO), jasmonic acid (JA), brassinosteroids (BRs), strigolactones (SLs) and systemin

peptides (Smith *et al.,* 2017). Although nitric oxide is well recognized as a signaling molecule and metabolic intermediary, it is not yet classified as a PGR due to its inorganic nature (Chandra *et al.,* 2018). Moreover, research on gibberellic acid (GA) biosynthesis, particularly its microbial pathways, remains relatively underexplored, with minimal progress reported in the past two decades. Among all PGRs, IAA is the most extensively studied due to its central role in plant growth promotion, mediated by PGPB (Rehman *et al.,* 2020). Both plants and microorganisms are capable of synthesizing IAA through various biosynthetic pathways, one of the most common being tryptophan (Trp)-dependent (Rehman *et al.,* 2020). Microbial IAA production is influenced by several physiological parameters, including temperature, pH, and the availability of carbon and nitrogen sources (Chandra *et al.,* 2018).

PGRs often undergo long-distance translocation within plants, a process that is essential for systemic signaling and inter-organ communication. This transport is facilitated through the phloem and xylem (Vanneste and Friml, 2009). For example, auxins synthesized in the apical buds are translocated basipetally to the roots via the phloem, while cytokinins produced in the roots are transported acropetally to the shoot meristems via the xylem. Additionally, certain PGRs, particularly weak organic acids, may passively diffuse across cellular membranes in their protonated form (Vanneste and Friml, 2009). As research continues to advance, it is expected that new classes of plant signaling molecules will be identified, thereby enhancing our understanding of the complex regulatory networks governing plant growth and environmental adaptation

(ii). Contribution of Microbial Activity to Nutrient Solubilization and Plant Growth

Phosphorus (P) is a critical macronutrient for plant growth, and its deficiency significantly impairs physiological development and crop productivity (Nikitha *et al.,* 2017). Certain PGPB

possess the enzymatic and metabolic capability to solubilize inorganic phosphorus compounds, such as tricalcium phosphate, hydroxyapatite, and rock phosphate, through the secretion of organic acids, primarily citric and gluconic acids (Verma *et al.,* 2017). These acids chelate phosphate-associated cations via their hydroxyl and carboxyl functional groups, enhancing phosphorus bioavailability in the rhizosphere (Verma *et al.,* 2017). Recent research has highlighted the role of specific microbial enzymatic pathways, particularly those involving glucose dehydrogenase (gdh), the pyrroloquinoline quinone (pqq) gene cluster, and organic acid biosynthesis systems, in mineral phosphate solubilization (Suleimanova *et al.,* 2023). For instance, the expression of the gdh gene, which encodes glucose dehydrogenase, has been positively correlated with enhanced phosphate solubilization in crops such as wheat and chickpea (Saha *et al.,* 2024). Under tricalcium phosphate (TCP) stress, gdh expression showed up to a 1.59-fold increase, as quantified by qRT-PCR, compared with uninoculated controls (Saha *et al.,* 2024). Furthermore, the pqq gene cluster, particularly the pqqC gene, is critical for the biosynthesis of PQQ, a redox cofactor essential for GDH activity (Saha *et al.,* 2024). This enzyme oxidizes glucose to gluconic acid, thereby acidifying the rhizosphere and mobilizing phosphate ions by displacing them from metal complexes. The presence and expression levels of pqqC have been closely linked to high phosphate-solubilizing efficiency in genera such as *Pseudomonas* and *Burkholderia*, positioning this gene as a potential molecular marker for selecting elite phosphate-solubilizing bacterial strains (An *et al.,* 2025).

Meta-analytical evidence further supports the application of phosphate-solubilizing bacteria (PSB) as bio-inoculants, reporting increases in the available phosphorus in soil ranging from 8% to 73%, with the outcomes influenced by soil physicochemical characteristics and crop species (Gullì *et al.,* 2025).

Zinc (Zn) is another essential micronutrient, acting as a catalytic or structural cofactor in numerous enzymes involved in photosynthesis, hormone synthesis, and stress resistance. Specific PGPB can increase zinc bioavailability by releasing siderophores and organic acids that solubilize Zn from otherwise insoluble forms, such as ZnO and $Zn_3(PO_4)_2$ (Eshaghi *et al.,* 2019). For example, the strains of *Burkholderia cepacia* and *Acinetobacter baumannii* demonstrated zinc solubilization of up to 1.44 ppm under *in vitro* conditions. In pot trials using *Zea mays,* inoculation with these strains significantly enhanced shoot and root development, compared with non-inoculated controls. High-performance liquid chromatography (HPLC) analyses identified the presence of oxalic, maleic, tartaric, and fumaric acids in the rhizosphere, supporting their role in zinc mobilization and uptake (Mahmud *et al.,* 2021).

Potassium (K), a major macronutrient, is essential for numerous plant processes including enzyme activation, osmoregulation, and stomatal function. Although potassium is naturally abundant in soils, a large proportion is sequestered in insoluble mineral forms, such as feldspar and mica, making it unavailable to plants (Verma *et al.,* 2017). Potassium-solubilizing bacteria (KSB), including *Bacillus aryabhattai* SK1-7 and *Pantoea vagans* ZHS-1, can release K+ ions through the secretion of organic acids such as citric, oxalic, and gluconic acids, which acidify the soil and chelate aluminum or silicon-based complexes (Rawat *et al.,* 2022). *B. aryabhattai* SK1-7 was shown to solubilize potassium at a rate of 32.6%, releasing 10.8 μg/mL of K^+ in vitro, while *P. vagans* ZHS-1 reached 20.3 mg/L under optimized fermentation conditions (Nawaz *et al.,* 2023). Field trials have reported increases in soil-available potassium ranging from 2.7% to 40.5%, depending on mineral composition and environmental context (Nawaz *et al.,* 2023). Moreover, KSB play an important role in enhancing plant tolerance to abiotic stress by

improving the cytosolic K^+/Na^+ ratio, which is critical for maintaining cellular homeostasis under saline conditions (Nawaz *et al.,* 2023).

2.13 Physiological Mechanisms of Plant-Microorganism Interaction

The beneficial interaction between plants and bacteria occurs within a dynamic and complex environment, where molecular and biochemical signaling plays a central role in mediating communication between the two partners (Bukhat *et al.,* 2020). This symbiotic relationship is sustained by a continuous exchange of chemical signals that coordinate interactions between plants and free-living bacteria, resulting in mutual benefits for both organisms (Song *et al.,* 2021).

Among these microorganisms, PGPB enhance plant development through a combination of direct and indirect mechanisms, as previously described. This section focuses on the physiological mechanisms that underlie plant–microorganism interactions, with the objective of elucidating how PGPB exert their beneficial effects at the functional and physiological levels. Particular attention is given to signaling pathways, stress mitigation responses, and the modulation of plant metabolism triggered by microbial activity.

2.13.1 Bacterial Contribution to Plant Nutrient Acquisition

(i). Nitrogen

Nitrogen is an essential element for all living organisms, as it forms a fundamental component of nucleic acids and proteins. Although atmospheric nitrogen (N_2) is abundant, most organisms are unable to assimilate it directly. Only a specific group of bacteria and archaea, collectively known as diazotrophs, possess the enzymatic machinery to convert N_2 into ammonia (NH_3) via biological nitrogen fixation (BNF), a process catalyzed by the oxygen-sensitive nitrogenase enzyme complex (Sharma *et al.,* 2016).

Diazotrophic bacteria do not freely excrete ammonia, as nitrogen fixation and assimilation are tightly coupled and regulated through complex molecular pathways (Geddes and Oresnik, 2016). These include transcriptional regulators, post-translational protein-modifying enzymes, and PII signal transduction proteins that modulate the expression of nitrogen metabolism genes. In diazotrophic proteobacteria, these pathways interact with the nitrogen fixation regulator NifA, ensuring nitrogenase expression is tightly regulated according to nitrogen demand (Cherkasov *et al.,* 2015). The ammonia produced during BNF diffuses across the bacterial membrane but is promptly recovered by the ammonium transporter AmtB, despite the high energetic cost of the process. Notably, BNF requires approximately 16 moles of ATP per mole of fixed N_2, underscoring its energy-intensive nature (Cherkasov *et al.,* 2015).

(ii). Phosphorus

After nitrogen, phosphorus (P) is the second most limiting essential mineral nutrient for plant growth, as it is only absorbed in its soluble monobasic ($H_2PO_4^-$) or dibasic (HPO_4^{2-}) forms (Song *et al.,* 2021). In soils, P is predominantly found in inorganic forms, either adsorbed onto soil mineral surfaces or in poorly available precipitates. It is also present in organic forms, where it is incorporated into biomass or associated with soil organic matter (Song *et al.,* 2021).

P is one of the nineteen essential elements for plant life and plays a central role in energy capture, storage, and transfer. It is a key structural component of DNA, RNA, and phospholipids in both plant and animal cells (Nikitha *et al.,* 2017). It participates in fundamental physiological processes including photosynthesis, root development, stem elongation, flower and seed formation, crop maturation, energy metabolism, cell division and expansion, nitrogen fixation in legumes, disease resistance, starch biosynthesis, and genetic information transfer (Khan *et al.,* 2013). Moreover, adequate phosphorus availability is critical for the formation of reproductive

primordia during early plant development (Khan *et al.,* 2013). Thus, phosphorus is indispensable for nearly all aspects of plant physiology.

Within the soil–plant system, approximately 90% of the total phosphorus is present in the soil matrix, while less than 10% is found in the soil solution (Rodríguez and Fraga, 1999). However, most soil phosphorus is bound to particles or minerals such as apatite, hydroxyapatite, and oxyapatite, making it largely unavailable to plants (Rodríguez and Fraga, 1999). Organic phosphorus compounds, such as inositol phosphates (phytate), phosphomonoesters, and phosphotriesters, must undergo mineralization to become bioavailable (Rodríguez and Fraga, 1999).

Phosphate-solubilizing bacteria (PSB) are key members of the plant microbiome that enhance the conversion of insoluble organic and inorganic phosphate forms into bioavailable phosphorus, particularly under phosphorus-deficient conditions (Bargaz *et al.,* 2021). This microbial activity is essential for improving phosphorus availability and supporting plant growth in nutrient-depleted soils. The primary mechanism employed by PSB involves acidification of the surrounding soil environment through the secretion of organic acids or proton release, thereby increasing phosphorus solubility (Bargaz *et al.,* 2021).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Collection of Samples

Ruminant (cow dung) and non-ruminant (Poultry droppings) animal waste manure was collected from three animal farms located in Benin-Sapele Road, Technical college road and Aduwawa in Benin City, Edo state. A total of 24 samples of ruminant and non-ruminant animal fecal waste samples was collected into sterile sampling plastic bowls and transported to the laboratory in ice-pack for bacteriological analysis.

3.2 Sterilization of Materials

Glass wares such as Petri dishes, test tubes, pipettes, conical flasks, measuring cylinder and beakers required for this study were washed using detergent and rinsed with distilled water. They were wrapped with aluminum foil paper and sterilized in the hot air oven in an inverted position at 160^{o}C for 3hr.

3.3 Preparation of Ruminant (Cow dung), Non-Ruminant (Poultry droppings) and Mixed Manure Treatments

Fresh fecal samples from ruminant and non-ruminant animals were collected separately in sterile containers and transported to the laboratory. The samples were air-dried under shade to reduce

moisture content, crushed, and sieved to obtain a uniform texture. Extraneous materials such as stones and bedding residues were removed.

The experimental soil was weighed into pots at 5 kg per pot. Manure was applied at a rate of 200 g per 5 kg of soil, equivalent to 4 percent weight by weight, which falls within commonly used organic amendment rates for pot experiments. The manure was thoroughly mixed into the soil to ensure even distribution.

For the ruminant treatment, 200 g of processed ruminant manure was incorporated into 5 kg of soil. For the non-ruminant treatment, 200 g of processed non-ruminant manure was incorporated into 5 kg of soil. For the mixed treatment, equal proportions of ruminant and non-ruminant manure were combined in a 1:1 ratio by weight, consisting of 100 g ruminant manure and 100 g non-ruminant manure, making a total of 200 g manure per 5 kg of soil. The mixture was blended thoroughly before incorporation into the soil.

All treatments were set up in triplicate. After incorporation, the soils were watered lightly and allowed to stabilize for 5 to 7 days before planting to permit initial microbial activity and nutrient equilibration. This standardized application rate ensured uniform nutrient input across treatments, allowing accurate comparison of microbial load and plant growth responses.

3.4 Preparation and Sterilization of Culture Media

All culture media (nutrient agar, tryptic soy agar, McConkey agar, and Mueller Hinton agar) were prepared and sterilized according to the manufacturer's instructions.

3.5 Isolation and Enumeration of Total Heterotrophic Bacterial and Coliform Counts

One gram (1 g) of the fecal sample was weighed into sterile beaker and 9 ml of distilled water was added to get the stock solution. The suspension was serially diluted using a ten-fold serial dilution.

After the serial dilution, 1 ml aliquot was inoculated into triplicate using tryptic soy agar (supplemented with fluconazole to prevent fungal growth) using the pour plate technique.

The plates were incubated at 30±2 ºC for 24 hr. Bacterial colonies were counted using a colony counter and the result was recorded. The number of colony forming unit per gram (cfu/g) was calculated using the formula below:

$$\frac{cfu}{g} = \frac{number\ of\ colonies\ x\ dilution\ fold/series}{volume\ of\ inoculum}$$

Bacterial colonies were purified and stored on nutrient agar slant for further use.

For coliform count, aliquots from appropriate dilutions were plated on selective and differential medium such as MacConkey agar or Eosin Methylene Blue agar using the pour plate or spread plate method. The plates were incubated at 37 °C for 24 hr. Typical coliform colonies were identified based on characteristic morphology, such as pink to red colonies on MacConkey agar due to lactose fermentation or colonies with a metallic sheen on Eosin Methylene Blue agar. Distinct colonies were counted and expressed as cfu/g using the same calculation formula.

Representative bacterial colonies were purified by repeated streaking and preserved on nutrient agar slants for further analysis.

3.6 Phenotypic identification of Bacteria isolates

Several tests such as Gram reaction, catalase, urease, indole, oxidase, sugar fermentation, citrate utilization, and triple sugar iron agar tests were carried out to presumptively identify the bacterial isolates.

3.6.1 Morphological identification

Gram staining

Gram staining techniques was used for differentiation between Gram-positive and Gram-negative bacteria. Gram positive bacteria retain the primary stain while Gram negative bacteria do not retain the primary stain when decolourized with alcohol. The Gram stain procedure is as follows:

A smear of the bacteria isolate was made on grease free slide and heat fixed by passing over flame. The smear was flooded with crystal violet which is the primary stain for 1min then washed off with distilled water. Subsequently the slides were flooded with Lugol's iodine solution for 30sec and then washed off with distilled water. 95% alcohol was used for decolorization for 10sec and washed off with distilled water. Finally, the smear was counter stained with safranin for 1min and washed off. The slides were allowed to air dry before observing under the microscope using an oil immersion objective lens of ×100 magnifications to view the slides.

