# Screening for bioactive metabolite-producing bacterial endophytes from *Elephantorrhiza elephantina*

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# DECLARATION

I, Benedict Ndou with student number 29292999 hereby declare and confirm that the work done for this dissertation titled ‘‘Screening for bioactive metabolite-producing bacterial endophytes from *Elephantorrhiza elephantina*’’ is my own work, unless where acknowledged. It has not been submitted to any institution for the purpose of obtaining a qualification. All materials used and quoted herein have been duly acknowledged.

# DEDICATION

This dissertation is dedicated to the unwavering support and love of my family, friends and girlfriend, whose encouragement and sacrifices have been the bedrock of my academic journey. To my parents, whose guidance and belief in my abilities have been a constant source of inspiration, I express my deepest gratitude.

I also dedicate this work to Malefu Nokufa Mabona, Sabela Ramafoko, and Dr. Matsobane Tlou, who have shared their knowledge and wisdom, enriching my academic experience. Your camaraderie and encouragement have made this journey both meaningful and enjoyable.

Finally, this dissertation is dedicated to the pursuit of knowledge and the countless individuals, whose contributions to the field have paved the way for my research. May this work contribute, even in a small way, to the collective understanding of the subject.

With heartfelt appreciation,

Benedict Ndou

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## ABSTRACT

**Background:** Medicinal plants are important source of natural compounds which are useful in the treatment of various diseases. The rich and diverse bioactive compounds in medicinal plants play a significant role in the commercial production of drugs. The escalating demand for medicinal plants in both developing and developed nations for primary healthcare, coupled with the exploration of pharmacologically novel drugs, has rendered these botanical species susceptible to extinction due to excessive harvesting and habitat degradation. Bacterial endophytes linked to medicinal plants are considered a viable strategy for generating bioactive compounds similar to those found in plants. This is due to their symbiotic association with the host plant and their possession of medicinal properties analogous to those of the plant hosts. The present study aimed to screen, isolate, identify and characterize metabolite-producing bacterial endophytes from *Elephantorrhiza elephantina*, extract bioactive metabolites, investigate the metabolites' biological activities and identify them using Liquid chromatography/mass spectrometry (LC/MS) analysis.

**Methods:** Screening of bacterial endophytes was done using the 16S rRNA metagenomics approach. Furthermore, bacterial endophytes were isolated from the surface sterilized rhizome of *E. elephantina*. To characterize and identify endophytic bacteria, the Gram staining technique and 16S rRNA gene sequencing were employed. Subsequent growth analysis and metabolite production was done, afterwards the extracts of selected endophytes underwent testing for antimicrobial activities against seven pathogenic strains. Additionally, their antioxidant potential was assessed through quantitative radical scavenging activity using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. Liquid chromatography-quadruple time-of-flight tandem mass spectrometry (LC-QToF-MS) was used to identify bioactive secondary metabolites.

**Results:** The microbial diversity of the leaves and rhizome consisted of phyla: Proteobacteria, Bacteroidota, Gemmatimonadota, Actinobacteriota, Verrucomicrobiota, Dependentiae, Firmicutes, and Armatimonodata. Five bacterial endophytes were isolated from the rhizome and identified using 16S rRNA, which were classified into three genera (*Stenotrophomonas, Microbacterium* and *Pseudomonas*). The crude extract of bacterial endophytes displayed antimicrobial activity against seven pathogenic strains and with the minimum inhibition concentrations ranging from 62.5 - 250 µg/mL. The endophytic bacteria's extract showed DPPH scavenging activity with the inhibition activity ranging from 43 % to 76%. Furthermore, the LC-QToF-MS revealed that the isolates produce secondary metabolites such as, avobenzone, dibutyl Phthalate, 9-Octadecenamide, cis-11-eicosenamide and beauvericin G2, that are known for antimicrobial, antioxidants and anti-inflammatory properties etc.

**Conclusion:** In this study, the 16S rRNA-based metagenome analysis of the bacterial populations in *E. elephantina* revealed that the roots have a higher density of bacteria when compared to leaves and that phylum Proteobacteria is the most dominant in both plant samples. The bacterial isolates correlated with the genera that were detected with the 16S rRNA-based metagenomics. *In vitro* activity assays revealed significant antimicrobial and antioxidant activity, which demonstrated the pharmaceutical potential for the isolates. Metabolites with known antioxidant and antimicrobial properties were identified from all isolates, which was in support of the activity assays. To our knowledge, this represents the initial documentation of bacterial endophytes linked to *E. elephantina*.

**Key words:** *Elephantorrhiza elephantina*, bacterial endophytes, 16S rRNA metagenomics, bioactive secondary metabolites, liquid chromatography-quadruple time-of-flight tandem mass spectrometry.

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| ACC | 1-Aminocyclopropane-1-carboxylic acid |
| BGC | Biosynthetic gene clusters |
| BLAST | Basic Local Alignment Search Tool |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| FDA | Food and Drug Administration |
| GC | Gas chromatography |
| HPLC | High-performance liquid chromatography |
| IAA | Indole-3-acetic acid |
| LC-qTOF-MS | Liquid chromatography quadrupole time-of-flight mass spectrometry |
| LB | Luria-Bertani |
| MIC | Minimum inhibitory concentration |
| NCBI | National Centre for Biotechnology Information |
| NGS | Next generation sequencing |
| NMR | Nuclear magnetic resonance spectrometry |
| NRPS | Non-ribosomal peptide synthetases |
| PBS | Phosphate buffer saline |
| PCR | Polymerase chain reaction |
| PGP | Plant growth promotion |
| PGPB | Plant growth promoting bacteria. |
| PKS | Polyketide synthases |
| RNA | Ribonucleic acid |

# Chapter 1: General Introduction and Literature Review

## 1.1 General Introduction

The term “medicinal plants” is used to describe wild plant species that have been used for centuries in herbal medicine for the treatment of various diseases. Medicinal properties are linked to the production of natural products such as alkaloids, flavonoids, terpenes, steroids, curcumins, saponins etc., that are responsible for the bioactivity recorded from the plant extracts (Alvin *et al*., 2014; Maroyi, 2017). Approximately 47% of all clinical drugs approved by the United States of America’s Food and Drug Administration (FDA) are derived from medicinal plants (Patridge *et al*., 2016). Moreover, as much as 80% of individuals in developing nations rely entirely on herbal remedies for their primary healthcare. Additionally, by the year 2006, more than a quarter of prescribed medications in developed countries were sourced from wild plant species (Hallmann and Berg, 2006; Maroyi, 2013). This, therefore, reflects a high demand for medicinal plants, which often translates to issues of unsustainable over-harvesting that poses a threat to plant conservation (Chen *et al*., 2016).

In general, plants live in association with a variety of microorganisms, collectively referred to as the plant microbiome. Certain microorganisms, such as endophytes, infiltrate and inhabit the internal tissues of the plant, constituting the endospheric microbiome (Dastogeer *et al*., 2020). Some of the endophytes are referred to as plant growth-promoters (PGPs) because they produce phytohormones (Jasim *et al*., 2014), play a role in nitrogen fixation (Greetatorn *et al*., 2020) and increasing resistance to abiotic and biotic stress factors, including invading plant pathogens (Schillaci *et al*., 2019). In recent years, endophytes isolated from medicinal plants have been reported to produce bioactive metabolites similar/related medicinal properties (Alvin *et al*., 2014; Trapp *et al*., 2015; Gouda *et al*., 2016; Chutulo and Chalannavar, 2018). Furthermore, Chatterjee and co-workers (2019) reported that biologically active compounds are frequently not the plant's own metabolic products but instead are produced by endophytes that inhabited both the intra- and inter-cellular tissues of the host plant. This suggests that endophytes can also be regarded as valuable sources of bioactive metabolites. The ability to growth of microbes on relatively inexpensive culture media coupled to the ease of genetic manipulation, suggest that the endophytes can be useful as easily accessible and sustainable producers of natural products in the search for novel therapeutics/drug leads (Gouda *et al*., 2016).

*Elephantorrhiza elephantina* (Burch.) is a medicinal plant that is traditionally used in Southern Africa for treatment of a variety of infections and diseases (Bisi-Johnson et al., 2010; Semenya and Potgieter, 2013; Olaokun *et al*., 2020). Studies have shown that *E. elephantina* produces a variety of phytochemical compounds including esters, fatty acids, phenolic compounds, flavonoids, tannins, and triterpenoids (Mpofu *et al*., 2014b; Maroyi, 2017). Furthermore, this plant is used as a food source and its tuberous rhizome is a popular source of tanning and dyeing materials (Jansen and Cardon, 2005). Due to its popularity, the plant is also sold as an herbal medicine in the informal "muthi" markets of South Africa (Chen *et al*., 2016; Maroyi, 2017; Jamshidi-Kia *et al*., 2018). The overutilization of *E. elephantina* rhizomes in traditional medicine has led to the plant becoming endangered, resulting in its inclusion on the Red Data List (Talukdar, 2002; Olaokun *et al*., 2020). This has prompted studies aimed at investigating the sustainable use of this plant, including the evaluation of its medicinal properties and the potential use of its leaves instead of the rhizome (Olaokun *et al*., 2020). Therefore, this study focused on screening and isolation of bacterial endophytes, as well as exploration of their biological activities and medicinal properties. The goal is to showcase the therapeutic potential of these microbes and underscore their importance as alternative sources for bioactive metabolites.