Expected observation: Gram positive bacteria appear purple or deep violet under the microscope due to retention of the crystal violet iodine complex, while Gram negative bacteria appear pink or red as a result of taking up the safranin counterstain.

Spore Staining

Spore staining was performed to detect the presence of endospores in bacterial isolates, which are highly resistant structures formed by certain Gram-positive bacteria such as *Bacillus* and *Clostridium* species. A small amount of bacterial culture was smeared onto a grease-free slide and heat-fixed by passing it briefly over a flame. The smear was then flooded with malachite green stain and heated gently to allow the stain to penetrate the tough spore coat. After staining for several minutes, the slide was rinsed with distilled water to remove excess stain. The smear was counterstained with safranin to color the vegetative cells. Under a microscope using oil

immersion, endospores appeared as green structures within red or pink vegetative cells. This method allowed differentiation between spore-forming and non-spore-forming bacteria, which is important for bacterial identification and understanding potential resistance and survival mechanisms.

3.6.2 Biochemical identification

Biochemical test was carried out so as to help in the identification of the bacteria isolates as phenotypic (cultural) characteristics is not sufficient. The various biochemical test carried out are shown below;

Motility Test

The motility test was performed to determine whether bacterial isolates were capable of self-propulsion, which is often mediated by flagella. A semi-solid agar medium was prepared, usually with a lower agar concentration (about 0.4–0.5%) to allow movement of motile bacteria. Using a sterile inoculating needle, a single bacterial colony was carefully stabbed into the center of the medium, taking care not to touch the sides of the tube. The inoculated tubes were then incubated at 37 °C for 24–48 hr.

Motile bacteria spread away from the line of inoculation, producing a diffuse, cloudy growth throughout the medium, whereas non-motile bacteria grew only along the stab line, leaving the surrounding medium clear. Motility is an important characteristic for bacterial identification and can indicate the ability of bacteria to move toward nutrients or away from harmful conditions. In the context of manure bacterial isolates, motility may also suggest how effectively these bacteria could colonize plant roots or soil environments, contributing to their plant growth-promoting potential.

Oxidase test

The oxidase test is used to detect the presence of cytochrome c oxidase, an enzyme involved in the bacterial electron transport chain. It is commonly applied in the identification of Gram-negative rods, particularly to distinguish oxidase positive organisms such as Pseudomonas species from oxidase negative members of the family Enterobacteriaceae (MacFaddin, 2000).

The test was carried out using freshly prepared 1 percent aqueous tetramethyl-p-phenylenediamine dihydrochloride as the oxidase reagent. A few drops of the reagent were placed on a piece of clean filter paper. Using a sterile wire loop, a portion of a fresh bacterial colony was smeared onto the reagent soaked paper. Development of a purple coloration within 10 sec was recorded as a positive result, indicating the presence of cytochrome c oxidase. Absence of color change within this time was considered a negative result.

Known oxidase positive and oxidase negative organisms were used as controls to validate the test procedure. Pseudomonas aeruginosa served as the positive control, while Escherichia coli served as the negative control.

Urease test

The urease test is used to determine the ability of an organism to produce the enzyme urease, which hydrolyzes urea into ammonia and carbon dioxide. The release of ammonia increases the pH of the medium, leading to a detectable color change. This test is widely applied in the differentiation of strongly urease positive organisms such as Proteus mirabilis from other members of the Enterobacteriaceae that do not produce urease (Cheesbrough, 2006).

Christensen's urea agar, a differential medium containing urea and the pH indicator phenol red, was used for the test. The sterile medium was dispensed into test tubes as slants. Each test isolate was inoculated aseptically onto the surface of the medium and incubated at 37 °C for 24 hr.

A change in colour of the medium from yellowish orange to pink or red indicated a positive urease reaction due to ammonia production and an increase in pH. Absence of colour change indicated a negative result.

Indole production test

The indole test was carried out to determine the ability of the isolates to degrade tryptophan and release indole in peptone water. Peptone broth was prepared by dissolving 5 g of commercially prepared peptone in 1 litre of distilled water. The medium was sterilized by autoclaving at 121 °C for 15 min. After sterilization, 4 ml portions were dispensed aseptically into sterile test tubes. Each bacterial isolate was inoculated into the broth and incubated at 37 °C for 24 hr.

Following incubation, a few drops of Kovac's reagent were added carefully to each tube. Kovac's reagent is composed of amyl alcohol, p-dimethylaminobenzaldehyde, and concentrated hydrochloric acid. The development of a red ring at the surface of the broth indicated a positive result, confirming indole production.

A negative result was indicated by the absence of a red ring, with the reagent layer remaining yellow or pale in colour.

Citrate utilization test

The citrate utilization test was performed to determine the ability of bacterial isolates to use citrate as their sole carbon source for growth. Simmon's citrate agar, a differential medium containing sodium citrate and the pH indicator bromothymol blue, was used for this test.

To prepare the medium, 22 g of commercially prepared Simmon's citrate agar was dissolved in 1 litre of distilled water and sterilized by autoclaving at 121 °C for 15 min. The sterile medium

was dispensed into test tubes and allowed to solidify as slants. Each test organism was inoculated onto the surface of the medium using a sterile straight inoculating wire. The inoculated tubes were incubated at 37 °C for 24 hr.

A positive result was indicated by a color change of the medium from green to bright blue, showing citrate utilization and an increase in pH. A negative result was indicated by the medium remaining green with no visible growth.

Catalase test

This is a test to detect the presence or absence of catalase enzyme. The catalase enzyme catalyses the breakdowns of hydrogen peroxide to release free oxygen gas and the formation of water. A few drops of freshly prepared 3% hydrogen peroxide were added onto the bacterial isolates smeared on a slide. The production of gas bubble indicated catalase enzyme production.

$2\ H_2O_2 \rightarrow 2\ H_2O + O_2$

KOH Test

The KOH (potassium hydroxide) test was used as a simple method to determine the Gram reaction of bacterial isolates. A drop of 3% KOH solution was placed on a clean microscope slide, and a small amount of bacterial culture was mixed into the solution. The mixture was stirred gently for about 60 seconds. Gram-negative bacteria released their cellular contents, producing a viscous, mucoid string, while Gram-positive bacteria did not form this string and the solution remained watery. This test provides a rapid alternative to Gram staining for distinguishing Gram-negative from Gram-positive bacteria and can be useful in routine identification or when rapid screening of multiple isolates is required.

Sugar fermentation and production of gases using Triple sugar iron agar (TSI)

Triple Sugar Iron (TSI) agar was prepared according to the manufacturer's instructions. The medium was dispensed into test tubes and allowed to solidify in a slant position. Using a sterile loop, the test bacterium was streaked on the slant surface and stabbed into the butt of the medium. The inoculated tubes were then incubated at 37 °C for 18–24 hr.

Results were interpreted based on color changes and other reactions: acid or alkaline production in the slant or butt indicated sugar fermentation, gas production was evidenced by cracks or bubbles in the agar, and hydrogen sulfide (H_2S) production was shown by blackening of the medium. A prepared laboratory chart was used for result interpretation.

Results were interpreted based on color changes and other reactions:

- Acid slant / acid butt (yellow/yellow): Fermentation of glucose and either lactose or sucrose.
- Alkaline slant / acid butt (red/yellow): Fermentation of glucose only.
- Alkaline slant / alkaline butt (red/red): No fermentation of sugars.
- Gas production: Visible as cracks, bubbles, or lifting of the agar.
- Hydrogen sulfide (H_2S) production: Blackening of the butt of the medium due to H_2S reacting with iron salts.

These observations allow differentiation of enteric bacteria based on sugar fermentation patterns, gas production, and H_2S formation.

3.7 Pathogenicity Test

Pathogenicity testing was done to observe the disease causing potentials of the selected isolates. These tests included: Gelatin liquefaction test, DNase test, lipase test and haemolysin test.

Gelatin liquefaction test

The gelatin liquefaction test is used to determine the ability of bacteria to produce the enzyme gelatinase, which hydrolyzes gelatin into soluble peptides and amino acids. Gelatinase production is associated with pathogenicity because it allows bacteria to degrade host connective tissue proteins, facilitating tissue invasion and dissemination in the host. This trait is commonly found in organisms such as Staphylococcus species, Enterobacteriaceae, and certain Gram-positive bacilli (Forbes *et al*., 2016).

Gelatin agar was prepared according to the manufacturer's instructions and dispensed aseptically into six sterile test tubes, which were allowed to solidify. Each test isolate was inoculated into the tubes and incubated at 37 °C for 24 hr. After incubation, the tubes were placed on ice for 30 min.

Interpretation of results:

- Positive: The gelatin remained liquid after cooling, indicating gelatinase production.
- Negative: The gelatin solidified, indicating absence of gelatinase.

DNase test

DNase Test Agar containing methyl green is a solid medium used to detect deoxyribonuclease (DNase) activity in microorganisms. The medium was prepared according to the manufacturer's instructions, poured into sterile Petri dishes, and allowed to solidify. Each test isolate was streaked onto the surface of the agar and incubated at 37 °C for 24 hr (Moremi *et al.,* 2016).

Interpretation of results: Positive: A change of the green colour in the medium to blue around the bacterial growth indicated DNase production. Negative: The green colour of the medium remained unchanged, indicating no DNase activity.

Lipase test

Spirit blue agar was prepared according to the manufacturers' instructions. It was cooled and 1ml of sterile olive oil were added to the agar and poured into petri dishes in a sterile environment. Colonies from the samples were streaked onto the plates and inoculated at 37°C for 24 hr. Becteria that produced lipase hydrolyzed the olive oil and produced a 'halo' around the zone of growth indicated a positive result while the colonies that didn't produce a halo around the region of streaking indicated negative results. This test is used to identify organisms that are capable of producing lipase.

Haemolysin production test

The haemolysin production test is based on the ability of certain bacteria to produce hemolysins—enzymes that lyse red blood cells. Hemolysin production is considered a virulence factor because it enables pathogenic bacteria to access nutrients such as iron from host blood and contributes to tissue damage during infection (Forbes *et al.,* 2016).

Principle: Bacteria that produce hemolysins will alter the appearance of blood in the agar surrounding their colonies.

Medium: Nutrient agar supplemented with 5% sheep blood was used. The blood was aseptically added to molten nutrient agar, mixed gently, allowed to cool, and poured into sterile Petri dishes to solidify.

Test isolates were streaked onto the surface of the blood agar and incubated at 37 °C for 24 hr.

Interpretation of results:

- Alpha (α) hemolysis: Partial lysis of red blood cells produces a greenish or darkened discoloration around the colonies.
- Beta (β) hemolysis: Complete lysis of red blood cells creates a clear, transparent zone around the colonies.

- Gamma (γ) hemolysis: No hemolysis occurs, and the medium remains unchanged in color.

(Maze *et al.,* 2018).

3.8 Antibiotic Susceptibility Testing

Antibiotic susceptibility of the bacterial isolates was determined using the modified Kirby-Bauer disk diffusion method. Muller Hinton agar (pH 7.2–7.4) was poured into sterile 90 mm Petri dishes to a depth of 4 mm. The test bacterial suspension was adjusted to 0.5 McFarland turbidity standard, and the agar surface was evenly inoculated by swabbing in three directions while rotating the plate to ensure uniform coverage. Antimicrobial discs used included Pefloxacin (5 µg), Gentamicin (10 µg), Ampiciox (10 µg), Zinnacef (30 µg), Amoxicillin (5 µg), Rocephin (30 µg), Ciprofloxacin (5 µg), Azithromycin (15 µg), Levofloxacin (5 µg), and Erythromycin (15 µg). The plates were incubated at 37 °C for 24 hr.

Interpretation of Results: The effectiveness of each antibiotic was determined by measuring the diameter of the zone of inhibition around each disc in millimeters. The results were classified as susceptible, intermediate, or resistant based on the standard reference ranges provided by the Clinical and Laboratory Standards Institute (CLSI) guidelines. Larger zones indicate higher susceptibility, while small or no zones indicate resistance (Kumburu *et al.,* 2017).

Multiple Drug Resistance Index (MDRI) of Bacterial Isolates

The Multiple Drug Resistance Index (MDRI) is a quantitative measure used to assess the resistance of bacterial isolates to multiple antibiotics. It provides insight into the potential risk posed by bacterial strains that have been exposed to high levels of antimicrobial agents, often indicating environments with heavy antibiotic use, such as animal manure or clinical settings.

MDRI is particularly useful for comparing resistance patterns among different isolates and evaluating the overall pressure of antimicrobial selection in a given environment.

Calculation of MDRI

The MDRI is calculated using the formula: MDRI = a / b

Where: a = the total number of antibiotics to which a single bacterial isolate shows resistance

b = the total number of antibiotics tested against that isolate

3.9 Molecular Identification Methods

These include multiplex reverse transcription-PCR (RT-PCR), qPCR. Random amplified polymorphic DNA (RAPD) analysis and restriction fragment length polymorphism (RFLP) analysis.

3.9.1 Molecular Identification of the bacterial Isolates

Molecular identification of bacterial species was carried out following the methods described by Samson *et al*. (2014). A small portion of bacterial biomass, approximately 10 mg of colony material, was placed into a sterile mortar. To this, 1 ml of DNA Extraction Buffer (DEB) composed of 100 mM Tris-HCl (pH 8.0), 51 mM EDTA (pH 8.0), 500 mM NaCl, 10 mM β-mercaptoethanol, and proteinase K at 0.05 mg/ml was added, and the mixture was macerated using a sterile pestle. The homogenate was transferred into a sterile 1.5 ml Eppendorf tube.

To the extract, 40 µl of 20% SDS was added, followed by brief vortexing and incubation at 65 °C for 10 minutes to lyse the cells and release DNA. At room temperature, 160 µl of 5 M potassium acetate was added, mixed by vortexing, and centrifuged at 10,000 rpm for 10 minutes to precipitate proteins and cell debris. The supernatant containing DNA was carefully transferred to a fresh tube, and 400 µl of cold isopropanol was added to precipitate the DNA. The mixture was gently mixed and incubated at -20 °C for 60 minutes.

DNA was recovered by centrifugation at 13,000 rpm for 10 minutes. The supernatant was decanted carefully to avoid disturbing the DNA pellet. The DNA pellet was then washed with 500 µl of 70% ethanol and centrifuged at 10,000 rpm for 10 minutes. After drying, the DNA was resuspended in an appropriate buffer for downstream PCR amplification and molecular identification.

3.9.2 Procedure of Deoxyribonucleic acid (DNA) Extraction

DNA was extracted from bacterial cells to allow for molecular analysis. The procedure involved lysing the cells to release genomic DNA and purifying it to remove proteins, cell debris, and other compounds that could inhibit downstream applications such as PCR. For environmental or difficult-to-lyse samples, physical disruption methods were used, including bead-beating with sterile micron-sized beads or rapid freeze-thaw cycles, which helped break the cell or spore walls and increased DNA yield. Chemical lysis was also employed, using a DNA extraction buffer containing Tris-HCl, EDTA, NaCl, SDS, and proteinase K, with incubation at elevated temperatures to digest proteins and lyse cells.