## 1.2 Elephantorrhiza elephantina

*Elephantorrhiza elephantina* is a well-known medicinal plant which is utilized for ethnomedicinal purposes. This plant belongs to the genus Elephantorrhiza, meaning "elephant root" and referring to the huge underground stem associated with most members of the genus (Radebe *et al*., 2022). In South Africa, *E. elephantina* is widely spread throughout most provinces including Limpopo, North-West, Mpumalanga, and KwaZulu-Natal. It is also found in neighbouring countries including Lesotho, Mozambique, Namibia, Zimbabwe, Botswana and Swaziland (Maroyi, 2017). This plant is known by various names among different indigenous groups and is documented in literature

(Grobler, 2010; Maroyi, 2017; Radebe *et al*., 2022). Some of these names include elephant's root (in English), intolwane (in Zulu and Xhosa), ugweje (in Ndebele), Mupangara (in Shona), and Mositsane (in Tswana and Sotho) (Grobler, 2010).

### 1.2.1 Plant description

*Elephantorrhiza elephantina* is a perennial low shrub that is a member of the *Leguminosae* (*Fabaceae*) family under the *Mimosoideae* subfamily (Table 1.1) (Grobler, 2010; Maroyi, 2017; Radebe *et al*., 2022). It has a short unbranched stem that grows up to 0.9 m above the ground level and grows from a thick woody rhizome (Grobler, 2010; Maroyi, 2017). It produces leaves that are dull-green colored and bipinnate compounded with 2 - 17 opposite pairs of pinnae and golden yellow flowers that bear reddish-brown fruits with dark brown and ellipsoidal-shaped seeds (Grobler, 2010; Olaokun *et al*., 2020). It often grows in large patches in hot and dry grasslands and open bushes.

**Table 1-1:** Taxonomy of *E. elephantina* (Radebe *et al*., 2022).

| **Rank** | **Scientific classification** |
|---|---|
| **Kingdom** | *Plantae* |
| **Phylum** | *Tracheophyla* |
| **Class** | *Magnoliopsida* |
| **Subclass** | *Caesalpinioideae* |
| **Order** | *Fabales* |
| **Family** | *Fabaceae* |
| **Genus** | *Elephantorrhiza* |
| **Species** | *Elephantina* |

### 1.2.2 Plant uses

*Elephantorrhiza elephantina* is a valuable Southern African medicinal plant that is extensively utilized as a source of food and traditional remedy. Its rhizome and leaves have been shown to possess a variety of medicinal properties that are utilized to treat or manage numerous diseases in both humans and animals (Maroyi, 2017). It has traditionally been used to treat syphilis, diarrhea, dysentery, skin diseases including acne, hemorrhoids, perforated peptic ulcers, and emetics (Mpofu *et al*., 2014a; Mpofu *et al*., 2014b; Maroyi, 2017). Small-scale farmers across South Africa have traditionally employed *E. elephantina* rhizome decoction as ethnoveterinary medicine for various animals, including pigs, horses, poultry, goats and sheep (Maroyi, 2017). This practice is utilized to address a range of ailments such as coughing, gastrointestinal parasites, gall sickness, heartwater, mange, pneumonia, helminthiasis, and more (Maphosa and Masika, 2012; Maroyi, 2017). Other than medicinal uses, this plant is most indigenously used for leather tanning, and dyeing, as a source of food, and nutritional supplement (Msimanga *et al*., 2012; Maroyi, 2017).

The medicinal properties of this plant are influenced by the production of a wide range of bioactive compounds with pharmacological and biological activities, including antioxidant, antifungal, antidiabetic, antibacterial, antinociceptive, anti-inflammatory, and antiplasmodial properties (Van Wyk, 2011; Semenya and Potgieter, 2013; Mpofu *et al*., 2014a; Mpofu *et al*., 2014b; Olaokun *et al*., 2020). The high demand for this plant species as herbal medicine, coupled with the primary focus on harvesting the rhizome, has placed *E. elephantina* under the risk of extinction (Olaokun *et al*., 2020). As a result, the idea to screen and isolate endophytic microbes with pharmaceutical properties from the plant emerges as an alternative strategy for preservation and sustainable use. Nevertheless, it is important to note that there is a lack of available information regarding the endospheric microbiome of this plant and the bioactive compounds they produce.

### 1.3 Plant microbiome

Plants host diverse communities of microorganisms including bacteria, fungi, protists, nematodes and viruses (the plant microbiome or microbiota) that colonize every accessible plant tissue including leaves, roots, and stems (Trivedi *et al*., 2020). Plants attract a variety of bacterial communities with which they will engage by producing root exudates (Santoyo *et al*., 2016). The microbes have different associations with plants, which may be symbiotic or pathogenic. The nature of interactions establishes the foundation for plant-microbial interactions which would determine the outcome of the plant's development, nutrient uptake, and adaptive fitness under adverse environmental conditions (Liu *et al*., 2019). These microorganisms may have originated from the environment around the plant i.e., the soil, water, and air (De Tender *et al*., 2017). The microbes include epiphytes that live on the plant surface, rhizospheric microbes, which inhabit the soil close to the roots and endophytes, which reside inside the plant tissues (Vishwakarma *et al*., 2020).

### 1.3.1 Endophytic bacteria

Endophytes colonize the plant host through wounds or openings or by the use of hydrolytic enzymes (Nair and Padmavathy, 2014). They can also be passed on to other plant hosts vertically through seeds and pollens (Arora and Ramawat, 2017). Endophytic bacteria may have originated in the microflora of the rhizosphere and phyllosphere and then entered the roots to reach the xylem tissues (Sturz and Nowak, 2000). Bacterial endophytes are categorized as obligate or facultative endophytes. Obligate endophytes rely exclusively on the plant's metabolism for survival, while facultative endophytes are capable of surviving both within the plant host and in the rhizospheric soil and atmosphere (Hardoim *et al*., 2008; Marella, 2014). Facultative endophytes have been isolated from different parts of the plant including flowers, buds, seeds, stems, rhizomes, and leaves (Christina *et al*., 2013).

The symbiotic interaction between endophytic bacteria and host plant offers several advantages to the plant host. It includes the synthesis of metabolites and phytohormones that increase the plant's capabilities to withstand both abiotic and biotic stressors while promoting overall plant growth (Strobel *et al*., 2004; Berg *et al*., 2005; Dias *et al*., 2008; Jasim *et al*., 2014). As an example, *Pseudomonas putida* JN596120 isolated from *Zingiber officinale* (ginger), was shown to produce Indole-3-acetic acid (IAA), 1-Aminocyclopropane-1-carboxylic acid (ACC), and siderophores, which greatly impact growth of plants (Jasim *et al*., 2014). Moreover, the metabolites generated by endophytic bacteria have been recognized for their potential benefits to humans. As a consequence, endophytic bacteria represent a promising resource for natural products and related activities, including antimicrobial and antioxidant properties, which can be useful in the agricultural and pharmaceutical sectors (Nair and Padmavathy, 2014; Gouda *et al*., 2016; Singh *et al*., 2017a). Illustratively, *P. viridiflava*, isolated in both the external and internal tissues of numerous grass species, demonstrated the synthesis of two previously undiscovered antimicrobial compounds termed ecomycins. These compounds exhibited notable bioactivities against a diverse range of human fungal pathogens (Miller *et al*., 1998).

### 1.3.2 Diversity of endophytic bacteria

The endophytic diversity of a plant is influenced by soil type, plant tissue and plant genotype (Ding and Melcher, 2016; Afzal *et al*., 2019). The same plant with different genotypes growing in different soil types displays differences in the diversity of endophytic microbes (Ding *et al*., 2013; Niu *et al*., 2017). In addition, different tissues of the same plant may contain different groups of bacterial endophytes (Johnston-Monje and Raizada, 2011). An additional crucial element influencing the diversity of endophytes within a plant pertains to the technique employed in isolating microorganisms (Christina *et al*., 2013). The most extensively studied genera of endophytic bacteria include members of *Pseudomonas*, *Bacillus*, *Azosprillum*, *Streptomyces, Methylobacterium*, *Rhizobium,* and *Herbaspirillum* (Chanway, 1996; Taghavi *et al*., 2009; Malfanova *et al*., 2013).