Following lysis, the mixture was centrifuged at high speed to remove cell debris, and the supernatant containing DNA was carefully transferred into a fresh tube. DNA was precipitated by adding cold isopropanol or absolute ethanol and incubating the mixture at -20 °C. After centrifugation, the DNA pellet was washed with 70% ethanol to remove residual salts and impurities. The pellet was air-dried briefly and then resuspended in sterile nuclease-free water or TE buffer for further analysis.

For samples with high levels of contaminants, additional purification steps were carried out using either microcentrifuge spin columns or phenol-chloroform extraction to ensure removal of proteins and PCR inhibitors. Care was taken throughout the process, as both the type of sample

and the purification method could result in low DNA recovery, sometimes as little as 10 percent. This protocol ensured that high-quality DNA suitable for molecular identification was obtained (Kumburu *et al.,* 2017).

3.9.3 PCR Product Sequencing

The PCR products obtained from bacterial DNA amplification were first purified using ExoSAP-IT to remove residual primers, nucleotides, and enzymes that could interfere with sequencing. The purified PCR products were then sent to Epoch Life Science (USA) for sequencing using the Sanger sequencing method. Sequencing was performed on an ABI 3730XL Genetic Analyzer (BigDye Terminator v3.1 chemistry), which generates nucleotide sequences by incorporating fluorescently labeled dideoxynucleotides during DNA synthesis.

The sequencing data were returned in FASTA format, representing the nucleotide sequences of the PCR products. The sequences were visualized, edited, and analyzed using BioEdit Sequence Viewer version 7.2.1 to confirm sequence quality and prepare the data for downstream applications such as alignment, identification, and phylogenetic analysis.

3.9.4 Sequencing Identification

Sequences were identified using Gen Bamk's Basic Local Alignment Search Tool (BLAST) algorithm on National Centre for Biotechnology and Information website. The corresponding sequences were identified using the online blast search at (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequences were aligned by multiple sequence alignment technique using CLUSTAL W and the phylogenetic tree constructed by the neighbor joining method using MEGA version 6.

3.10 Phylogenetic tree construction

Phylogenetic corresponding of the 16SrRNA gene of the isolates was extrapolated with the downloaded sequence of other species from gene bank. Sequences were aligned by multiple

sequence alignment technique using CLUSTAL W and the phylogenetic tree constructed by the neighbor joining method using MEGA version 6.

3.11 Screening for Plant Growth-Promoting Bacteria (PGPB)

3.11.1 Screening for Ammonia production

Freshly grown bacterial cultures from animal manure were inoculated in 10 ml nutrient broth and incubated at 30°C for 48h in a rotator shaker. After incubation, 0.5 ml of Nessler's reagent was added to each tube. The development of a yellow to brown colour indicated a positive reaction for ammonia production (Kumar *et al*., 2012).

3.11.2 Screening for Indole Acetic Acid (IAA) production

This was determined by reaction of liquid culture of animal manure bacterial isolates grown in 500 mg/L. L-Tryptophan (the precursor for IAA biosynthesis) placed in tryptic soy broth (1 g/L MES hydrate, pH 6) and Salkowki's reagent. Inoculated broth was incubated at 30°C for 72h in a rotary shaker. After incubation broth was centrifuged at 3000rpm for 15min. Then 1.0 ml of the supernatant was mixed with 2.0 ml of Salkowski reagent (50 ml of 35% Perchloric acid + 1 ml of 0.5 M $FeCl_3$ solution), and the mixture was then incubated at room temperature for 25mins. Development of pink color after incubation at room temperature indicated IAA production. The absorption of the positive reaction was determined at 535nm using a spectrophotometer.

3.12 Plant Morphological studies

The experiment set up was observed every week to record changes in the morphological defects of the *Telfairia occidentalis* plant such as seed germination, length of vine, leaf area, leaf length and fresh and dry weight. *Telfairia occidentalis* was selected for this study because it is an economically and nutritionally important leafy vegetable widely cultivated in Nigeria and other parts of West Africa. It is highly responsive to soil amendments, making it an ideal model for

evaluating the effects of animal manure and plant growth-promoting bacteria. Its fast growth, measurable morphological traits, and sensitivity to nutrient changes allow for clear assessment of the growth-promoting properties of organic fertilizers, providing practical and applicable results for improving crop productivity in local agricultural systems.

The study evaluated the bacteriological properties and plant growth-promoting potential of animal manure, which contains both nutrients and beneficial microorganisms. Using *Telfairia occidentalis* allowed assessment of how these manure-derived bacteria and nutrients affect plant growth. Specifically, the manure served as a natural amendment, supplying nitrogen, phosphorus, and other essential nutrients, as well as plant growth-promoting bacteria (PGPB) that can enhance germination, root development, leaf expansion, and biomass accumulation. Observing the morphological traits of the plants provided a direct measure of the efficacy of the manure treatments in promoting healthy growth.

Telfairia occidentalis seeds were sown in sterile pots or containers filled with a standard growth medium, such as a mix of soil and sand, or in pots containing treated soil amended with different manure samples. The plants were grown under controlled environmental conditions, which included:

- Temperature: 25–30 °C
- Light: Natural sunlight or 12–16 hr of artificial light per day
- Watering: Regular watering to maintain consistent soil moisture
- Humidity: Ambient laboratory or greenhouse humidity levels

This design allowed the effects of different manure treatments on growth parameters such as germination rate, vine length, leaf area, and biomass to be measured accurately, isolating the contributions of nutrients and plant growth-promoting bacteria from other variables.

3.12.1 Percentage Seed Germination

The seeds of *Telfairia occidentalis* were grown to assess the influence of different types of animal manure, both individually and in combination, on early plant development and overall growth. Monitoring seed germination provided a direct measure of the potential of manure-associated bacteria and nutrients to promote plant growth, which aligns with the main aim of the study. A seed was considered germinated when the radicle visibly emerged through the seed coat. The number of germinated seeds was recorded until no further germination occurred. The total percentage germination was computed at the end of the germination period using the formula:

Percentage germination = (Number of germinated seeds / Total number of seeds planted) × 100.

All observations were documented immediately after counting to avoid errors.

3.12.2 Length of Vine

The length of the vines of *Telfairia occidentalis* was measured weekly to monitor the vegetative growth of the plants. Vine length is a key morphological indicator of plant health, vigor, and the ability to acquire resources such as light, water, and nutrients. Longer vines generally reflect better nutrient uptake and overall plant development.

In this study, measuring vine length was directly related to the aim of evaluating the plant growth-promoting properties of bacteria associated with animal manure. Manure contains both essential nutrients and beneficial microorganisms that can enhance plant growth by improving nutrient availability, producing growth-stimulating hormones (such as indole-3-acetic acid), solubilizing phosphorus, and reducing stress on the plant. By tracking vine length, the experiment quantified the effects of different manure treatments on vegetative growth, thereby linking observed plant performance to the bacterial and nutrient contributions of the manure.

3.12.3 Leaf length

Leaf length was determined by measuring fully developed leaves on each plant. The length of each leaf was taken from the point of attachment at the petiole to the tip of the leaf blade using a ruler or measuring tape. Only mature, healthy leaves were selected to ensure uniformity. Measurements were recorded consistently at the same growth stage to minimize variability (Harris *et al.,* 2015).

Measuring leaf length provided a direct indicator of how effectively the plants utilized the nutrients and growth-promoting bacteria present in the animal manure. Manure contains nitrogen, phosphorus, and other essential nutrients, as well as beneficial microorganisms such as plant growth-promoting bacteria (PGPB), which can enhance nutrient uptake, produce phytohormones like indole-3-acetic acid, and improve overall plant metabolism. Longer leaves reflect enhanced photosynthetic capacity and improved biomass accumulation, indicating that the manure and its microbial content positively influenced plant growth.

This parameter aligns with the study objectives by demonstrating the impact of different types of manure, alone or in combination, on plant development (Objectives 2, 3, and 4). Leaf length thus served as a measurable outcome to evaluate the effectiveness of animal manure as a source of both nutrients and growth-promoting microorganisms for *Telfairia occidentalis*.

3.12.4 Leaf Number Determination and Leaf Area

The number of leaves produced per plant was obtained by counting. Leaf area was determined by multiplying the length by the breath of the leaf.

3.12.5 Fresh and Dry Weight Determination

At the end of the experiment, the fresh and dry weights of the plants were measured to assess biomass accumulation. Fresh weight was determined by collecting plant leaves from each

replicate and weighing them immediately using an electronic balance. To determine dry weight, the same plant leaves samples were placed in an oven and dried at 70 °C for 24 hr to remove all moisture. After drying, the leaves samples were cooled and weighed again using an electronic balance (Kochhar *et al*., 2017).

3.13 Data Analysis

All experimental results were presented as the mean of three replications. The means were calculated using Microsoft Excel 2016. Statistical differences between treatments were determined using two-way analysis of variance (ANOVA) to account for the effects of manure type and environmental variation, as the samples were collected from different locations and the growing environment was not fully controlled. The ANOVA was performed using SPSS version 20, and the level of significance was set at a 95% confidence interval ($p < 0.05$). Post-hoc comparisons were carried out using Duncan multiple range test (DMR) test to identify statistically significant differences between treatment means.

CHAPTER FOUR

RESULTS

The results presented in Table 4.1 show variations in the heterotrophic and coliform bacterial counts among the ruminant, non-ruminant, and mixed animal fecal samples. The mixed animal fecal matter exhibited the highest bacterial load, with a heterotrophic count of 27.80± 2.81 × 10^5 cfu/g and a coliform count of 13.69± 1.80 × 10^4 cfu/g. The ruminant manure showed moderate heterotrophic bacterial and coliform counts of 15.40±2.04 × 10^5 cfu/g and 7.65±1.37 × 10^4 cfu/g respectively while the non-ruminant manure had the lowest heterotrophic bacterial and coliform counts of 13.30±1.80 × 10^5 cfu/g and 6.40±1.17 × 10^4 cfu/g respectively. Significant difference ($p<0.05$) across columns were obtained for values of both heterotrophic and coliform counts in the various treatment samples.

Table 4.2 reveal the results obtained for the cultural, morphological and biochemical characteristics of bacterial isolates from ruminant and non-ruminant animal fecal matter.

Analyses revealed the presence of resident bacteria isolates such as *Enterobacter* sp*, Escherichia* sp*, Klebsiella* sp*, Pseudomonas* sp*., Salmonella* sp and *Bacillus* sp.

The results in Table 4.3 show the distribution and frequency of bacterial isolates identified from ruminant and non-ruminant animal fecal matter. A total of six bacterial species were isolated, with *Escherichia coli* showing the highest frequency of occurrence (25%), indicating its dominance and common presence in animal feces. *Klebsiella pneumoniae* followed with 20% occurrence, while *Enterobacter cloacae*, *Pseudomonas aeruginosa*, and *Salmonella enterica* each occurred at 15%, reflecting moderate distribution across the samples. *Bacillus velezensis* had the lowest occurrence (10%).

Table 4.1: Heterotrophic and coliform bacteria count of ruminant, non-ruminant and mixed animal fecal matter

Faecal Sample	Heterotrophic count ($x10^5$ cfu/g)	Coliform count ($x10^4$ cfu/g)
Ruminant (Cow dung)	15.40 ± 2.04^b	7.65 ± 1.37^b
Non-ruminant (Poultry droppings)	13.30 ± 1.75^a	6.40 ± 1.17^a
Mixed	27.80 ± 2.81^c	13.69 ± 1.80^c

Values are presented as mean ± SEM; n=3. Mean values with different superscripts within column are significantly different ($P<0.05$).

Table 4.2: Cultural, morphological and biochemical characteristics of bacterial isolates from ruminant and non-ruminant animal fecal matter

Characteristics	1	2	3	4	5	6
Cultural						
Elevation	Flat	Flat	Flat	Raised	raised	Flat
Margin	Undulate	Undulate	Entire	Entire	Entire	Undulate
Color	Cream	Cream	Cream	lemon	Cream	Cream
Shape	Irregular	Irregular	Circular	Circular	Circular	Irregular
Size	Large	Large	Small	Medium	Medium	large
Gr. diff. agar	MCC	EMB	MCC	PCA	SSA	BCA
Colour	Purple	Green	Pink	green	black	Straw
Morphological						
Gram stain	-	-	-	-	-	+
cell type	Rod	Rod	Rod	rod	rod	Rod
Arrangement	disperse	disperse	disperse	disperse	pair/chains	disperse
Color	pink	pink	pink	pink	pink	purple
Spore staining	-	-	-	-	-	+
Biochemical						
KOH test	+	+	+	+	+	-
Catalase	+	+	+	+	+	+
Indole	-	+	-	-	-	-
Citrate	+	-	+	-	-	+
Oxidase	-	-	-	+	+	-
Motility	+	+	-	+	+	+
Urease	+	-	+	+	-	-
Glucose	+	+	+	-	+	+
Sucrose	+	+	+	-	-	+
Lactose	+	+	+	-	-	+
Mannitol	+	-	+	-	-	+
Gas formation	+	+	+	-	+	-
H_2S formation	-	-	+	-	+	-
Identity	*Enterobacter cloacae*	*Escherichia coli*	*Klebsiella pneumoniae*	*Pseudomonas aeruginosa*	*Salmonella enterica*	*Bacillus velezensis*

Key: +: Positive, -: Negative, MCC – MacConkey Agar, EMB – Eosin Methylene Blue Agar, PCA – Plate Count Agar, SSA – Salmonella Shigella Agar, BCA – Baird-Parker Agar

Table 4.3: Distribution of bacterial isolates from ruminant and non-ruminant animal fecal matter

S/N	Bacterial isolates	Frequency of occurrence	Percentage (%) of occurrence
1	*Enterobacter cloacae*	3	15.0
2	*Escherichia coli*	5	25.0
3	*Klebsiella pneumoniae*	4	20.0
4	*Pseudomonas aeruginosa*	3	15.0
5	*Salmonella enterica*	3	15.0
6	*Bacillus velezensis*	2	10.0

Table 4.4 show the phenotypic virulence determinants expressed by bacterial isolates obtained from ruminant and non-ruminant animal fecal matter. The table highlights the production of hemolysin, DNase, gelatinase, and lipase enzymes, which are indicators of pathogenic potential and metabolic versatility. Among the isolates, *Escherichia coli*, *Klebsiella pneumoniae* and *Salmonella enterica* demonstrated the highest expression of multiple virulence factors, testing positive for hemolysin, DNase and lipase, indicating strong pathogenic potential. *Salmonella enterica* also exhibited gelatinase activity, further emphasizing its virulence profile. *Pseudomonas aeruginosa* produced hemolysin, gelatinase and lipase, but lacked DNase activity, while *Enterobacter cloacae* was positive only for DNase and lipase. *Bacillus velezensis* showed minimal virulence traits, expressing only DNase and lipase activities.