Over 200 genera of bacterial endophytes have been identified and documented in various plant components (Arora and Ramawat, 2017). These genera span across 16 phyla and include both cultivable and previously uncultured bacteria. The phyla include Acidobacteria, Actinobacteria, Bacteroidetes, Cyanobacteria, Dependentiae, Proteobacteria, Firmicutes, Gemmatimonates, and Verrucomicrobia (Wang *et al*., 2019; Arora and Ramawat, 2017). The most abundant endophytic bacteria have been found to represent three major phyla viz., Firmicutes, Actinobacteria, and Proteobacteria (Singh *et al*., 2017a; Vandana *et al*., 2021). Furthermore, these phyla have been identified as significant components of endospheric communities, indicating a broad similarity in endospheric bacterial communities at the phylum level across various host species. The prevalent phyla of endophytic bacteria have been demonstrated to be similarly abundant in various environmental samples, including water, phyllosphere, rhizospheric, and soil samples (Yaish *et al*., 2016; Moronta-Barrios *et al*., 2017; Mhete *et al*., 2020).

## 1.4 General methods for screening, isolation, identification, and characterization of bacterial endophytes

The diversity of endophytic bacteria can be efficiently studied using culture-independent methods such as denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (TRFLP) and metagenomic analysis carried out using cloning and sequencing of the 16S rRNA gene using various universal primers. The culture-dependent methods involve isolation and purification of visible colonies (Ali *et al*., 2021; Enespa and Chandra, 2022). According to Strobel *et al*. (2004), factors such as the environmental settings of the plant, ethnobotanical history, season, and age should be considered when selecting a plant of interest as these all have been shown to influence the biodiversity of bacterial endophytes.

### 1.4.1 Screening for endophytic bacteria using culture-independent methods

Methods that do not rely on cultivating cultures to investigate endophytic diversity typically involve extracting all genomic DNA of bacterial origin from plant tissues. Initially, the plant tissue undergoes surface sterilization to eliminate surface bacteria. The plant tissues, having undergone surface sterilization, are subsequently homogenized for the extraction of bacterial genomic DNA, as outlined by Sessitsch *et al*. (2002). The obtained genomic DNA can be subject to various molecular fingerprinting techniques. Primarily, a marker gene, typically the 16S rRNA gene, is amplified from the genomic DNA to assess bacterial diversity, as illustrated by Garbeva *et al*. (2001). The resultant collection of amplified gene fragments, showing the whole plant bacterial endophyte population, is then scrutinized utilizing community DNA fingerprinting methods such as amplified rDNA restriction analysis (ARDRA), denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), and terminal restriction fragment length polymorphism (T-RFLP) (Garbeva *et al*. 2001), Wang *et al*. (2019), and Anguita-Maeso *et al*. (2020). These techniques have been successfully used to study microbial communities from different environmental samples including marine and plant species (Moeseneder *et al*., 2001; Pei *et al*., 2017; Dergousoff *et al*., 2020). However, these approaches are limited by factors such as low reproducibility; DGGE can only analyse small fragments (up to 500 base pairs); and requirement of laborious and technically demanding methodological procedures, as well as high expenses (Wittwer and Makrigiorgos, 2018; Amiteye, 2021).

The advent of next-generation sequencing (NGS) technologies has transformed microbial community (microbiome) research, allowing for culture-independent high-throughput sequencing methods that are both cost-effective and fast (Glenn, 2011; Laudadio *et al*., 2019). The use of high-throughput sequencing of the 16S rRNA gene has emerged as a crucial technique for identifying constituents of microbial communities within an environmental sample (Mosher *et al*., 2014). The 16S rRNA gene-based metagenome analysis stands out as a powerful NGS system, capable of producing more than 40,000 high-quality reads, with average lengths extending to several thousand base pairs and an average quality exceeding 99% (Mosher *et al*., 2014; Laudadio *et al*., 2019). This approach is a straightforward and cost-effective method to profile the taxonomic composition of a microbial community (Peterson *et al*., 2021). This technology has been successfully used in studying microbial diversity and abundance in various environmental samples including waste water (Makuwa *et al*., 2023), plant samples (Akinsanya *et al*., 2015; Stevens *et al*., 2021) and the human gut (Peterson *et al*., 2021).

Numerous investigations have underscored the limitations of metagenomic approaches, which necessitate extensive sequence coverage and struggle to distinguish closely related bacterial taxa. Furthermore, genomes obtained from *de novo* metagenomic assemblies might be incomplete or could embody populations of chimeric species, in contrast to the precision offered by high-quality reference genomes derived from pure cultures (Laudadio *et al*., 2019; Anguita-Maeso *et al*., 2020). These factors curtail the precision of high-resolution taxonomic classification and functional analysis when employing metagenome-derived genomes. Furthermore, metagenomics techniques tend to miss out on many species due to factors such as various DNA extraction methods, PCR primers and conditions used to amplify the target, data analysis pipeline and database (Laudadio *et al*., 2019, Hinsu *et al*., 2021). The amplification process employed to amplify the rRNA gene has the potential to introduce sequence artifacts, leading to subsequent biases in the quantification of taxa within the resulting taxonomic profiles (Acinas *et al*., 2005; Tremblay *et al*., 2015). Furthermore, the choice of primers binding to the 16S rRNA gene during amplification or the existence of introns has been demonstrated to significantly impact the characterization of microbiome communities (Peterson *et al*., 2021). Some of these missed microorganisms tend to grow well on appropriate media, implying that metagenomics can also miss out commonly present organisms (Hinsu *et al*., 2021). Therefore, a combination of culture-based and culture-independent methods is useful in bacterial population studies (Reiter and Sessitsch, 2006; Ryan *et al*., 2008; Manias *et al*., 2020).

### 1.4.2 Isolation of endophytic bacteria using culture-dependent methods

Numerous studies have focused on endophytic bacteria, primarily employing culture-based techniques for isolation (Pei *et al*., 2017; Wang *et al*., 2019; Cun *et al*., 2022). The diversity of cultivable endophytic bacteria is contingent upon the isolation methods and growth parameters applied (Christina *et al*., 2013). The method used for isolation needs to be both sensitive to retrieve the majority of cultivable bacterial endophytes and robust enough to eliminate contaminants and epiphytes from the plant tissue surface (Zhang *et al*., 2017; Nasrollahi *et al*., 2020). The most widely used protocol for endophytic bacteria isolation entails surface sterilization of plant tissues, followed by maceration, serial dilution, and plating on culture media (Zhang *et al*., 2017; Ali *et al*., 2021). Sterilizing agents such as hydrogen peroxide, sodium hypochlorite, and ethanol are commonly utilized in sequence to enhance sterilization effectiveness (Lodewyckx *et al*., 2002). The treated tissue undergoes multiple rinses with sterile distilled water to remove any residual chemicals. The success of the sterilization process is validated by culturing a small portion of the last rinse's distilled water on culture media, with the absence of bacteria confirming successful sterilization. Furthermore, substantial growth of endophytic bacteria on agar media following surface sterilization indicates minimal impact on the endophytic population by the sterilization method (Eevers *et al*., 2015).

The choice of growth media impacts both the abundance and variety of endophytes that can be obtained from a particular plant tissue, as no single medium can fulfill the nutritional and growth needs of all bacteria (Reiter and Sessitsch, 2006; Eevers *et al*., 2015). The inability to culture bacteria is not solely attributed to growth media, as some bacteria can enter a viable but nonculturable (VNC) state, rendering them incapable of dividing (Sessitsch *et al*., 2002). Moreover, even when endophytic bacteria have been successfully isolated, maintaining them on growth media can sometimes prove to be difficult (Eevers *et al*., 2015; Trivedi *et al*., 2011). However, it is advisable to employ a broader range of growth media for the isolation of endophytic bacteria, including those that are rich in both macro- and micronutrients, as well as custom-formulated media (Stevens *et al*., 2021). This includes growth media with low concentrations of mineral salts (Janssen *et al*., 2002; Davis *et al*., 2005), the supplementing of media with plant (host) extracts (Eevers *et al*., 2015; Martinez-Klimova *et al*., 2017), and the use of multiple solid media or a medium known to support a wide range of microorganisms (Pan *et al*., 2019; Anguita-Maeso *et al*., 2020).

### 1.4.3 Characterization and identification of endophytic bacteria

The isolated endophytes are characterized and identified using morphological, physiological, biochemical and molecular approaches. Staining and microscopy techniques such as Gram staining, endospore staining and Zeihl-Neelsen staining are generally regarded as the initial step in the identification of bacterial isolates (Beveridge *et al*., 2007; Manias *et al*., 2020). This step is followed by simple biochemical test including catalase testing, oxidase testing, and substrate utilization tests (Manias *et al*., 2020). Furthermore, a number of molecular markers have been used to identify microbial taxa and their phylogenetic classification (Ma *et al*., 2016). Among various molecular markers, the 16S rRNA is widely employed for bacterial identification and establishing their phylogenetic relationships (Srinivasan *et al*., 2015). Furthermore, the 16S rRNA has proven to be a valuable tool for generating phylogenetic information at the genus level (Clarridge, 2004).

Historically, the assessment of bacterial communities and diversity relied on culturing microbes from the environment. Nevertheless, insights from culture-dependent studies have been constrained in understanding bacterial community structure and diversity. This limitation stems primarily from the fact that a significant proportion of bacteria cannot be cultivated in laboratory conditions, primarily due to a lack of understanding of their specific growth requirements (Martinez-Klimova *et al*., 2017). Furthermore, it is worth noting that less than 1% of bacteria can be successfully cultured, which hampers our understanding of the full diversity and comprehensive roles of endophytic bacteria (Vartoukian *et al*., 2010). As a result, a large portion of the microbial population remain unexplored. Advancement in culture-independent techniques such as high-throughput sequence analysis of the 16S rRNA genes from environmental samples, has increased our knowledge on the composition, richness, and microbial diversity of uncultured microbes across different environmental samples (Qaisrani *et al*., 2019). These studies have facilitated microbiologist to make more focused attempts to isolate and culture previously uncultured microbes. Hence, integrating both culture based and culture-independent approaches enhances the probability of comprehensively studying the structure and function of a plant's endophytic bacterial community (Sessitsch *et al*., 2004; Hallmann and Berg, 2006).

## 1.5 Bacterial growth and Secondary metabolite production

Secondary metabolites, also known as specialized metabolites or natural products, are organic compounds of low molecular mass synthesized by plants, bacteria, or fungi of certain taxonomic groups (Singh *et al*., 2017a). While these metabolites are not essential for the survival of bacteria, they significantly contribute to enhancing their interactions with the environment, making them more effective in various ecological contexts (Brader *et al*., 2014). Bacterial endophytes are known to produce a variety of secondary metabolites, including terpenoids, alkaloids, polyketides, nonribosomal peptides (NRPs), phenols, enzymes, and phytohormones. These compounds play a crucial role in facilitating plant-bacteria interactions and colonization (Rutledge and Challis, 2015; Gouda *et al*., 2016; Singh *et al*., 2017a).

Secondary metabolites derived from bacterial endophytes exhibit diverse biological activities such as antimicrobial, anti-carcinogenic, antioxidant, anticancer, anti-inflammatory, and immunosuppressive properties (Christina *et al*., 2013; Trapp *et al*., 2015; Gouda *et al*., 2016; Martinez-Klimova *et al*., 2017; Singh *et al*., 2017a; Photolo *et al*., 2020). As a result, these metabolites have potential applications in pharmaceuticals and agricultural industries (Alvin *et al*., 2014; Singh and Sharma, 2020). Bacterial endophytes from medicinal plants have been reported to produce bioactive compounds similar to those found in their host plants, presenting a sustainable and promising source of bioactive molecules (Alvin *et al*., 2014; Trapp *et al*., 2015; Gouda *et al*., 2016; Martinez-Klimova *et al*., 2017; Singh *et al*., 2017a). For instance, *Bacillus spp*. isolated from *Alternanthera brasiliana* were found to produce identical bioactive compounds as their host plant. The synthesis of these bioactive compounds in endophytes may be linked to the evolution of host-microorganism interactions, possibly involving the transfer of genetic information from higher plants to endophytes through horizontal gene transfer or vice-versa (Slot and Rokas, 2011).

### 1.5.1 Biosynthetic gene clusters

Important bioactive secondary metabolites including antibacterials such as vancomycin and erythromycin, antifungal agents, e.g., nystatin and amphotericin B and anticancer agents such as bleomycin and doxorubicin, have been isolated from bacteria (Figure 1.1 A) (Sekurova *et al*., 2019). These bioactive secondary metabolites are biosynthesized by multi-modular enzymes, such as polyketide synthases (PKS) terpene synthases, NRPS-independent siderophore synthetases, and nonribosomal peptide synthetases (NRPS) (Nikolouli and Mossialos, 2012; Hwang *et al*., 2020). The genes encoding biosynthetic machinery for secondary metabolites are organized in biosynthetic gene clusters (BGCs) (Figure 1.1 B), analyses of which can provide insight regarding the chemical classes of the compounds they specify (Martinet *et al*., 2019). Furthermore, more than 11000 BGCs have been identified from 9000 bacterial genomes. However, a majority of the gene clusters are yet to be linked to the corresponding metabolites and factors that influence the activities of the BCGs remain largely unknown.

Therefore, there is a need to further study the produced secondary metabolites in bacteria. Optimal growth and biochemical reactions of microbes are contingent upon suitable cultivation conditions encompassing factors such as temperature, pH, and oxygen concentration. However, a considerable number of biosynthetic genes remain inactive under standard culture conditions. Consequently, it becomes imperative to modify the cultivation conditions to induce the activation of these dormant BGCs (Pan et *al*., 2019). Several approaches such as variation in growth conditions, engineering the transcription and translation machinery, manipulating global regulators, and reporter-guided mutant selection, have been used to activate these silent BGCs (McKenzie *et al*., 2010; Onaka *et al*., 2011; Gomez-Escribano *et al*., 2012; Guo *et al*., 2015). As an example, when Streptomyces sp. is cultured at a temperature below 30°C, the secondary metabolites produced consist of chlortetracycline. However, when the culture temperature is increased to 35°C, only tetracycline is synthesized (Cui *et al*., 1996). Moreover, secondary metabolites are typically produced in response to stress conditions, and it is commonly observed that the accumulation of these secondary metabolites occurs during the later stages of the stationary phase or fermentation process (Nigam and Pandey, 2009; Jaishankar and Srivastava, 2017).

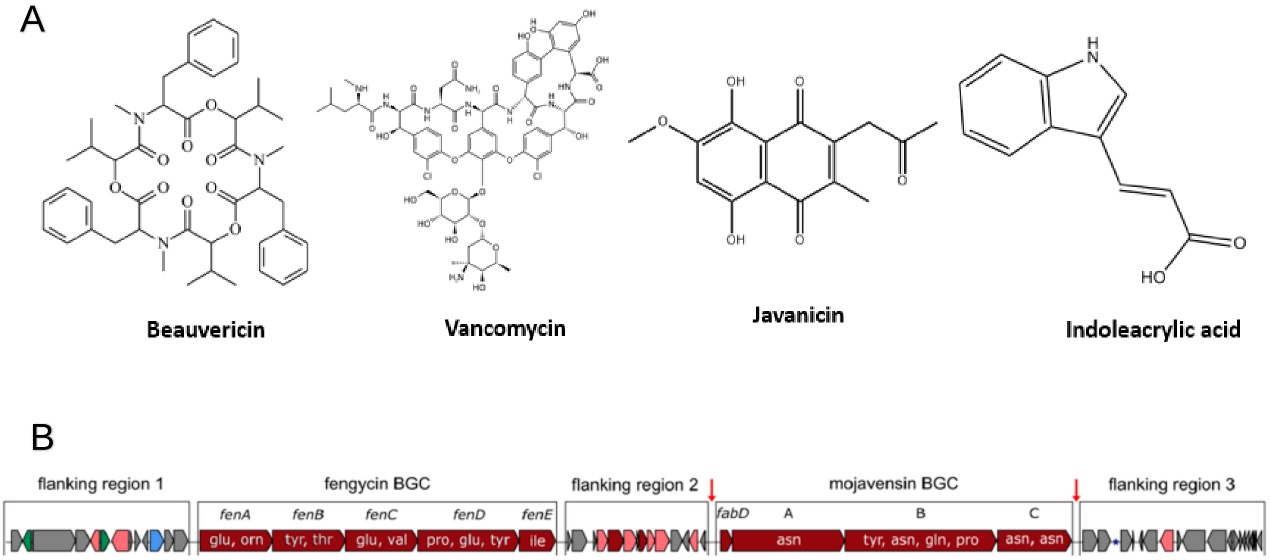


Figure 1-1: (A) Selected bioactive metabolites with medicinal importance isolated from endophytes (Rutledge and Challis, 2015; Gouda *et al*., 2016). (B) Schematic representation of fengycin and mojavensin BGCs and their microsynteny in *Bacillus halotolerans* Hil4 (Thomloudi *et al*., 2021).