Table 4.5 shows the results for the antibiotic sensitivity pattern of bacterial isolates obtained from ruminant and non-ruminant animal fecal matter. The data indicate variable responses of the isolates to different antibiotics, revealing patterns of resistance, susceptibility, and intermediate sensitivity. Among the isolates, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* showed the highest sensitivity to most antibiotics tested, with inhibition zones ranging between 12–27 mm, and relatively low Multiple Drug Resistance Index (MDRI) values of 0.2 and 0.3, indicating high susceptibility levels. *Enterobacter cloacae* also demonstrated good susceptibility to several antibiotics, including Cefuroxime (CRX), Azithromycin (AZ), Levofloxacin (LEV) and Erythromycin (E), though it was resistant to Pefloxacin (PEF), Amoxicillin (AM), and Ampiclox (APX) (MDRI = 0.4). In contrast, *Escherichia coli* and *Bacillus velezensis* exhibited higher resistance profiles, each with an MDRI of 0.5, indicating resistance to multiple antibiotics such as Pefloxacin, Ampiclox, and Amoxicillin, though they remained sensitive to Rocephin, Cefuroxime, Levofloxacin, and

Table 4.4: Phenotypic virulence determinants of bacterial isolates from ruminant and non-ruminant animal fecal matter

Isolates	Hemolysin	DNAse	Gelatinase	Lipase
Enterobacter cloacae	0(-)	3(+)	0(-)	3(+)
Escherichia coli	5(+)	5(+)	0(-)	5(+)
Klebsiella pneumoniae	4(+)	4(+)	0(-)	4(+)
Pseudomonas aeruginosa	3(+)	0(-)	3(+)	3(+)
Salmonella enterica	3(+)	3(+)	3(+)	3(+)
Bacillus velezensis	0(-)	2(+)	0(-)	2(+)

Key: - = Negative, + = Positive

Table 4.5: Antibiotics sensitivity pattern of Bacterial isolates from ruminant and non-ruminant animal fecal matter

	Antibiotic zone of inhibition (mm)										
Bacterial isolates	**PEF**	**CN**	**APX**	**Z**	**AM**	**R**	**CPX**	**AZ**	**LEV**	**E**	**MDRI**
Enterobacter cloacae	10 (R)	20 (S)	14 (R)	16 (R)	11 (R)	20 (S)	25 (S)	20 (S)	20(S)	20(S)	0.4
Escherichia coli	10 (R)	25 (S)	13 (R)	16 (R)	14 (R)	22 (S)	26 (S)	10 (R)	25 (S)	24(S)	0.5
Klebsiella pneumoniae	12 (R)	19 (S)	19 (S)	20 (S)	12 (R)	20 (S)	24 (S)	22 (S)	23 (S)	20(S)	0.2
Pseudomonas aeruginosa	12 (R)	20 (S)	19 (S)	27 (S)	24 (R)	16 (I)	23 (S)	20 (S)	19 (R)	19(S)	0.3
Salmonella enterica	20 (S)	20 (S)	18 (S)	21 (S)	22 (R)	20 (S)	25 (S)	20 (S)	21 (S)	18(I)	0.1
Bacillus velezensis	10 (R)	20 (S)	12 (R)	13 (R)	20 (R)	30 (S)	20 (S)	10 (R)	15 (R)	25(S)	0.5

Key

Antibiotic key	Disc code
Pefloxacin 5 µg	PEF
Gentamycin 10µg	CN
Ampiclox 10µg	APX
Zinnacef 30µg	Z
Amoxicillin 5µg	AM
Rocephin 30µg	R
Ciprofloxacin 5µg	CPX
Azithromycin 15µg	AZ
Levofloxacin 5µg	LEV
Erythromycin 15µg	E

S = Susceptible, R = Resistance

Erythromycin. *Salmonella enterica* showed moderate sensitivity with an MDRI of 0.1, suggesting minimal multidrug resistance.

Figure 4.1 shows the percentage (%) seed germination of *Telfairia occidentalis* after 1 week of planting *Telfairia occidentalis*. *Telfairia occidentalis* seed germination in ruminant animal fecal soil was 60%, seed germination for non-ruminant animal fecal soil was 65%, while for mixed animal fecal soil was 70%, compared to the control which was 40% respectively.

The results presented in Table 4.6 show the effect of different animal fecal matter treatments on the number of leaves of *Telfairia occidentalis* over an 8 weeks cultivation period. The data reveal a progressive increase in leaf number across all treatments with time, but with notable differences among the manure types. Plants cultivated with mixed animal manure produced the highest number of leaves at all growth stages, increasing from 10.25 ± 4.21 at week 2 to 33.75 ± 4.48 at week 8. This was followed by the non-ruminant manure, which also supported substantial leaf growth, reaching 29.06 ± 3.91 by week 8. Ruminant manure resulted in moderate leaf development (26.50 ± 7.09 at week 8), while the control (no manure) recorded the lowest leaf count throughout the study period (20.50 ± 5.40 at week 8).

Table 4.7 illustrates the influence of different animal fecal matter treatments on the leaf length of *Telfairia occidentalis* over an 8-week growth period. Across all treatments, leaf length increased progressively with time, but the rate of increase varied depending on the type of manure applied. Plants grown with mixed animal manure exhibited the greatest leaf elongation, with leaf length increasing from 5.38 ± 0.40 cm at week 2 to 25.13 ± 0.68 cm at week 8. This was followed by plants treated with non-ruminant manure, which also showed substantial growth, reaching 20.50 ± 0.49 cm by week 8. The ruminant manure treatment produced moderately long leaves (19.0 ± 1.11 cm at week 8), while the control plants (no manure) recorded the shortest leaves at all growth stages, reaching only 14.0 ± 0.22 cm at week 8.

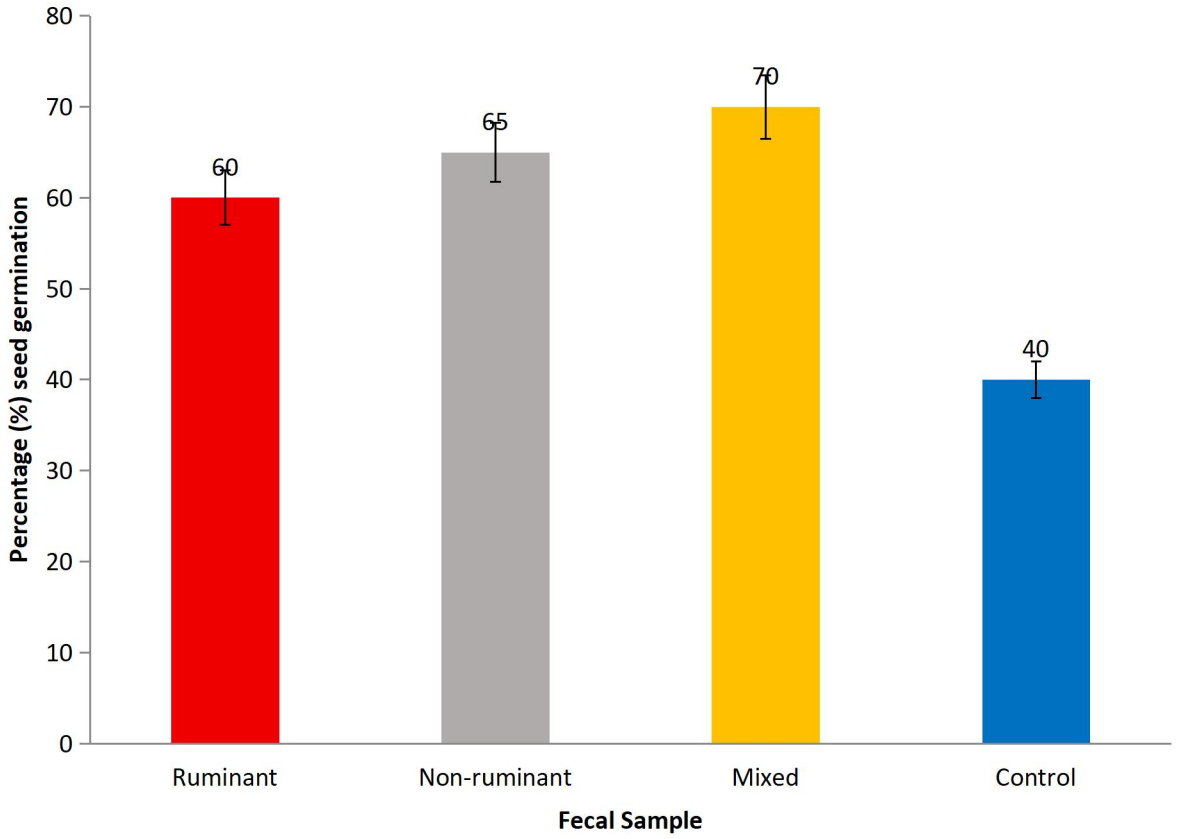

Figure 4.1: Percentage (%) seed germination of *Telfairia occidentalis* after 1 week of planting

Table 4.6: Number of leaves of *Telfairia occidentalis* cultivated with animal fecal matter

	Number of Leaves			
Fecal Sample	**Week 2**	**Week 4**	**Week 6**	**Week 8**
Control	4.13±5.82	10.36±5.66	14.0±5.71	20.50±5.40
Ruminant	5.50±7.16	13.01±7.23	19.5±7.81	26.50±7.09
Non-ruminant	7.25±3.83	15.06±3.52	22.38±3.74	29.06±3.91
Mixed sample	10.25±4.21	19.86±4.30	27.36±4.63	33.75±4.48

Values are mean ± SEM; n=3

Table 4.7: Length of leaf of *Telfairia occidentalis* cultivated with animal fecal matter

	Length of leaf (cm)			
Fecal Sample	**Week 2**	**Week 4**	**Week 6**	**Week 8**
Control	3.88±0.45	5.50±0.56	9.13±0.32	14.0±0.22
Ruminant	4.63±1.06	7.75±1.20	14.25±1.23	19.0±1.11
Non-ruminant	4.15±0.50	8.38±0.68	17.63±0.52	20.50±0.49
Mixed sample	5.38±0.40	10.0±0.59	23.88±0.17	25.13±0.68

Values are mean ± SEM; n=3

Table 4.8 shows the effect of different animal fecal matter treatments on the vine length of *Telfairia occidentalis* over an 8-week cultivation period. The data indicate a steady increase in vine length across all treatments as growth progressed, with notable variations depending on the manure type applied. Plants grown with mixed animal manure exhibited the greatest vine elongation throughout the study, increasing from 34.38 ± 0.40 cm at week 2 to 76.05 ± 0.68 cm at week 8. This was followed by the non-ruminant manure treatment, which resulted in a vine length of 62.48 ± 0.49 cm at week 8, and the ruminant manure, which reached 59.47 ± 1.11 cm at week 8. In contrast, the control plants (without manure) showed the shortest vine lengths, attaining only 55.13 ± 0.22 cm by week 8.

Figure 4.2 shows the leaf area of *Telfairia occidentalis* cultivated with animal fecal matter. At week 2 the average leaf area of *T. occidentalis* was between 4.9 cm^2 to 6.4 cm^2, while at week 8 the average leaf area of *T. occidentalis* increased from 13.4 cm^2 to 20.2 cm^2. The highest leaf area was observed in plants treated with mixed animal manure (6.4 cm^2 – 20.2 cm^2), while the lowest was recorded in ruminant animal manure (4.9 cm^2 – 13.3 cm^2); besides the control, which showed a range of 43 cm^2 – 10.3 cm^2.

Figure 4.3 shows the fresh and dry weight of *Telfairia occidentalis* cultivated with animal fecal matter. The fresh weight of *T. occidentalis* was between 59.4 g to 76.6 g, while the dry weight was from 48.2 g to 57.8 g. The highest fresh and dry weight was observed in plants treated with mixed animal fecal soil (76.6 g and 57.8 g), while the lowest was ecorded in ruminant animal fecal soil (59.4 g and 48.2 g); besides the control which was 55.1g and 41.1 g respectively.

The results shown on Table 4.9 describes the plant growth-promoting (PGP) traits exhibited by bacterial isolates obtained from ruminant and non-ruminant animal fecal matter. Among the

Table 4.8: Length of vine of *Telfairia occidentalis* cultivated with animal fecal matter

	Length of vine (cm)			
Fecal Sample	**Week 2**	**Week 4**	**Week 6**	**Week 8**
Control	23.05±0.45	30.50±0.56	41.13±0.32	55.13±0.22
Ruminant	29.63±1.06	38.75±1.20	48.25±1.23	59.47±1.11
Non-ruminant	28.15±0.50	39.38±0.68	51.63±0.52	62.48±0.49
Mixed sample	34.38±0.40	46.0±0.59	57.88±0.17	76.05±0.68

Values are mean ± SEM; n=3

isolates, *Enterobacter cloacae* and *Pseudomonas aeruginosa* demonstrated positive results for all three PGP traits, indicating strong capabilities for nitrogen fixation, phytohormone (IAA) synthesis, and ammonia production, making them the most promising growth-promoting bacteria. *Escherichia coli* was positive for IAA and ammonia production but lacked nitrogen-fixing ability, while *Klebsiella pneumoniae* exhibited only nitrogen fixation activity. *Salmonella enterica* and *Bacillus velezensis* were positive only for ammonia production.

Table 4.10 shows the outcome of the molecular characterization of bacterial isolates obtained from animal manure samples using 16S rRNA gene sequencing. This molecular approach was used to confirm the identity of the bacterial isolates through comparison with known sequences in the GenBank database. The analysis revealed that the isolates showed high sequence similarity to known bacterial strains. The isolate with accession number CP033084 showed an 88.5% similarity with *Pseudomonas aeruginosa* strain PA3, while the isolate CP101666 exhibited 92.1% similarity to *Escherichia coli* strain NCCP 15734. Similarly, the isolate CP097595 displayed similarity (97.3%) with *Bacillus velezensis* strain UA0297, and the isolate CP174450 showed 96.8% similarity with *Klebsiella pneumoniae* strain BUH3.