### 1.5.2 Factors that influence secondary metabolite production

The production of secondary metabolites in bacteria is greatly affected by various fermentation parameters such as pH, aeration, temperature, precursors and culture media (carbon and nitrogen source,) which often vary from organism to organism (Pan *et al*., 2019). The carbon source serves a dual role by serving as the foundation for biomass construction and as the energy source for all heterotrophs. Additionally, it contributes carbon units essential for the formation of secondary metabolites. Similarly, the nitrogen source is indispensable for the synthesis of crucial proteins and nucleic acids, providing N-containing units necessary for the production of secondary metabolites (Singh *et al*., 2017b; Pan *et al*., 2019). The choice of carbon and nitrogen sources in the culture medium is known to exert a significant influence on microbial secondary metabolism (Ma *et al*., 2009). Consequently, microorganisms cultured in media containing different components may display distinctively adapted metabolic patterns and express specific sets of biosynthetic gene clusters. This, in turn, results in differential biosynthesis of specialized metabolites (Song *et al*., 2020). Additionally, when microorganisms are exposed to diverse types of media supplemented with various halogens, they may activate synthesis pathways aimed at restoring osmotic balance. This activation can lead to the expression of previously dormant or hidden biosynthetic gene clusters (BGCs) (Wang *et al*., 2011).

The detection and characterization of these secondary metabolites can be accomplished through various techniques, including nuclear magnetic resonance (NMR) spectroscopy, gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS), capillary electrophoresis (CE), mass spectrometry (MS), and other methods (Akter *et al*., 2022). LC-MS has gained recognition as a dependable method frequently employed in metabolomics studies due to its high sensitivity and its capacity to identify a broad spectrum of secondary metabolites (Madala *et al*., 2013). LC-MS combines the chromatographic capabilities of HPLC with the precision of an MS detector to identify multiple metabolites in a single analysis (Das *et al*., 2014).

## 1.6 Bio prospects of endophytic bacteria

Endophytes are occasionally credited for imparting medicinal properties to their hosts (Rahman *et al*., 2017). There is evidence suggesting that endophytes play a pivotal role in the synthesis of diverse metabolites. Consequently, we propose that endophytes associated with *E. elephantina* hold significant importance in this context. Endophytes linked with ethnomedicinal plants present a promising reservoir of natural products applicable in addressing oxidative stress and serving as novel antimicrobial agents (Nongkhlaw and Joshi, 2015). For instance, *Methylobacterium radiotolerans* MAMP 4754, isolated from the medicinal plant *Combretum erythrophyllum*, produced antioxidant and antimicrobial agents like 2,4-dinitrophenyl acetate, recognized for their antimicrobial activity against various pathogens, including *Bacillus spp*., *Pseudomonas aeruginosa*, and *Enterococcus faecalis* (Photolo *et al*., 2020). The emergence of novel antimicrobial metabolites from endophytes is increasingly considered an alternative strategy to counter the escalating levels of drug resistance. Additionally, bacterial endophytes may hold an advantage over other microorganisms due to their ability to defend, communicate with, and colonize their plant hosts. This results in the production of a wide array of structurally diverse secondary metabolites compared to epiphytes or soil microbes (Strobel, 2003).

The extensive species diversity of endophytes, coupled with their adaptability to diverse environments, represents a valuable and largely untapped reservoir of novel secondary metabolites with potential applications in pharmaceutical or agricultural contexts (Bacon and White, 2000). Moreover, given their symbiotic association with plants, bioactive compounds derived from endophytes are anticipated to exhibit reduced toxicity to humans. This characteristic holds significant implications for the medical field, as potential antimicrobial and antioxidant agents isolated from endophytes may exert their effects without adverse impacts on human cells (Zin *et al*., 2017). Recently, novel antibiotics have been identified within endophytes inhabiting diverse plant species (Martinez-Klimova *et al*., 2017). Illustratively, *Bacillus subtilis* is known to synthesize polyketide antibiotics, namely bacillomycin, fengycin, iturin, lichensyn, mycosubtilin, plipastin, pumilacidin, and surfactin (Stein, 2005).

## 1.7 Hypothesis

*E. elephantina* harbours diverse bioactive metabolite-producing endophytic bacteria. These bacterial endophytes produce chemically distinct and bioactive natural products with antioxidant and antimicrobial activity.

## 1.8 The aim of this study

The aim of this study was to screen, isolate, identify and characterize metabolite-producing bacterial endophytes

Specific objectives were then set as:

- to screen for bacterial endophytes using 16S rRNA-based metagenomics and isolate bacterial endophytes from *E. elephantina,*
- to characterize and identify the isolates using gram staining, 16S rRNA sequencing, and phylogenetic analysis,
- to investigate the bacterial growth and secondary metabolite production of endophytic isolates,
- to investigate the bioactivity of the secondary metabolites using *in vitro* activity assays, and
- to identify secondary metabolites from the bacterial endophytes using LC/MS.

# *Chapter 2:* Screening, isolation, and phylogenetic characterization endophytic bacteria from *Elephantorrhiza elephantina*

## 2.1 Introduction

The plant microbiome consists of a multitude of beneficial, commensal, and pathogenic microorganisms, each playing crucial roles in the health and growth of plants (Raaijmakers and Mazzola, 2016; Lemanceau *et al*., 2017). The term "plant microbiome" encompasses epiphytic, phyllospheric, rhizospheric, and endophytic microbes collectively. The composition of the microbiome is shaped by intricate interactions involving the hosts and environmental factors, including salinity, soil moisture, soil organic matter, root exudates, soil type, soil structure, and soil pH (Fierer, 2017). The advancement of high-throughput sequencing techniques has greatly enhanced our understanding of microbial community composition, taxa-specific distributions, and their functions (Song *et al*., 2020). The plant microbiome has earned both scientific and commercial significance, driven by its proven potential to enhance plant protection and growth, as well as to facilitate the development of innovative pharmaceuticals (Yadav and Yadav, 2017).

Endophytes from medicinal plants have many potential applications in crop production and bioprospecting for bioactive compounds (Singh *et al*., 2017). The most frequently isolated and characterized genera of endophytic bacteria include *Pseudomonas, Bacilli, Microbacterium, Stenotrophomonas, Burkholderia, and* Micrococcus (Liu Lufeng, 2019; Ali *et al*., 2021). The diversity of endophytic bacteria can be effectively explored through a combination of culture methods and metagenomic analysis. This involves cloning and high-throughput sequencing of the 16S rRNA gene, utilizing a range of universal primers (Ali *et al*., 2021). The prokaryotic 16S rRNA genes contain nine conserved hypervariable regions (V1-V9) that facilitate sequencing, taxonomic and phylogenetic classification (Huse *et al*., 2008). In this study, we reported on the 16S rRNA-aided analysis of the metagenome for bacterial endophytes in *Elephantorrhiza elephantina,* isolation and molecular identification/characterization of the bacterial isolates.

*Elephantorrhiza elephantina* is a perennial herb characterized by an underground tuber and is classified within the *Fabaceae* family under the *Elephantorrhiza* genus (Maroyi, 2017). Its distribution primarily spans southern African countries, including Botswana, Namibia, Lesotho, and various provinces in South Africa such as Limpopo, North West, Mpumalanga, Free State, Eastern Cape, Northern Cape, and KwaZulu-Natal (Grobler, 2010; Maroyi, 2017; Olaokun *et al*., 2020). Ethnomedicinally, the roots and leaves of this plant are utilized for addressing a range of conditions, including diarrhea, diabetes, chest complaints, bladder problems, skin issues, fever, infertility in women, and hemorrhoids (Grobler, 2010; Maroyi, 2017; Asong *et al*., 2019; Olaokun *et al*., 2020; Radebe *et al*., 2022). The literature underscores that *E. elephantina* is known to produce diverse secondary metabolites, encompassing, esters, phenolic compounds, flavonoids, glycosides, phytosterols, saponins, tannins, and triterpenoids (Msimanga *et al*., 2012; Maroyi, 2017; Radebe *et al*., 2022). These compounds manifest a range of beneficial properties, including anthelmintic, antibacterial, antifungal, antiinflammatory, antinociceptive, antiplasmodial, antioxidant, antibabesial, and antirickettsial activities (Mabona *et a*l., 2013; Mpofu *et al*., 2014; Asong *et al*., 2019; Olaokun *et al*., 2020). Given the plant's various traditional uses and demonstrated pharmacological activities, there is a compelling motivation to explore the bacterial endophytes associated with *E. elephantina* that contribute to metabolite production.

Several research reports have demonstrated that endophytic bacteria associated with medicinal plants produced bioactive compounds that were similar or related to the host's secondary metabolites (Alvin *et al*., 2014; Trapp *et al*., 2015). Hence, we could hypothesise that endophytes isolated from this medicinal plant are likely to possess analogous medicinal properties by producing bioactive metabolites that are either related or similar to those found in the host plant. To date, there have been no reports on endophytes from this medicinal plant and their associated medicinal potential. As a result, this chapter provides a comprehensive account of the isolation and identification of bacterial endophytes derived from *E. elephantina*, shedding light on their potential medicinal properties.

## 2.2 Methods and Materials

### 2.2.1 Materials

Tween 80, ethanol (99.5%, HPLC grade), sodium hypochlorite (NaOCl), Luria-Bertani (LB) agar and glycerol were procured from Merck Life Science (Pty) Ltd (Modderfontein, South Africa). Quick-DNA$^{TM}$ Fungal/Bacterial Zymo Miniprep kit, One*Taq*® Quick-Load® 2X master mix with standard buffer, universal primers, agarose and 1 kb quick loading DNA marker were purchased from Inqaba Biotechnical Industries (Pty) Ltd (Pretoria, South Africa). Phosphate-buffered saline (PBS) tablets, and GeneJet gel extraction kit were bought from Thermo Fisher Scientific Inc (Waltham, USA).

### 2.2.2 Plant sample collection and surface sterilization

Healthy disease-free plant samples (roots and leaves) were collected from *E. elephantina*, in Mahikeng (25°57’ 27,5”S 25°26’30,6”E), North West province, South Africa. The identification of the plant and sample collection was performed with the assistance of Dr. Madeleen Struwig (S.D. Phalatse Herbarium, North-West University-Mahikeng Campus).

The plant samples underwent a comprehensive cleansing process involving washing with tap water to remove soil debris, then segmented into smaller units, afterwards treatment with Tween 80 detergent for ten minutes accompanied by shaking, and subsequent rinsing with distilled water. Following this, the plant samples were soaked in 70% ethanol for a minute immersed in 70% ethanol for a minute, followed by treatment with 1% (v/v) of sodium hypochlorite for a duration of ten minutes. The samples were then rinsed 3-5 times with autoclaved sterile distilled water and then the water from the final wash was plated on LB agar plates (g/L; peptone 10, sodium chloride 10, yeast extract 5, and agar 15, pH 7.0) as a control to determine the effectiveness of the surface sterilization. This was done following aseptic techniques under laminar hood.

### 2.2.3 16S rRNA based-metagenomics analysis

The surface sterilized plant parts were macerated in 10 mL of sterile phosphate buffered saline (PBS) (g/L; sodium chloride 8, potassium chloride 0.2, disodium hydrogen phosphate 1.44 and potassium dihydrogen phosphate 0.24, pH 7.4). The plant samples

(10 mL of each plant macerated sample) were sent to Inqaba Biotechnical Industries (Pretoria, South Africa), a commercial next generation sequencing (NGS) service provider, for sequencing. Firstly, genomic DNA was extracted from the plant samples using the Quick-DNA$^{TM}$ Fungal/Bacterial Miniprep kit following the manufacturer's protocol. Then the extracted DNA samples were used as template in a PCR, using a universal primer pair 27F (5′-AGAGTTTGATCTGGCTCAG-3′) and 1492R (5′-AAGGAGGTGWTCCARCC-3′) targeting the V1 - V9 region of the bacterial 16S rRNA gene (Huse *et al*., 2008). The following conditions were used for PCR: 1X initiation denaturation cycle at 92°C for 2 min, 30X denaturation cycles at 92°C for 30 sec, 30X primer annealing cycles at 52°C for 30 sec, 30X extension cycles at 72°C for 2 min, and a 1X final elongation cycle at 72°C for 2 min. The resultant amplicons were assigned Pacbio M13 barcodes through limited cycle PCR for multiplexing purposes, as detailed on www.pacb.com. Following this, the barcoded amplicons were quantified, equimolarly pooled, and subjected to a bead-based purification step using AMPure PB. Subsequently, a PacBio SMRTbell library was generated from the pooled amplicons following the manufacturer's protocol. Sequencing primer annealing and polymerase binding were executed according to the SMRTlink software protocol to prepare the library for sequencing on the PacBio Sequel IIe system. Raw subreads underwent processing through the SMRTlink (v10.2) circular consensus sequences (CCS) algorithm to generate highly accurate reads (>QV40). These high-fidelity reads were then subjected to quality control assessment using DADA2 (https://benjjneb.github.io/dada2/index.html) and taxonomic classification through qiime2 (https://docs.qiime2.org/2021.11/). Raw sequence reads of the 16S rRNA gene amplicon data reported in this study were submitted to the Sequence Read Archive (SRA) of the NCBI under the Bio Project PRJNA1087695 with the accession numbers SAMN40452947 and SAMN40452948.

### 2.2.4 Isolation of endophytic bacteria and Gram staining

The endophytic bacteria were isolated from the rhizome utilizing a method outlined in Jasim *et al*., (2014). In summary, the outer surface of the plant material was trimmed off and the sample homogenized in PBS. The samples were serially diluted up to $10^{-3}$ with PBS buffer and 0.1 mL of the dilution plated on LB agar and incubated at 30°C up to five days. The plates were monitored daily to track bacterial colony development, after which sub-culturing were performed until achieving pure colonies. Glycerol stocks (50%, glycerol diluted in sterile LB broth) for each bacterial isolate were prepared and stored at -80 °C for future utilization. Subsequently, the isolated pure colonies underwent Gram staining, following the methodology outlined by Collins *et al*., (2004), to ascertain morphological features including shape and Gram stain reaction. The prepared slides were examined utilizing an OLYMPUS CH20BIMF200 compound bright-field microscope (Lasec® Group, Cape town, South Africa) with 100x magnification.

### 2.2.5 Genomic DNA extraction and polymerase chain reaction amplification and sequencing

The genomic DNA of each isolate was extracted from an overnight starter culture utilizing a Quick-DNA™ Fungal/Bacterial Miniprep kit, following the manufacturer’s protocol. The extracted genomic DNA was then quantified using a NanoDrop™ ND-2000 UV-Vis spectrophotometer (Thermo Fisher Scientific Inc, Waltham, USA).

The amplification of the 16S rRNA gene for each bacterial endophyte followed the protocol outlined by Tsuchida *et al.,* (2002). Universal primers, 27F (5′-AGAGTTTGATCTGGCTCAG-3′) and 1492R (5′-AAGGAGGTGWTCCARCC-3′), were employed for the amplification. The PCR reaction was conducted in 25 µL total volumes using the following conditions: an initial cycle at 92°C for 2 min, 30 denaturation cycles at 92°C for 30 sec, 30 primer annealing cycles at 52°C for 30 sec, 30 extension cycles at 72°C for 2 min, and a final elongation cycle at 72°C for 2 min, concluding with termination at 4°C. Subsequently, the resulting amplicons were subjected to electrophoresis on a 1% agarose gel at a constant 100 V and 200 mA. The resulting gel was analysed using Bio-Rad gel documentation (Lasec® Group, Cape town, South Africa). Positive amplicons were excised from the gel, purified using the GeneJet gel extraction kit, and then sequenced at Inqaba Biotechnical Industries (Pretoria, South Africa) using Sanger sequencing.

### 2.2.6 Sequence and phylogenetic analysis

The CLC Bio Main Workbench was employed to assemble both forward and reverse sequencing reads, generating a consensus sequence for each isolate. Subsequently, a Basic Local Alignment Search Tool (BLAST) search was conducted on the National

Center for Biotechnology Information (NCBI) GenBank nucleotide sequence database (https://www.ncbi.nlm.nih.gov/genbank/) to identify potential matches between the query sequence and those in the database, as outlined by Altschul *et al.* (1997). Sequences of species closely resembling the BLAST query for each sample, along with their closely related taxa, were retrieved for subsequent phylogenetic analysis. Alignment of these sequences was accomplished using the Clustal X 2.1 version multiple sequence alignment tool (Thompson *et al*., 1994). Phylogenetic and molecular evolutionary analyses were performed using MEGA X, employing the maximum likelihood method with 1,000 bootstrap replicates, as detailed by Kumar *et al*., (2018).

## 2.3 Results

### 2.3.1 16S rRNA based metagenomics analysis

Table 2.1 shows the results obtained from the bacterial metagenome from the rhizome and the leaves of *E. elephantina*. For the rhizome, a total of 693 read counts was obtained and assigned to 7 phyla, 11 classes, 18 orders, 21 families, and 23 genera while for the leaves, a total of 2459 read counts were obtained and assigned to 8 phyla, 8 classes, 12 orders, 17 families, and 19 genera.