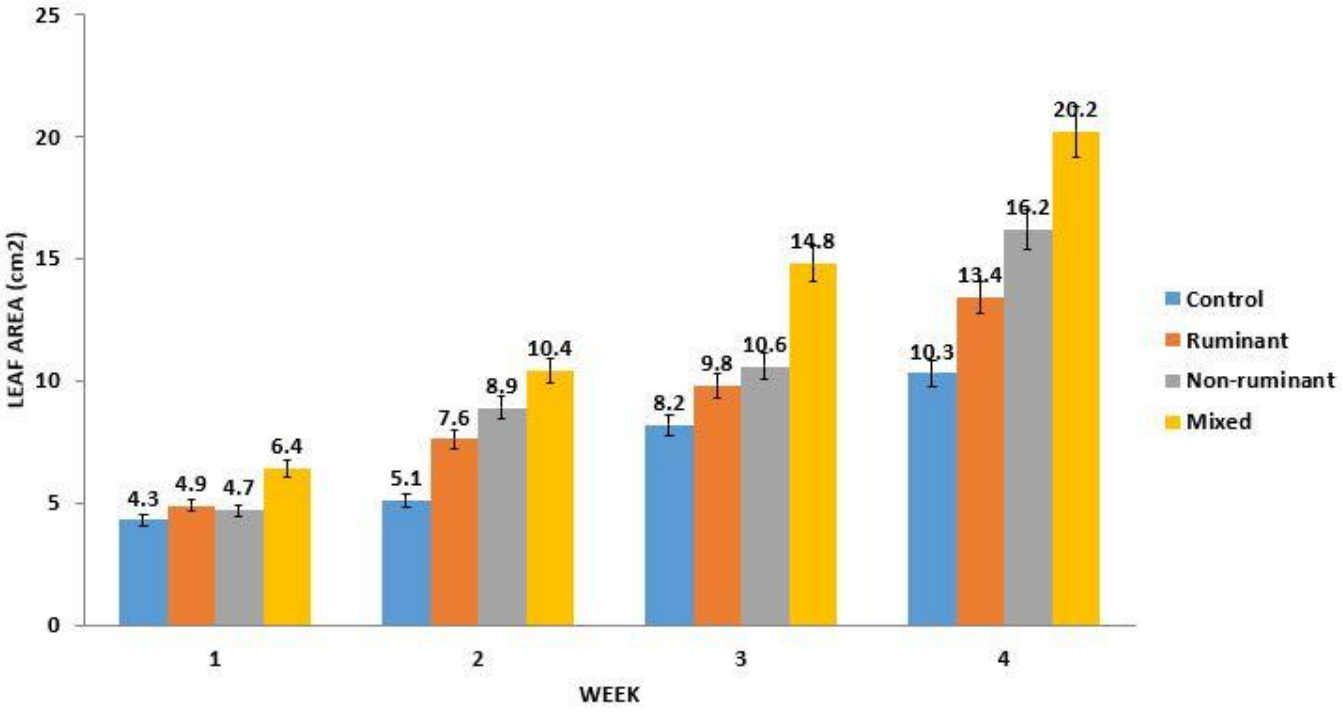

Figure 4.2: Leaf area of *Telfairia occidentalis* cultivated with animal fecal matter

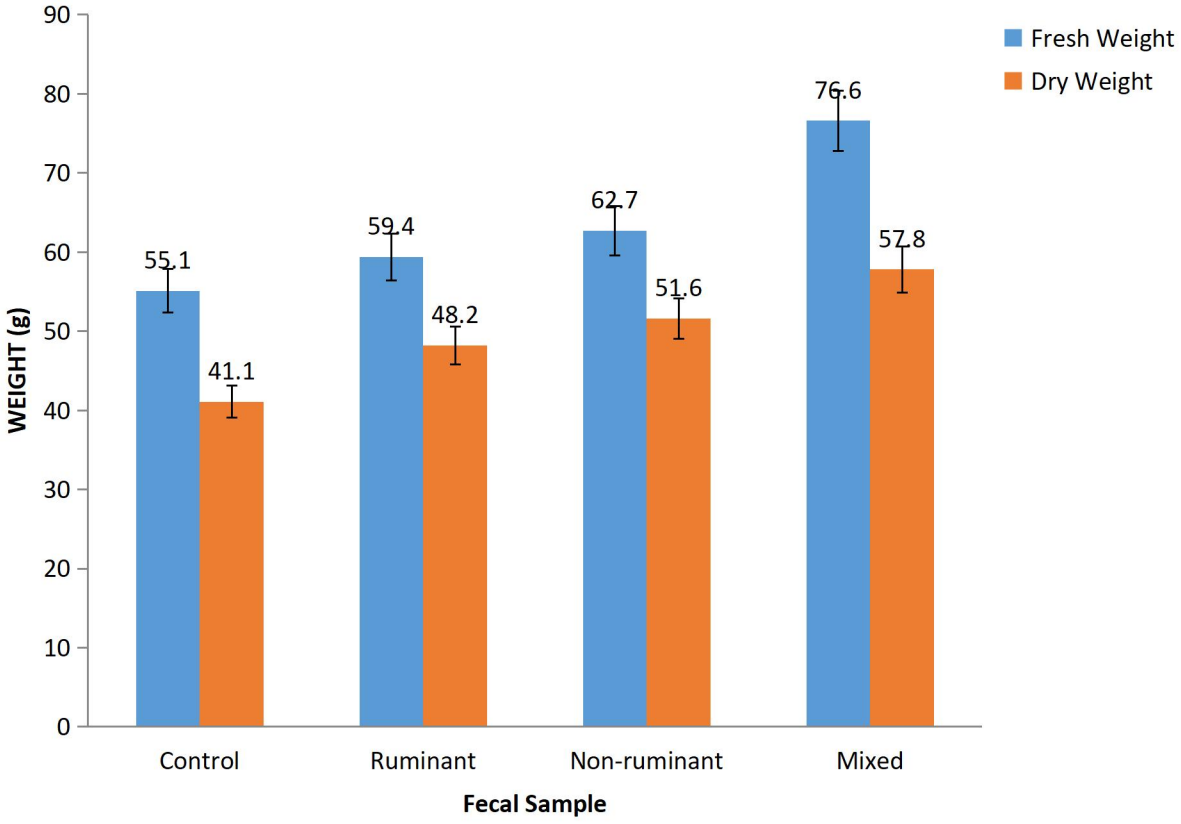

Figure 4.3: Fresh and dry weight of leaves of *Telfairia occidentalis* cultivated after 8 weeks

Table 4.9: Characteristics of bacteria isolates

Isolates	NFA	IAA	AMMONIA	%
Enterobacter cloacae	+	+	+	100
Escherichia coli	-	+	+	66.66
Klebsiella pneumoniae	+	-	-	33.33
Salmonella enterica	-	-	+	33.33
Pseudomonas aeruginosa	+	+	+	100
Bacillus velezensis	-	-	+	33.33

Key: +: Positive, -: Negative, IAA- Indole Acetic Acid, NFM- Nitrogen Fixation Activity

Table 4.10: Molecular characterization of bacterial isolates using 16Sr RNA Sequencing

Sample ID	Scientific name	Max score	Total score	Query cover (%)	E-value	Percentage identity (%)	Accession number
Isolate 1	*Pseudomonas aeruginosa*	1489	5946	99	0.00	97.38	CP034369
Isolate 2	*Escherichia coli*	1618	11237	100	0.00	99.77	AP026463
Isolate 3	*Bacillus velezensis*	1509	1509	100	0.00	97.73	LC881989
Isolate 4	*Klebsiella pneumoniae*	1343	10683	100	0.00	96.68	CP124824

CHAPTER FIVE

5.0 DISCUSSION

The study investigated the efficacy potentials of Plant Growth-Promoting bacteria isolated from both ruminant (cow dung) and non-ruminant (poultry droppings) animal wastes in facilitating the growth of *T. occidentalis,* commonly known as fluted pumpkin. Findings in this study indicated that mixed animal fecal matter exhibited the highest bacterial loads for both heterotrophic and coliform counts, with mean values of $27.80 \pm 2.81 \times 10^5$ CFU/g and $13.69 \pm 1.80 \times 10^4$ CFU/g, respectively. In comparison, the ruminant samples showed moderate counts ($15.40 \pm 2.04 \times 10^5$ CFU/g and $7.65 \pm 1.37 \times 10^4$ CFU/g respectively), while non-ruminant samples recorded the lowest bacterial loads ($13.30 \pm 1.75 \times 10^5$ CFU/g and $6.40 \pm 1.17 \times 10^4$ CFU/g respectively). Statistical analysis confirmed significant differences among the groups ($P < 0.05$).

The markedly higher microbial loads observed in the mixed samples may be attributed to the synergistic combination of microbial communities from different animal sources. Such mixing creates a broader range of nutrients and organic substrates that favor the proliferation of diverse microbial species. Blaustein *et al.* (2015) similarly reported that the co-mingling of animal wastes from different livestock species tends to elevate total heterotrophic and coliform counts due to cumulative microbial inputs and cross-contamination effects during handling. The values obtained in the present study are consistent with those reported in comparable investigations of Manyi-Loh *et al*. (2014) who investigated fresh livestock manure and made findings for total heterotrophic counts to typically range between 10^5 and 10^9 CFU/g, and coliform counts varied from 10^3 to 10^6 CFU/g depending on animal species and management practices.

The intermediate values obtained for ruminant manure may be related to the physiological and dietary characteristics of ruminant animals. Ruminants possess a complex gastrointestinal system

supporting a rich consortium of microorganisms involved in cellulose degradation and fermentation, which may contribute to high bacterial densities in their fecal matter (Manyi-Loh *et al*., 2014). However, the comparatively lower bacterial counts in non-ruminant feces could be associated with differences in digestive processes, diet composition, and manure handling conditions. Chen *et al.* (2014) noted that poultry and swine manure often exhibit variable bacterial loads depending on litter management, moisture content, and exposure duration prior to sampling. Thus, the relatively low counts in non-ruminant manure in this study could result from partial drying or storage before analysis.

Environmental factors such as temperature, aeration, and manure age are also critical determinants of microbial abundance. Studies by Larney *et al.* (2003) and Klein *et al.* (2011) demonstrated that composting and aerobic stabilization significantly reduce heterotrophic and coliform populations due to heat inactivation and microbial succession. From an environmental and public health perspective, the elevated heterotrophic and coliform counts observed in mixed animal manure highlight potential risks associated with its direct use as fertilizer without proper treatment. Manyi-Loh *et al.* (2014) emphasized that untreated manure can serve as a reservoir for pathogenic bacteria, potentially contaminating soil, water, and crops. Consequently, appropriate manure management practices such as composting, anaerobic digestion, or prolonged storage are essential to reduce microbial loads and ensure biosafety before agricultural application (Larney *et al.,* 2003; Klein *et al*., 2011).

The results obtained in this study revealed the present of six bacterial isolates from ruminant and non-ruminant animal wastes including *Enterobacter cloacae*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella enterica* and *Bacillus velezensis*.

The first three isolates (identified as *Enterobacter cloacae*, *Escherichia coli* and *Klebsiella pneumoniae*) are facultative, Gram-negative rods that grew on EMB and MacConkey media and displayed lactose/fermentation profiles characteristic of Enterobacteriaceae (Davin-Regli and Pagès, 2015). Similar case study by Ongeng *et al.* (2011) revealed *E. coli* as an abundant faecal indicator organism in both poultry and ruminant wastes.

P. aeruginosa is not always a dominant manure constituent, culture-based and molecular surveys demonstrate that it can inhabit manure and manure-amended soils and that strains from these matrices may display notable genetic diversity (Youenou *et al*., 2014). *Salmonella enterica* isolates was detected in assayed animal manures in this study. Numerous experimental and field studies such as that of Toth *et al.* (2011) demonstrated the persistence of *Salmonella* spp. in manure and manure-amended soils, and their capacity for prolonged survival under a range of environmental conditions. This has clear implications for biosafety when manure is used as fertiliser (Toth *et al.,* 2011). The detection of *S. enterica* in the present study thus aligns with prior observations that faecal wastes can serve as reservoirs for zoonotic enteric pathogens.

Bacillus velezensis also identified in animal manure samples in this study aligns with well-established ecology of *Bacillus* spp. in organic wastes and composts as reported by Pan *et al*. (2012). The recovery of *B. velezensis* from faecal material is therefore both typical and agronomically relevant, since such organisms may influence subsequent manure stabilization and plant-growth interactions.

The distribution and frequency of bacterial isolates recovered from ruminant and non-ruminant fecal matter in the study revealed *Escherichia coli* was the most frequently isolated bacterium, accounting for 25% of the total isolates. This was followed by *Klebsiella pneumoniae* at 20%, *Enterobacter cloacae*, *Pseudomonas aeruginosa* and *Salmonella enterica* each representing 15%,

while *Bacillus velezensis* was the least frequently recovered at 10%. These findings indicate that coliform bacteria, particularly *E. coli* and *K. pneumoniae*, are predominant in the fecal microbiota of the sampled animals' manures.

The prominence of *E. coli* in ruminant and non-ruminant fecal matter aligns with numerous studies reporting its ubiquity in livestock wastes. For instance, Manyi-Loh *et al*. (2014) highlighted that *E. coli* is frequently detected in dairy cattle and poultry manure, reflecting its abundance in the gastrointestinal tract and its capacity to survive in manure environments. Similarly, Ongeng *et al*. (2011) observed that *E. coli* serves as a reliable indicator for faecal contamination and pathogen load in manure-amended soils under tropical field conditions.

Klebsiella pneumoniae and *Enterobacter cloacae* were also common, comprising 20% and 15% of isolates, respectively. Their presence in livestock manure has been widely documented, as these organisms can persist in faecal environments and contribute to the microbial diversity of animal wastes (Davin-Regli and Pagès, 2015). Their survival in manure is influenced by nutrient availability, moisture, and temperature, factors that are critical in determining the microbiological quality of manure intended for agricultural application.

Pseudomonas aeruginosa and *Salmonella enterica*, each accounting for 15% of isolates, represent opportunistic pathogens of environmental and public health significance. *P. aeruginosa* is known to colonize moist niches in manure and bedding, while *S. enterica* can survive in faecal material for extended periods, posing potential risks if manure is applied to crops without adequate treatment (Youenou *et al.,* 2014; Tóth, 2011). The detection of *Bacillus velezensis*, though less frequent (10%), is noteworthy given its role in organic matter decomposition and its contribution to the early stages of composting, reflecting its functional relevance in manure stabilization (Pan *et al.,* 2012).

Phenotypic screening results for four virulence-associated enzyme activities (hemolysin, DNase, gelatinase, lipase) was assayed for among the six bacterial species isolated from ruminant and non-ruminant animal faecal matter. Findings revealed that *Escherichia coli* was positive for three activities (Hemolysin, DNAse and Lipase), indicating a broad virulence profile in this context. *Klebsiella pneumoniae* exhibited three positive traits (hemolysin, DNase, lipase) with gelatinase negative, which reflects substantial virulence potential. *Salmonella enterica* showed four positive traits (hemolysin, DNase, gelatinase and lipase) underscoring its pathogenic potentials. *Pseudomonas aeruginosa* was positive for hemolysin, gelatinase and lipase but negative for DNase; Enterobacter cloacae displayed DNase and lipase but not hemolysin or gelatinase activity; and *Bacillus velezensis* showed only DNase and lipase activity. These varying profiles indicate that all isolates carry at least some virulence associated enzymatic functions, but the more frequent and multiple positive attributes in *E. coli, K. pneumoniae* and *S. enterica* suggest higher pathogenic potential relative to the others.

The strong performance of E. coli across three assays supports its well known status as a frequent agent of opportunistic infection in agricultural environments. In dairy manure contexts, Lambertini *et al*. (2015) reported detection of virulence factor-rich *E. coli* in cattle manure and farm environments, highlighting the ability of the organisms to persist and express virulence traits in animal waste matrices. The capacity of *E. coli* to produce hemolysin, DNase and lipase is consistent with its ability to damage host tissues or compete effectively in nutrient rich environments such as manure (Lambertini *et al*., 2015). The fact that gelatinase was negative in some isolates may reflect that not all strains carry this enzyme or that expression is environment dependent.