Table 2-1: Taxonomic distributions of the read counts from metagenomics analysis using 16S rRNA amplicons of bacterial diversity from the rhizome and the leaves of *E. Elephantina.*

| | **Rhizome** | **Leaves** |
|---|---|---|
| **Phyla** | 7 | 8 |
| **Classes** | 11 | 8 |
| **Orders** | 18 | 12 |
| **Families** | 21 | 17 |
| **Genera** | 23 | 19 |

#### 2.3.1.1 Phylum-level distribution of the leaves and rhizome

At the phyla level, Proteobacteria, Bacteroidota, Gemmatimonadota, Actinobacteriota, Verrucomicrobiota, Dependentiae, Firmicutes, and Armatimonodata were detected with

Proteobacteria being predominant in both the leaves and rhizome. Furthermore, 17.6% and 4.51% of unknown bacteria were observed in both the rhizome and leaves respectively. Phyla Verrucomicrobiota and Dependentiae were observed only in the leaves while phylum Armatimonadota was only observed in the rhizome (Figure 2.1).

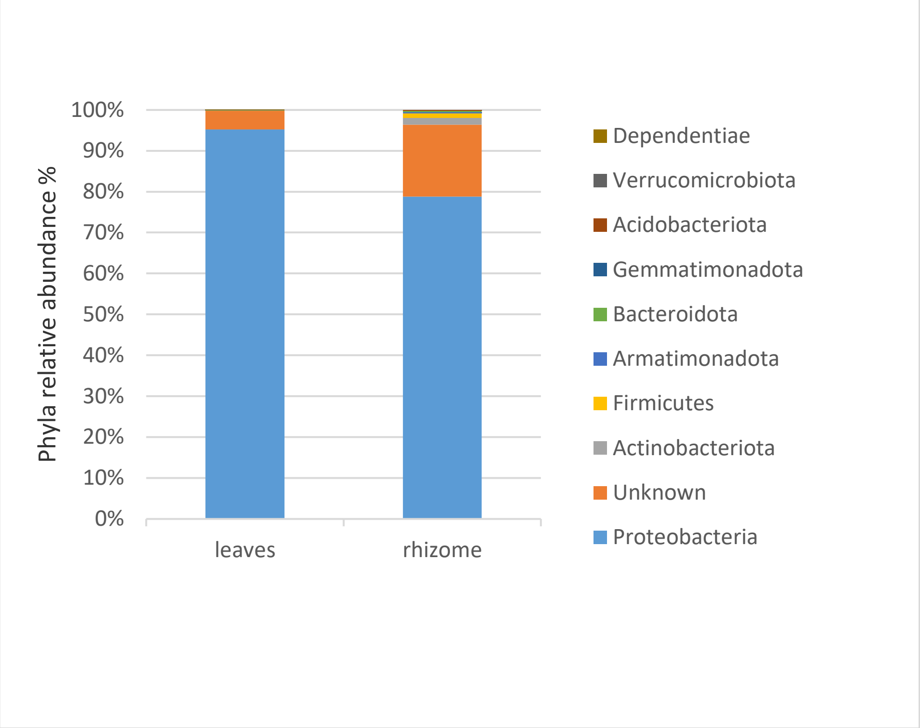


Figure 2-1: Chart showing the relative taxonomic abundance of bacterial phyla, in leaves and rhizome samples based on analysis of full-length 16S gene amplicons.

#### 2.3.1.2 Diversity and structure of the endophytic bacterial communities

The genera *Novosphingobium*, *Mesorhizobium*, *Methylobacterium*, and *Ralstonia*, made up most of the microbiota in both the leaves and rhizome, which all belong to the phylum Proteobacteria. At the genus level, the rhizome had greater bacterial diversity than the leaves (Figure 2.2).

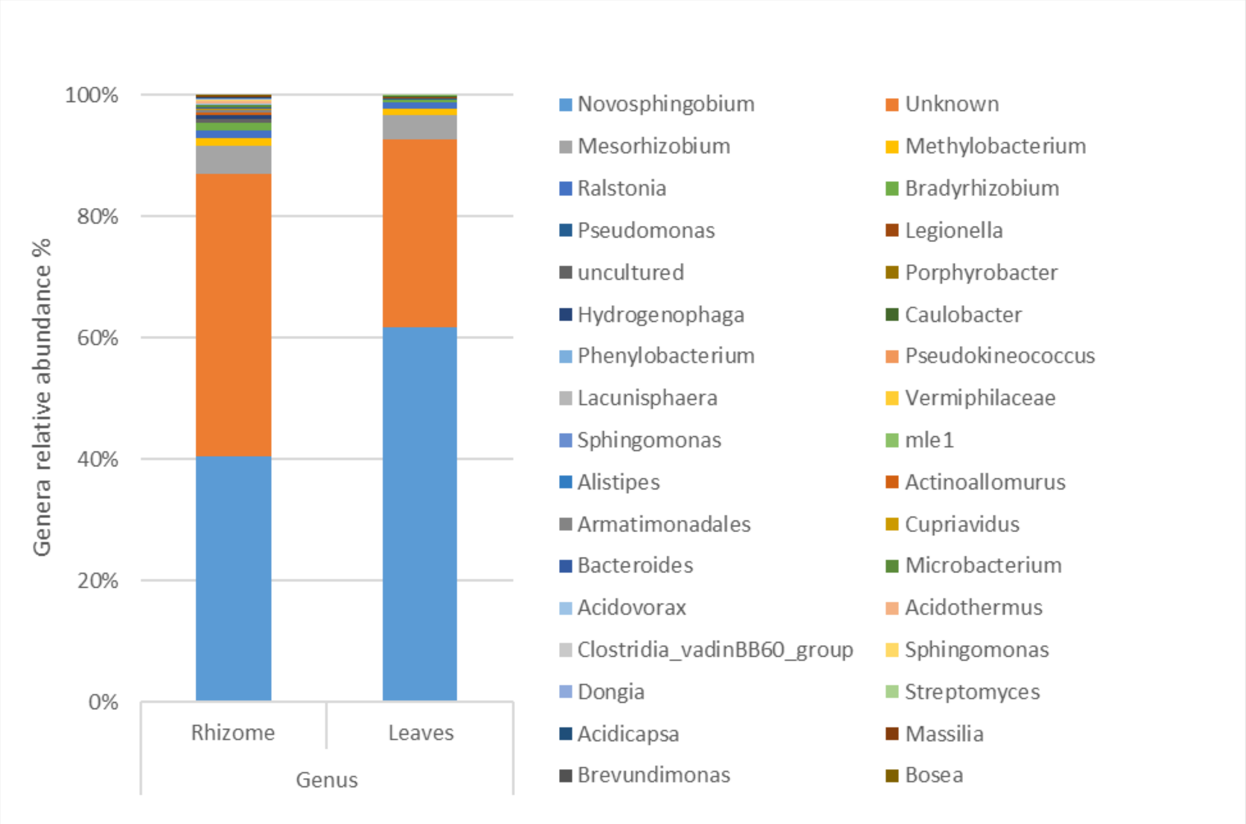


Figure 2-2: Chart showing the relative taxonomic abundance of the bacterial genera, in the rhizome and leaves samples based on analysis of full-length 16S gene amplicons.

### 2.3.2 Isolation of bacterial and identification

The bacterial isolation resulted in the identification of 10 pure colonies that were named EER1 to EER10. Molecular identification of the bacterial isolates was done by sequencing the 16S rRNA gene using universal primers 27F and 1492R. The PCR products were sequenced, and data analysis revealed that the 16S rRNA sequences of EE-R1-EE-R3 and EE-R5, EE-R6, EE-R7, EE-R9, and EE-R10 showed its (16S rRNA) maximum identity of 99% or greater to *Stenotrophomonas maltophilia*, *Microbacterium oxydans*, *Pseudomonas alcaliphila*, *Pseudomonas chengduensis*, and *Pseudomonas alcaligenes*, respectively (Table 2.2). EE-R4 and EE-R8 were mixed cultures and were discarded. The 16S rRNA sequences acquired were submitted to NCBI and assigned the accession numbers listed in Table 2.2.