K. pneumoniae showing three positive traits (hemolysin, DNase, lipase) aligns with literature noting that this species can harbour multiple virulence factors including hemolysins and lipases, even when isolated from environmental or food-animal sources (Gündoğan *et al.,* 2010). Although the cited study was in meat rather than manure, the principle that *K. pneumoniae* retains multiple virulence traits in non clinical settings is relevant. The absence of gelatinase in the isolates may simply reflect strain variation; this does not reduce the significance of the other positive traits in assessing risk.

*Salmonella enterica'*s three positive traits complement a broader body of work showing this species employs multiple extracellular enzymes and virulence determinants when persisting in animal wastes, manure or compost. For instance, van Asten *et al*. (2005) documented a range of classical virulence plasmid and toxin based determinants in *Salmonella* spp., underlining their capability for host invasion and survival. The presence of hemolysin, DNase, gelatinase and lipase in the test isolates suggests that these environmental strains may retain pathogenic potential, relevant to manure management and land application practices.

The detection of hemolysin and lipase in *P. aeruginosa* but absence of DNase aligns with phenotypic surveys of environmental *Pseudomonas* isolates, which often display protease/gellatinase/lipase activities associated with nutrient acquisition rather than full complement of virulence genes typical of clinical strains (Fazlul *et al.,* 2019). The finding of DNase negativity indicates either absence of that gene in this strain or lack of expression under the test conditions.

Antibiotic susceptibility profiles of the six test bacterial species isolated from animal faecal matter in this study revealed differential resistance patterns among isolates. *Enterobacter cloacae*

exhibited resistance (R) to Pefloxacin (PEF), Ampiciox (APX), Zinnacef (Z), Amoxicillin (AM) but remained susceptible (S) to Gentamycin (CN), Rocephin (R), Ciprofloxacin (CRX), Azithromycin (AZ), Levofloxacin (LEV), and Erythromycin (E). *Escherichia coli* showed resistance to four antibiotics (PEF, APX, Z, AZ) and susceptibility to six others (CN intermediate/S, AM, CRX, LEV, E). *Klebsiella pneumoniae* had resistance to PEF, CN, AM and susceptibility to the rest; *Pseudomonas aeruginosa* also had mixed resistance (PEF, LEV) and susceptibility to most compounds; *Salmonella enterica* showed intermediate (I) to PEF, resistance to APX, Z, AM, AZ, E, but susceptibility or intermediate to others; *Bacillus velezensis* exhibited resistance to PEF, APX, Z, AZ, LEV but susceptibility to CN, AM, R, E.

The relatively higher MDRI values for *E. coli* and *B. velezensis* at 0.5 suggest that these isolates have been exposed to selective pressure of multiple antibiotic classes, likely due to management practices involving prophylactic or therapeutic use of antibiotics in livestock (Fazlul *et al.,* 2019). This observation resonates with the conceptual model that intensive animal production imposes selection for multi resistant bacteria, which subsequently appear in manure and can reach soils or crops (Fazlul *et al.,* 2019). The lower MDRI for *Salmonella enterica* (0.1) may reflect either fewer exposures to selective agents or a lower propensity for acquiring multiple resistances under the farm conditions studied.

The variation in antibiotic sensitivity between isolates also highlights the importance of species-specific characteristics. For instance, *Pseudomonas aeruginosa* displayed susceptibility to many tested agents, which may reflect intrinsic resistance mechanisms or niche adaptation; yet the resistance to PEF and LEV (fluoroquinolones) suggests that even opportunistic environmental species in manure can harbour clinically relevant resistance. The presence of resistance to fluoroquinolones (such as PEF, LEV) or broad-spectrum agents (e.g., APX, Z) in manure derived

isolates raises concerns about the potential for soil or crop contamination and downstream transmission to humans.

From an agronomic and public health perspective, the existence of such resistance patterns in isolates derived from animal faeces implies that raw manure or inadequately treated manure presents a risk when applied to soils without appropriate stabilisation or treatment. Resistant bacteria can persist or transfer resistance genes to indigenous soil microbiota or to crops (Adekiya and Agbede, 2017).

Findings in this study revealed improved germination, vegetative growth (leaves, leaf area, vine length), and biomass accumulation under manure amendment for cultivated *T. occidentalis*. This aligns with several similar researches showing positive effects of animal manures on vegetable growth performance. Adekiya and Agbede (2017) demonstrated that poultry and cow manures increase leaf area, number of leaves, shoot and root biomass of leafy vegetables and early vegetative crops. Organic manures improve soil chemical (e.g., increased available N, P, K), physical (improved water-holding capacity, aggregation, aeration) and biological (enhanced microbial activity) soil attributes which in turn favour plant growth (Loss *et al.,* 2019). The comparatively superior performance of the mixed-faecal treatment in this study may be attributed to a more balanced nutrient breakdown, improved diversity of decomposer microbial populations, or improved soil physical environment due to diverse manure types (Usman *et al.,* 2015). Some studies suggest that mixed manures can produce synergistic effects on soil fertility and crop growth due to complementary nutrient release patterns (Usman *et al.,* 2015).

Moreover, the consistency of improvement from week 2 to week 8 suggests that manure amendment effects were not short-lived but sustained over a considerable period, allowing the

plant to exploit improved soil conditions and accumulate biomass steadily. This sustained effect aligns with findings that manure has residual effects improving soil fertility over multiple cropping cycles (Bilalis and Karamanos, 2010).

From a practical viewpoint, for cultivation of *T. occidentalis*, the data suggest that applying animal faecal matter (especially mixed source) may significantly improve early establishment (higher germination), shoot development (leaf number, size, vine length) and final vegetative biomass all of which likely translate into enhanced yield of leaves (the edible part) in this leafy vegetable. The results thus support incorporating such organic amendments into soil fertility management systems, especially in resource-limited contexts where synthetic fertilizers are costly or unavailable. However, it is also important to note that manure type, quality, timing, rate of application, decomposition stage and soil conditions will influence actual outcomes. The literature emphasizes that manure characteristics (C:N ratio, nutrient content, degree of composting) and crop-specific responses must be considered (Loss *et al*., 2019). In this study, the greater effect of mixed manure suggests that combining manure types may offer benefit, but further work to optimize rates, timings and combinations would be valuable.

Free-living or associative nitrogen fixers among non-legume-associated bacteria remain an important focus for agricultural bioinoculants (e.g., Rhizobium, Azotobacter) and the finding here indicates that manure-derived isolates may harbour this ability. Zerihun *et al.* (2019) in their study discovered that isolates of *Enterobacter* from rhizospheres have been shown to grow on N-free media and indicate nitrogen-fixation potential. Meanwhile IAA production by isolates (*E. cloacae*, *E. coli*, *P. aeruginosa*) detected in this study aligns with the role of auxin-producing bacteria in stimulating root elongation, lateral branching and enhanced nutrient uptake as shown in multiple PGPR study of Gupta and Kaushal (2017). The capacity to produce ammonia

(observed in all positive cases) may contribute indirectly to plant growth by elevating levels of ammoniacal N in the rhizosphere, thus improving nitrogen availability for plants or microbe–plant interactions.

Relating to the context of animal manure, the study by Dalaq and Zakia (2019) demonstrated that bacterial isolates from bovine manure exhibited multiple PGP traits including IAA production, nitrogen-fixation potential and ammonia production, while also noting that these manures may contain opportunistic pathogens or antibiotic-resistant strains. This supports the idea that manure is not only a nutrient source, but also a potential reservoir of beneficial bacteria. In their study, Dalaq and Zakia (2019) found that twenty strains were positive for both nitrogen fixation and IAA production and that all sorted bacteria except one have potential for plant experiments as biofertilizers. Thus, the results obtained in this study are consistent with the growing body of literature suggesting that manure-associated microbial communities include PGP bacteria with traits useful for enhancing plant growth.

From a practical agronomic perspective, the detection of multiple PGP traits in manure-derived isolates suggests that when animal manures are incorporated into soils, they may serve dual functions as nutrient amendments and as carriers of beneficial microbes which can support plant growth beyond mere nutrient supply. The positive results for *E. cloacae* and *P. aeruginosa*, for instance, imply that these isolates might be exploited (subject to biosafety verification) as bioinoculants in manure-based fertilisation programmes. However, caution is needed as manure microbial communities can also include opportunistic pathogens and antibiotic-resistant bacteria as emphasized in the study of Dalaq and Zakia (2019). Hence, the potential PGP benefits of

manure-associated bacteria must be balanced with appropriate risk management, especially if whole (untreated) manure is applied to soils supporting edible crops.

In this study, the observed percent identities were below the species-level threshold; however, the genera indicated by GenBank matches *Pseudomonas, Escherichia, Bacillus* and *Klebsiella* are all commonly reported as constituents of animal feces, manure, and soils amended with manure. Numerous culture-based and molecular surveys such as that of Youenou *et al.* (2014) have recovered these genera from livestock wastes and have discussed their ecological roles: *Bacillus* spp. in organic matter turnover and composting; *Enterobacteriaceae* (including *Escherichia* and *Klebsiella*) as faecal indicators and potential opportunistic pathogens; *Pseudogmonas* as diverse environmental colonizers of soil and manure. Thus the overall taxonomic pattern observed in animal manure samples in this study is consistent with the published manure microbiology literature.

5.1 Contribution to Knowledge

The study has contributed to knowledge in the following ways:

1. Ruminant and non-ruminant faecal animal wastes harbour Plant Growth-Promoting Bacteria that can enhance plant growth and improve soil fertility.
2. The mixed (combined) ruminant and non-ruminant animal waste had a better plant growth promoting potentials compared to the individual animal waste.
3. The presence of potential pathogens in the ruminant and non-ruminant animal manure, indicated that these wastes could pose a risk to public health if improperly handled or applied to soil.

4. The bacterial isolates exhibited multiple virulence factors such as hemolysin, DNase, gelatinase, and lipase-demonstrating their pathogenic potential and highlighting the importance of monitoring manure for organisms capable of causing disease.

5.2 Recommendations

Based on findings from this study to maximise the benefits of both ruminant and non-ruminant manures while mitigating environmental and public health risks, a set of best practices should be implemented. Pre-treatment of manures, such as composting, anaerobic digestion, or windrow management, is essential to stabilise nutrients, reduce the load of pathogenic microorganisms, and minimise odour.

Education and training of farmers, extension workers, and agricultural stakeholders are also critical. Awareness programs can encourage the adoption of sustainable manure management practices, highlight environmental risks, and improve compliance with regulatory standards.

Ultimately, the adoption of these best practices requires a holistic approach that considers nutrient cycling, pathogen control, environmental protection, and economic viability. By integrating proper treatment, careful application, soil monitoring, and education, agricultural systems can effectively utilise animal manures to support sustainable crop production while minimising negative impacts on the environment and public health.

5.3 Conclusion

This study demonstrated that animal manure particularly the mixed combination of ruminant and non-ruminant wastes serves as a rich source of beneficial and plant growth–promoting bacteria such as *Pseudomonas aeruginosa*, *Bacillus velezensis*, *Escherichia coli* and *Klebsiella pneumoniae*. The mixed manure treatment produced the best germination rate, leaf development, and vine elongation in *Telfairia occidentalis*. Overall, the findings affirm that properly managed animal manure is an effective, sustainable alternative to chemical fertilizers for improving soil health and promoting eco-friendly agriculture.

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APPENDIX

APPENDIX I

Media preparation

Nutrient Agar

Twenty-eight grammes (28g) of nutrient powder was dissolved in 1 litre of distilled water in a conical flask covered with cotton wool and aluminum foil paper. It was mixed thoroughly and sterilized by autoclaving at 121 ºC for 15 min. The medium was cooled to 45 º - 50 ºC and then dispensed aseptically into sterile Petri dishes.

Tryptic soy Agar (TSA)

Thirty grams of TSA powder was dissolved in 1 litre of distilled water in a conical. It was then covered with cotton wool and aluminium foil. It was mixed thoroughly and sterilized by autoclaving at 121 ºC for 15 min. the medium was allowed to cool and dispensed asceptically into sterile Petri dishes.

MacConkey Agar

Fifty grams (52 g) of the medium was weighed into a conical flask and one liter of distilled water was added. After complete dissolution, the medium was autoclaved at 121ºC for 15 miN. The medium was cooled to 45 º - 50 ºC and then dispensed aseptically into sterile Petri dishes.

Mueller-Hinton agar

Thirty grams (38g) of mueller-hinton agar powder was dissolved in 1000ml of distilled water in a conical flask covered with cotton wool and aluminum foil paper. It was mixed thoroughly and sterilized by autoclaving at 121 ^{o}C for 15 min. The medium was cooled to 45 ^{o} - 50 ^{o}C and then dispensed aseptically into sterile petri dishes.

APPENDIX II

SEQUEENCE OF THE 16S rRNA GENE OF EACH BACTERIA ISOLATE

>ISOLATE 1

CAACCCCAGGGCGGTCGACTTATCGCGTTAGCTGCGCCACTAAGATCTCAAGGATCC
CAACGGCTAGTCGACATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTG
CTCCCCACGCTTTCGCACCTCAGTGTCAGTATCAGTCCAGGTGGTCGCCTTCGCCACT
GGTGTTCCTTCCTATATCTACGCATTTCACCGCTACACAGGAAATTCCACCACCCTCT
ACCGTACTCTAAATCAGTAGTATTGGATGCAGTTCCCAGGTTGAGCCCGGGGATTTC
ACATCCAACTTGCTGAACCACCTACGCGCGCTTTACGCCCAGTAATTCCGATTAACG
CTTGCACCCTTCGTATTACCGCGGCTGCTGGCACGAAGTTAGCCGGTGCTTATTCTGT
TGGTAACGTCAAAACAGCAAGGTATTAACTTACTGCCCTTCCTCCCAACTTAAAGTG
CTTTACAATCCGAAGACCTTCTTCACACACGCGGCATGGCTGGATCAGGCTTTCGCC
CATTGTCCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTCAGTTC
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ACCCCACCAACTAGCTAATCCGACCTACGCTCATCTGATAGCGTGAGGTCCGAAGAT
CCCCCACTTTCTCCCCACAAGACATATGCGGTATAACCGCCCGTTTCCGGACGATAT
CCCCCACTACCAGGCAGATTCCTAAGCATTACTCACCCCTCCGCCGCTGATCCACGA
GAAACTCCCTTCATCCGACCCACTTGCCTGAGTTAGGCCAGCCGCCAGCGTTCAATC
TGAGCCATGATCAAACTCTCCG

>ISOLATE 2

CCCCCCAAGCGGTCGACTTAACGCGTTAGCTCCGGAAGCCACGCCTCAAGGGCACA
ACCTCCAAGTCGACATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGC
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ACGAGACTCAAGCTTGCCAGTATCAGATGCAGTTCCCAGGTTGAGCCCGGGGATTTC
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>ISOLATE 3

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GTGAGCCGTTACCTCACCAACTAGCTAATGCGCCGCGGGTCCATCTGTAAGTGGTAG
CCGAAGCCACCTTTTATGTCTGAACCATGCGGGTTCAAACAACCATCCGGTATTAGC
CCCGGGTTCCCGGAGTTATCCCAGCCTTACAGGCCGGTTCCCACGTGTTACTCACCC
GTCCGCGGTAACATCAGGGAGCAAGCTCCCATCTGTCCGCTCGATTTGCATGATTAG
GCAGGCGGCCGCGTTCGTCCTAAGCCAGGAATAAACTCTTAGC

>ISOLATE 4

CAAGGCGGGTCGATTTAACGCGTTAGCTCCGGAAGCCACGCCTCAAGGGCACAACC
TCCAAATCGACATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCC
CCACGCTTTCGCACCTGAGCGTCAGTCTTTGTCCAGGGGGCCGCCTTCGCCACCGGT
ATTCCTCCACATCTCTACGCATTTCACCGCTACACCTGGAATTCTACCCCCCTCTACA
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CCCCTTTGGTCTTGAGACATTATGCGGATAGCCTACCGTTTCCAGTAGTAATCCCCCT
CCATCAGGGAGATCCCCAAACATTAACTTCACCCGTCCGCCGCTCGTCACCCGAAAG
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APPENDIX III

ZR Fungal/Bacterial DNA Extraction Protocol

For optimal performance, add beta-mercaptoethanol (user supplied) to the Fungal/Bacterial DNA Binding Buffer to a final dilution of 0.5% (v/v) i.e., 500ul per 100ml.

(1) Add 50-100mg (wet weight) fungal or bacterial cells that have been resuspended in up to 200ul of water or isotonic buffer (e.g. PBS) or up to 200mg of tissue to a ZR BashingBead™ Lysis Tube. Add 750ul Lysis Solution to the tube.

(2) Secure in a bead beater fitted with a 2ml tube holder assembly (e.g., Disruptor Genie™) and process at maximum speed for 5 minutes.

Processing times may be as little as 40 seconds when using high-speed cell disruptor (e.g., the portable Xpedition™ Sample Processor, page 5, FastPrep®-24, or similar). See manufacturer's literature for operating information.

(3) Centrifuge the ZR BashingBead™ Lysis Tube in a microcentrifuge at 10,000 x g for 1 minute

(4) Transfer up to 400ul supernatant to a Zymo-Spin™ IV Spin Filter (orange top) in a Collection Tube and centrifuge at 7,000rpm (~7,000 xg) for 1 minute.

Snap off the base of the Zymo-Spin IVTM Spin Filter prior to use

(5) Add 1,200ul of Fungal/Bacterial DNA Binding Buffer to the filtrate in the Collection Tube from step 4.

(6) Transfer 800ul of the mixture from Step 5 to a Zymo-Spin™ IIC Column in a Collection Tube and Centrifuge at 10,000 x g for 1 minute

(7) Discard the flow through from the Collection Tube and repeat step 6.

(8) Add 200ul DNA Pre-Wash Buffer to the Zymo-Spin™ IIC Column in a new Collection Tube and centrifuge at 10,000 x g for 1 minute.

(9) Add 500ul Fungal/Bacterial DNA Wash Buffer to the Zymo-Spin™ IIC Column and Centrifuge at 10,000 x g for 1 minute.

(10) Transfer the Zymo-Spin™ IIC Column to a clean 1.5ml microcentrifuge tube and add 100ul (25ul minimum) DNA Elution Buffer directly to the column matrix Centrifuge at 10,000 x g for 30 seconds to elute the DNA.

Ultra-pure DNA is now ready for use in your experiment

PROTOCOL FOR DNA AMPLIFICATION USING THE GENOMIC GUILDED SEQUENCE METHOD

(1) Gently vortex and briefly centrifuge the PCR master mix (2x) after thawing

(2) Place a thin walled PCR tube on ice and add the following components for each 25µl reaction

Quick load One Taq One Step PCR master mix (2x)	12.5µl
Forward primer (10µM)	1.25µl
Reverse primer (10µM)	1.25µl
Template DNA	5µl
Nuclease free water	5µl
Total volume	25µl

(3) Gently vortex the samples and spin down

(4) PCR where perform using the following thermal cycling condition outlined below.

Step	**Temperature** ^{o}C	**Time**	**Number of Cycles**
Initial denaturation	94	3mins	1
Denaturation Annealing Extension	94 TM-5 72	30sec 30sec 1min	35
Final extension	72	7mins	1
Hold	4	∞	

(5) 10µl of the PCR product were analysed on a 1.0% agarose gel electrophoresis, stained with ethidium bromide

PREPARATION OF 1.0% AGAROSE GEL

1) Weigh 1.0g of agarose powder
2) Add 100ml of TBE running buffer
3) Heat for 3 mins in a microwave to dissolve the powder
4) Allow to cool for 56^{o}c
5) Add 2µl of ethidium bromide
6) Cast the gel solution in gel mould in which the gel comb has been appropriately inserted
7) Allow the agarose gel to solidify for 45mins at room temperature

Key

TrisBase

Boric acid

EDTA

AGAROSE GEL ELECTROPHORESIS/DOCUMENTATION

Load 10µl of ready to use DNA ladder (molecular marker) mixed with loading dye in the first well of the solidified gel immersed in TBE buffer in Gel electrophoresis chamber.

If quick load PCR master mix is used, Load 10 µl of PCR product(s) (amplicon) in each wells of agarose gel; If not, 2 µl DNA loading dye is mixed with 8 µl of PCR product(s) (amplicon) and then loaded into each well.

Run at 90 volts for 60 minutes.

View under gel documentation system with UV trans-illuminator

SEQUENCING

Sequencing were carried out using the following Protocol

PCR products are cleanup using ExoSAP Protocol as follows:

1. Prepare the Exo/SAP master mix by adding the following to a 0.6ml micro-centrifuge tube:
    a. Exonuclease I (NEB M0293) 20U/ul 50.0 µl
    b. Shrimp Alkaline Phosphatase (NEB M0371) 1U/ul 200.0 µl
2. Prepare the following reaction mixture:

PCR Mixture	10.0µl
Exo/SAP Mix	2.5µl

3. Mix well and incubate at 37°C for 30 minutes.
4. Stop the reaction by heating the mixture at 95°C for 5 minutes.

Sequencing is then done with the ABI V3.1 Big dye kit according to manufacturer's instructions. http://mvz.berkeley.edu/egl/inserts/Big_Dye_v3.1_Protocol_Manual.pdf

The labelled products were then cleaned with the Zymo Seq clean-up kit using the following protocol

1. Add 240µl of **Sequencing Binding Buffer** to 5-20 µl sequencing reaction.

2. Transfer mixture to a provided **Zymo-Spin™ IB-96 Plate** mounted onto a

Collection Plate

3. Centrifuge at ≥ 3,000 x *g* (5,000 x *g* max.) for 2 minutes.

4. Add 300 µl **Sequencing Wash Buffer** to each well of the plate. Centrifuge at ≥

3,000 x *g* for 5minutes

5. Add 15-20 µl water directly to the column matrix of the filter plate. Place the

Zymo-Spin™ IB-96 Plate on top of the supplied **96-Well PCR Plate** and mount

the assembly onto the **Collection Plate,** then centrifuge at ≥ 3,000 x *g* for 2 minutes to elute the DNA.

Note: A formamide solution (≤ 20%) may also be used to elute the DNA.

Ultra-pure DNA is now ready to be loaded into the sequencer

The cleaned products are injected on the ABI3500XL analyzers with a 50cm array, using POP7.

Analysis of sequences

Sequences data generated were analyzed with NCBI and MEGA 11 software and phylogenetic trees were constructed using neighbor joining.

NCBI GenBank Submission

The sequences were formatted according to GenBank requirements and submitted via the NCBI online submission portal.

APPENDIX IV

MOLECULAR ANALYSIS

Plate 4.1 shows the agarose gel electrophoresis of bacterial isolates amplicated with 1.3% Agarose gel electrophoresis amplified at 950bp. L is 1kb plus ladder (100bp-10,000Kbp) DNA ladder (Molecular marker). Identified isolates from the gel electrophoresis include *Pseudomonas aeruginosa* (isolate 1), *Escherichia coli* (isolate 2), *Bacillus velezensis* (isolate 3) and *Klebsiella pneumoniae* (isolate 4) which were all positive for 16S rRNA gene with bands at 950bp.

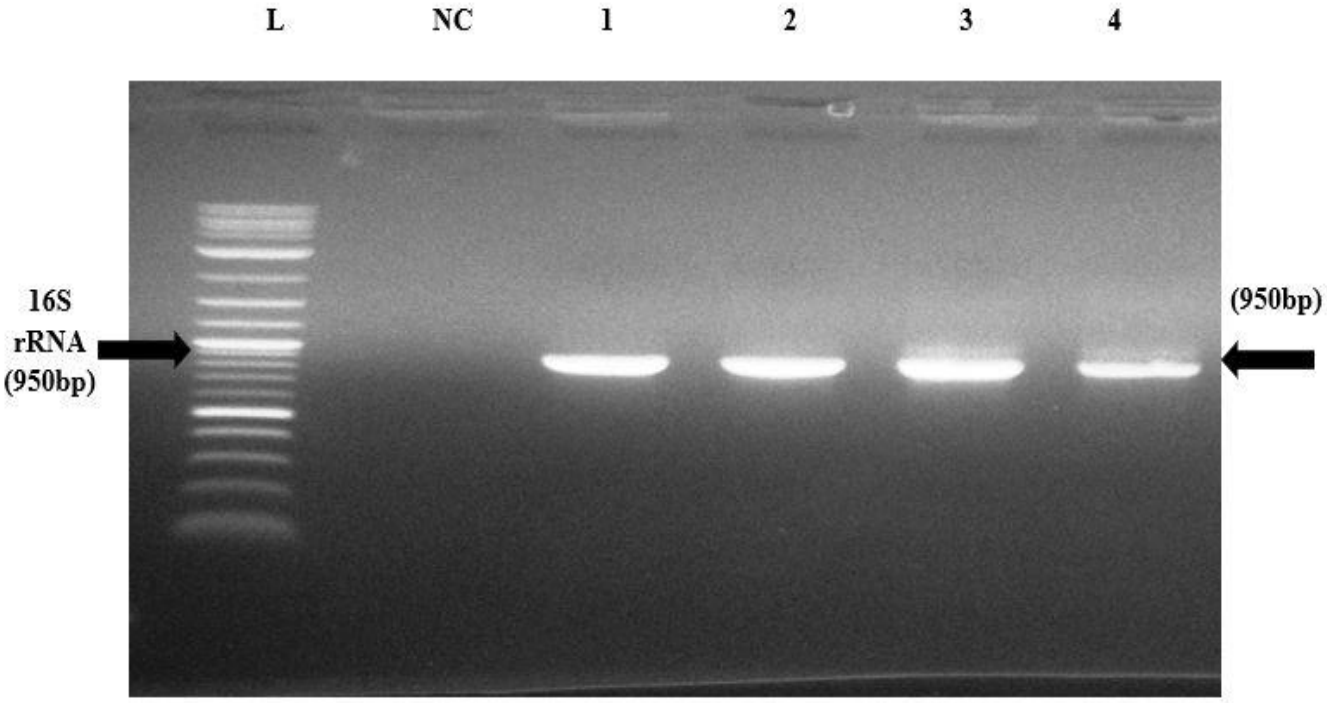

Plate 4.1: Agarose gel electrophoresis of bacterial isolates amplicated with 1.3% Agarose gel electrophoresis amplified at 950bp

Key: L is 1kb plus ladder (100bp-10,000Kbp) DNA ladder (Molecular marker). Samples: Isolate 1 - *Pseudomonas aeruginosa*, Isolate 2 - *Escherichia coli*, Isolate 3 - *Bacillus velezensis* and Isolate 4 - *Klebsiella pneumoniae* are all positive for 16s rRNA gene with bands at 950bp. NC is no DNA template.

APPENDIX V

PHYLOGENETIC ANALYSIS OF BACTERIAL ISOLATE

Figure 4.4 shows the phylogenetic tree of Isolate 1 based on its ITS gene nucleotide sequence. constructed using the Neighbor-joining method in NCBI and MEGA-12 software, the tree shows that Isolate 1 clusters closely with *Pseudomonas aeruginosa* strain B14130 (CP033084). The bootstrap values at the forks indicate the confidence of these groupings, confirming that the isolate is genetically similar to this reference strain.

Figure 4.5 illustrates the phylogenetic placement of Isolate 2. The analysis shows that the isolate shares a close sequence similarity with *Escherichia coli* strain NCCP 15734 (CP097001). The branching pattern and bootstrap support indicate that the isolate is reliably grouped with this reference strain, validating its identification.

Figure 4.6 reveals the phylogenetic tree depicting Isolate 3, which clusters closely with *Bacillus velezensis* strain UA0297 (CP097595). High bootstrap values at the forks demonstrate strong support for this relationship, confirming that Isolate 3 belongs to the *Bacillus* genus and shares significant genetic similarity with the reference strain.

Figure 4.7 shows the phylogenetic analysis of Isolate 4 which is closely related to *Klebsiella pneumoniae* strain BUH3 (CP174450). The tree structure and bootstrap values provide confidence in the classification of Isolate 4, indicating that it shares a high sequence identity with this reference *Klebsiella* strain.

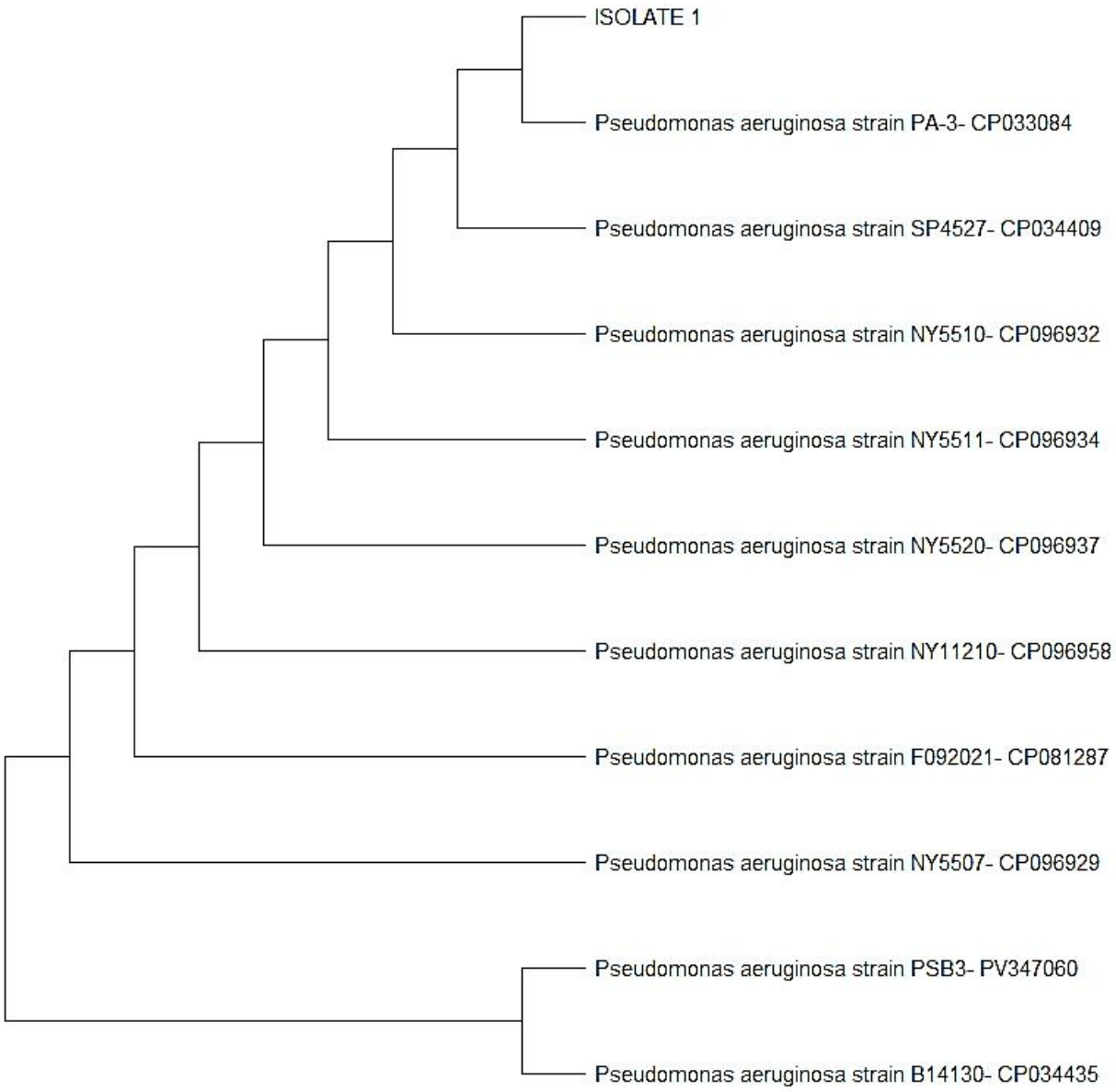

Figure 4.4: Phylogenetic analysis of bacterial isolate 1 based on the nucleotide sequence part of the ITS gene. The phylogenetic tree was constructed by the Neighbor-joining method program in the National Center for Biotechnological information (NCBI) and MEGA-12 software. The number at the forks shows the number of respective groups to the right out of 100 bootstraps samples. Isolate 1 has a similar sequence with *Pseudomonas aeruginosa* strain B14130 with accession number CP033084.

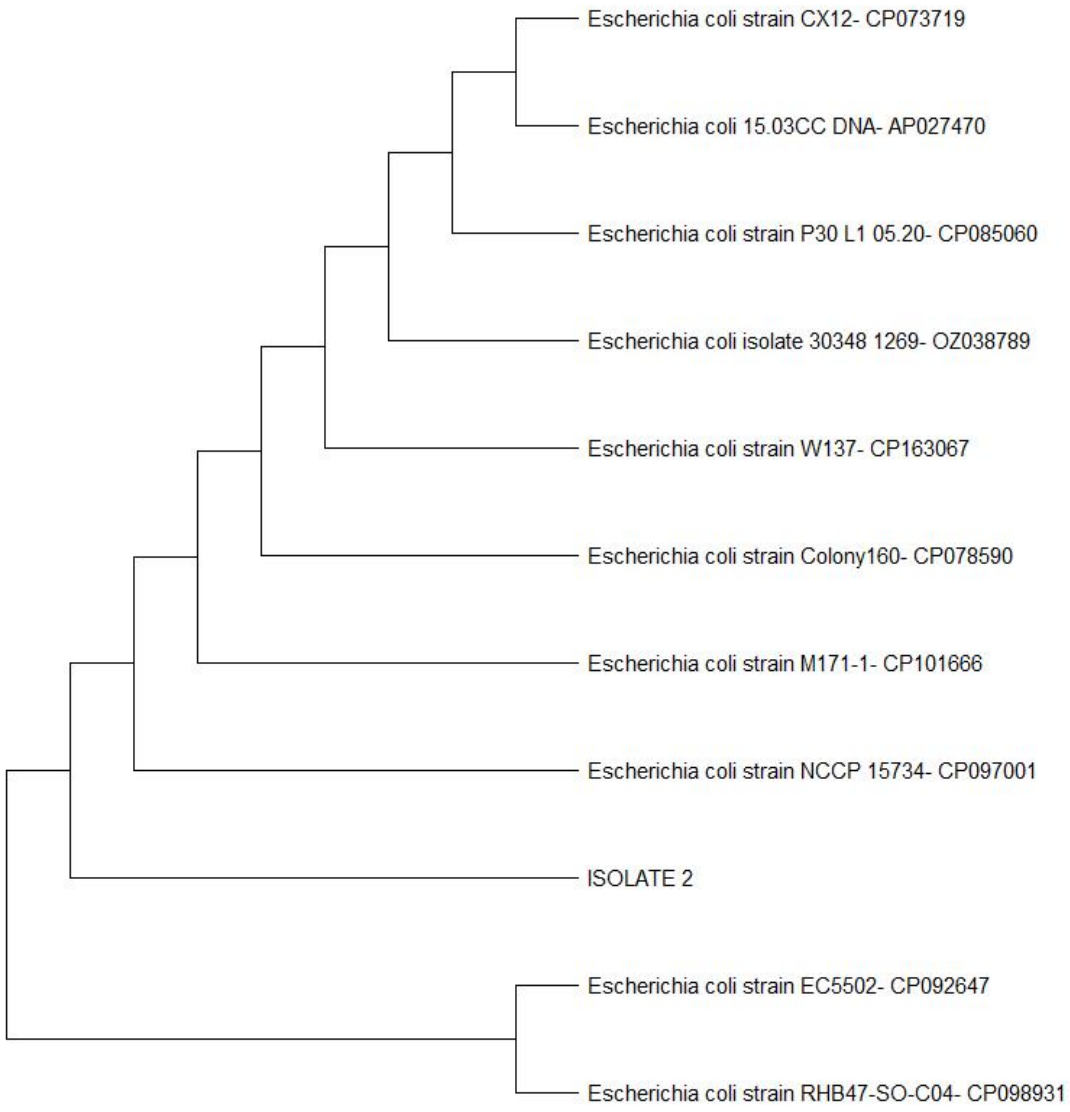

Figure 4.5: Phylogenetic analysis of bacterial isolate 2 based on the nucleotide sequence part of the ITS gene. The phylogenetic tree was constructed by the Neighbor-joining method program in the National Center for Biotechnological information (NCBI) and MEGA-12 software. The number at the forks shows the number of respective groups to the right out of 100 bootstraps samples. Isolate 2 has a similar sequence with *Escherichia coli* strain NCCP 15734 with accession number CP097001

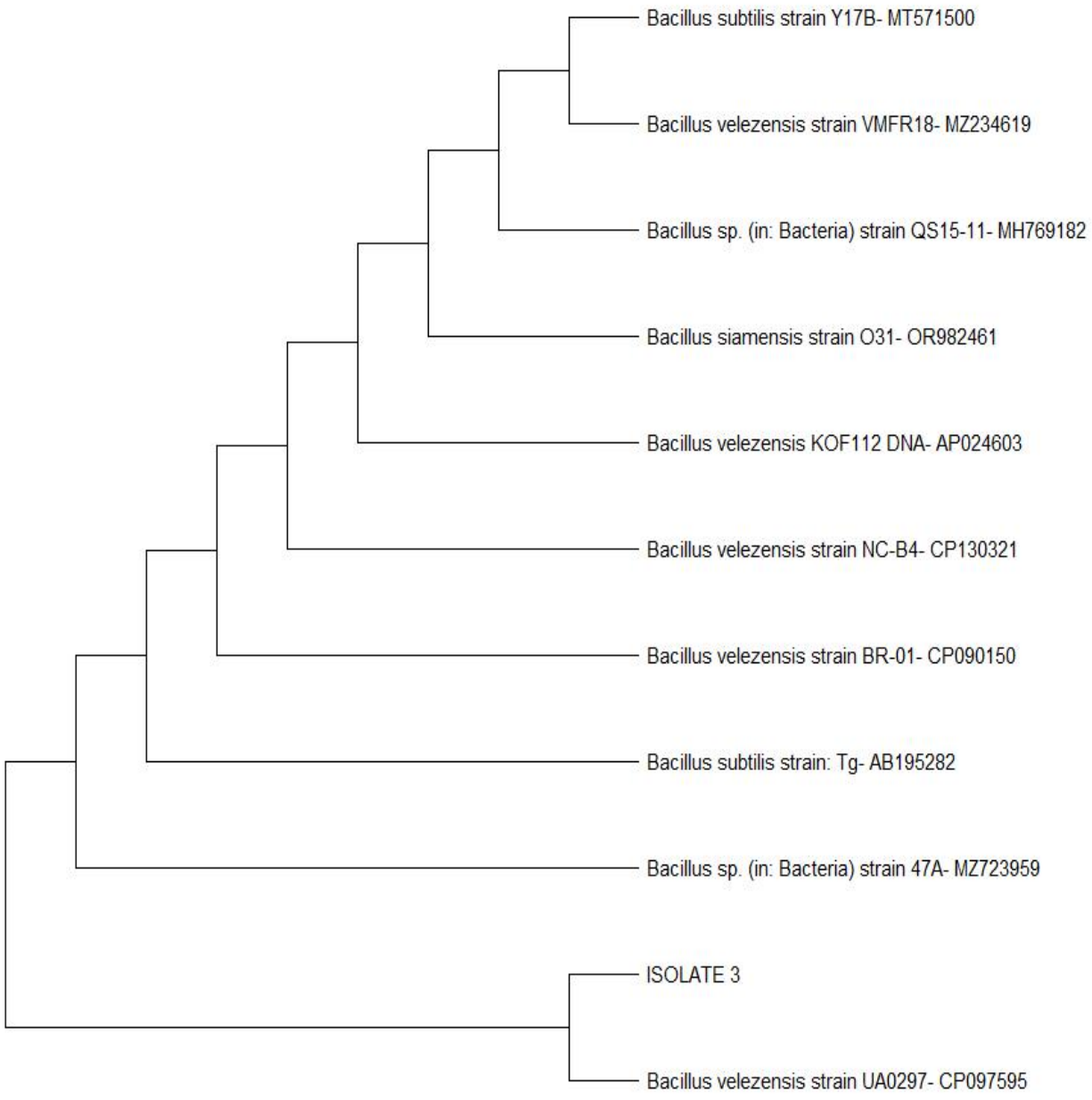

Figure 4.6: Phylogenetic analysis of bacterial isolate 3 based on the nucleotide sequence part of the ITS gene. The phylogenetic tree was constructed by the Neighbor-joining method program in the National Center for Biotechnological information (NCBI) and MEGA-12 software. The number at the forks shows the number of respective groups to the right out of 100 bootstraps samples. Isolate 3 has a similar sequence with *Bacillus velezensis* strain UA0297 with accession number CP097595

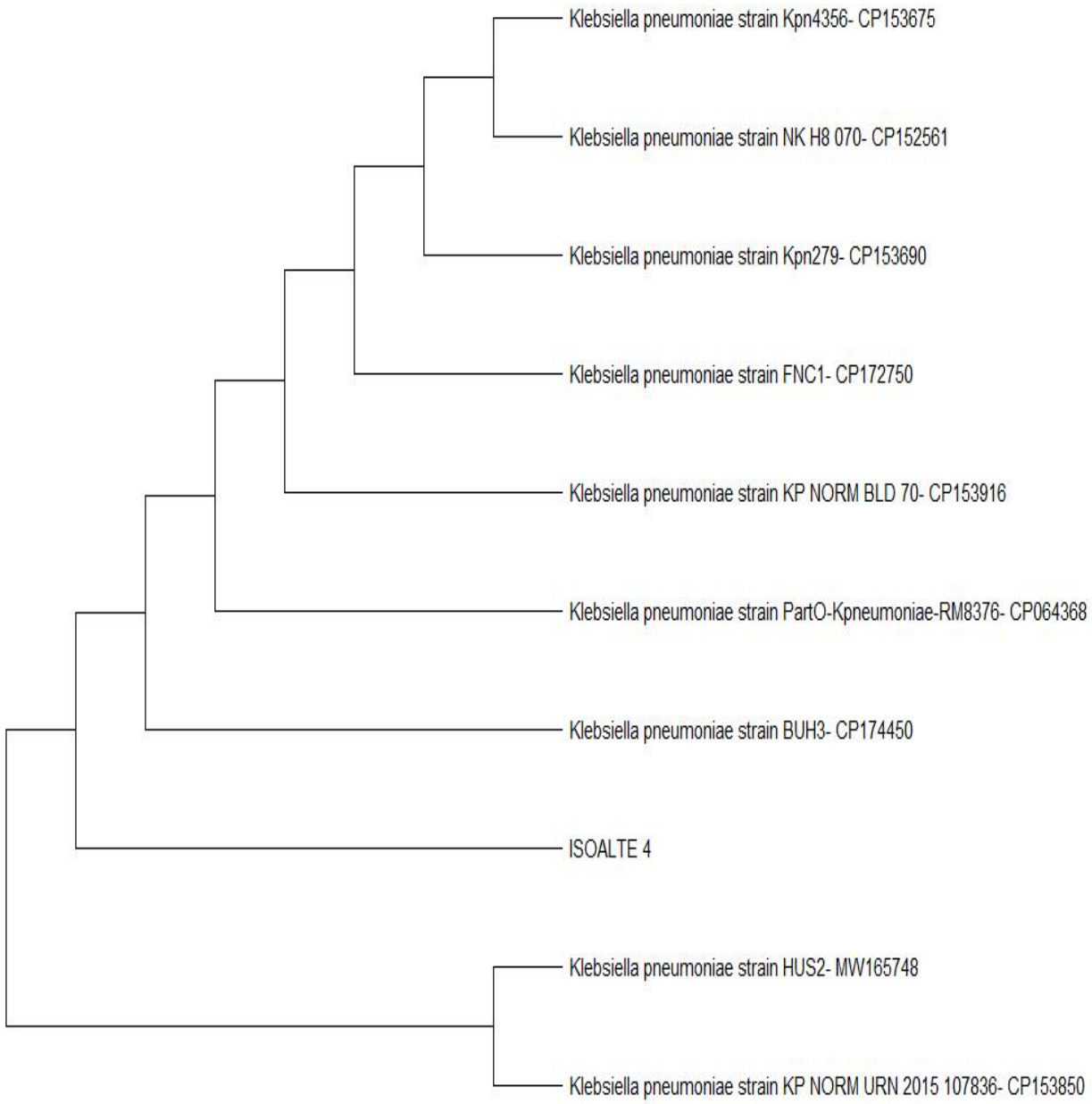

Figure 4.7: Phylogenetic analysis of bacterial isolate 4 based on the nucleotide sequence part of the ITS gene. The phylogenetic tree was constructed by the Neighbor-joining method program in the National Center for Biotechnological information (NCBI) and MEGA-12 software. The number at the forks shows the number of respective groups to the right out of 100 bootstraps samples. Isolate 4 has a similar sequence with *Klebsiella pneumoniae* strain BUH3 with accession number CP174450.