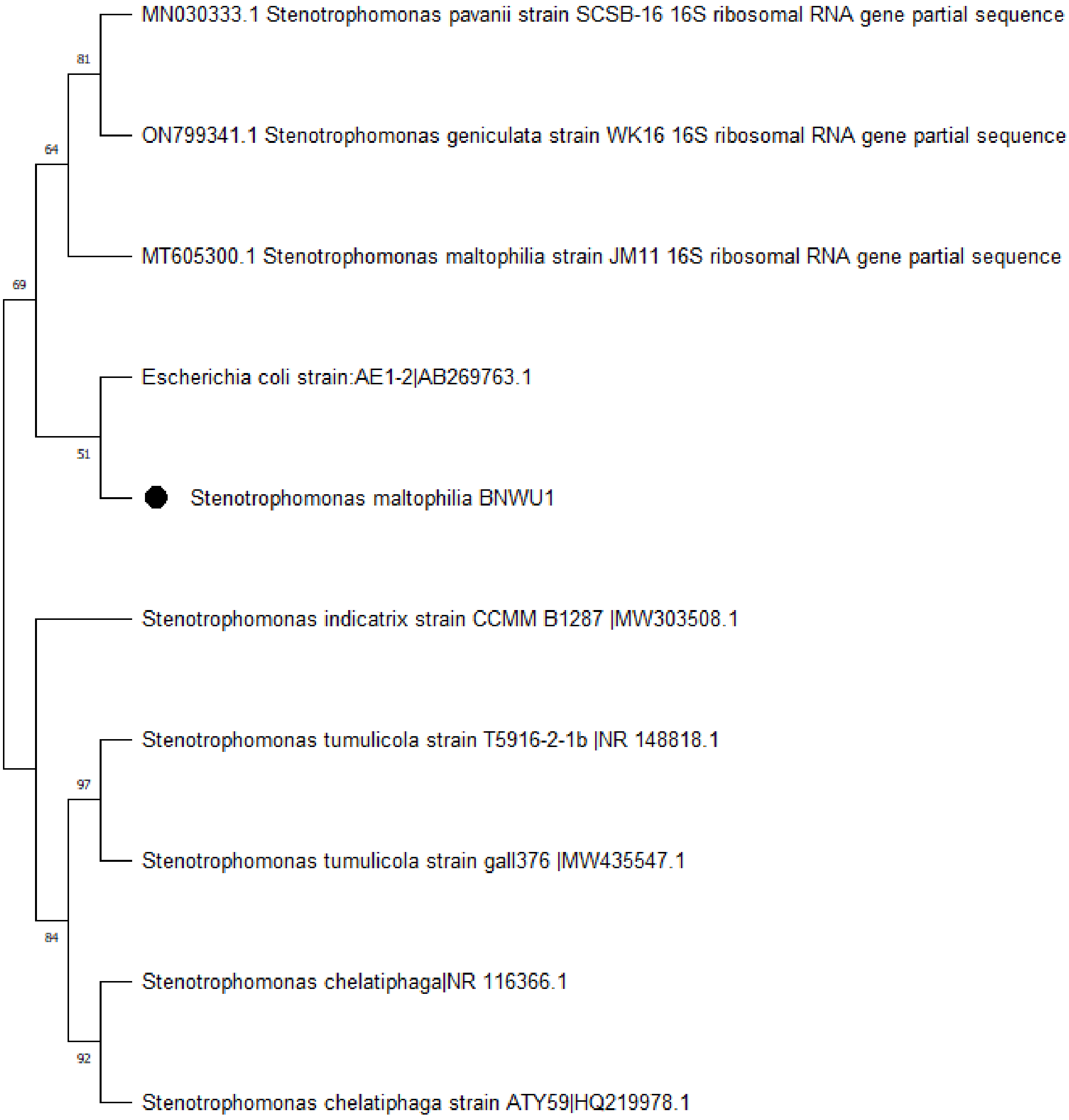
Table 2-2: NCBI BLAST results of the 16S rRNA gene sequences of bacterial endophytes isolated from *E. Elephantina* and assigned accession numbers.

| Isolates | Assigned bacterial name | Gen bank accession number | NCBI Results | | | |
|---|---|---|---|---|---|---|
| | | | Closest NBCI related bacterial species with accession number | Query coverage % | *E*-value | Identity similarity % |
| EE-R1 | *Stenotrophomonas maltophilia* strain BNWU1 | OQ859587 | *Stenotrophomonas maltophilia* (OQ588742.1) | 100% | 0.0 | 99.57% |
| EE-R6 | *Microbacterium oxydans* strain BNWU2 | OQ859588 | *Microbacterium oxydans* (MT533951.1) | 100% | 0.0 | 99.76% |
| EE-R7 | *Pseudomonas sp.* Strain BNWU3 | OQ860052 | *Pseudomonas alcaliphila* (MH127731.1) | 100% | 0.0 | 100.00% |
| EE-R9 | *Pseudomonas sp.* Strain BNWU4 | OQ859589 | *Pseudomonas chengduensis* (MN099372.1) | 100% | 0.0 | 99.88% |
| EE-R10 | *Pseudomonas sp.* Strain BNWU5 | OQ859590 | *Pseudomonas alcaligenes* (MT323223.1) | 99% | 0.0 | 99.88% |

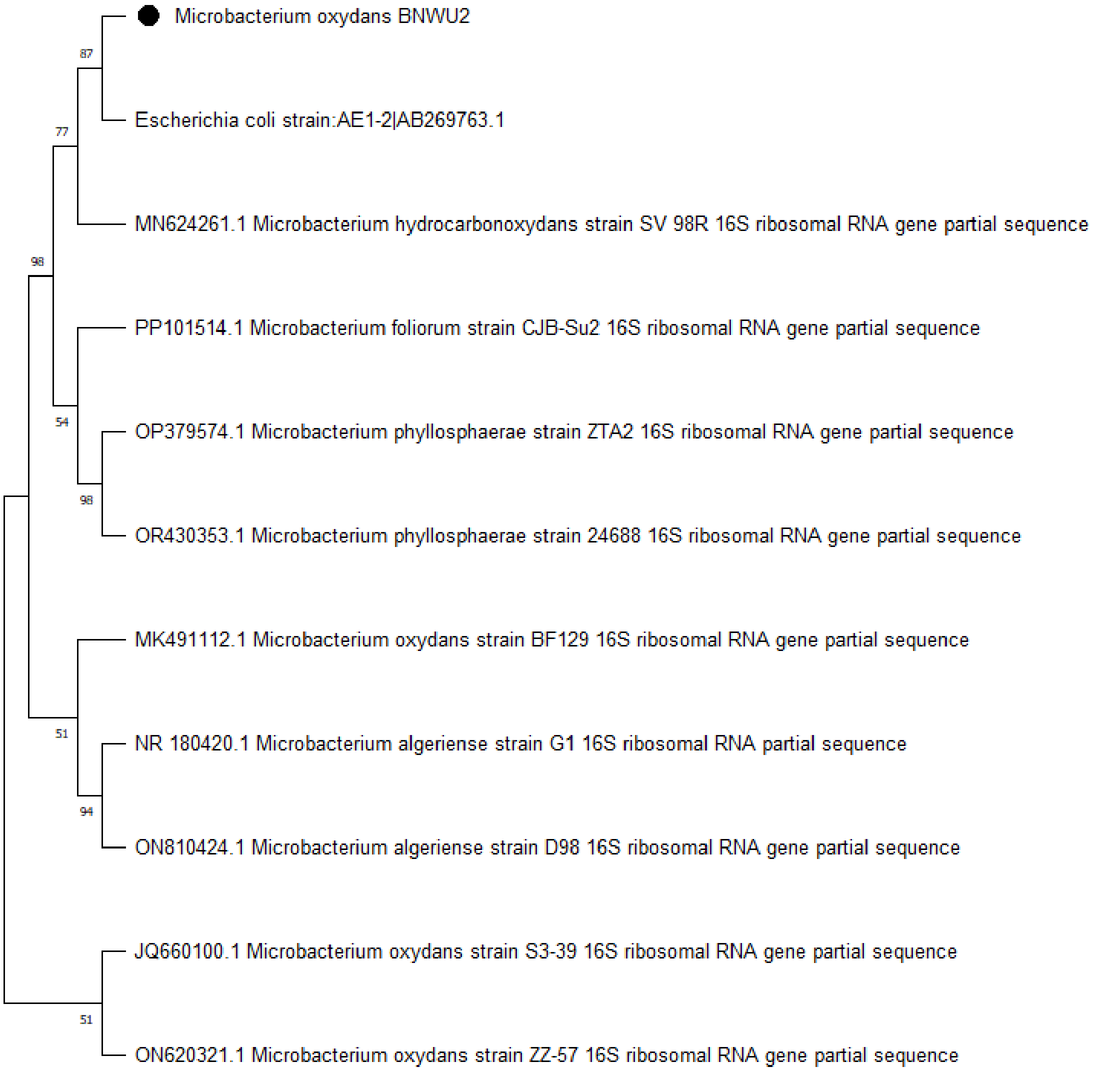
### 2.3.3 Morphological and phylogenetic analysis

The phylogenetic analysis of the 16S rRNA sequence of the isolates along with the sequences retrieved from the NCBI was carried out with MEGA X using the maximum likelihood method with 1,000 bootstrap replicates. The results showed that all the endophytic bacterial isolates grouped with various closely related bacterial species. Each genus was analyzed in separate phylogenetic trees (Figure 2.3) and it was observed that, *Stenotrophomonas* sp. BNWU1 (Fig 2.3A) has a monophyletic relationship with *Stenotrophomonas maltophilia* strain JM11, *S. pavanii* strain SCSB and *S. geniculate* strain WK16 supported by 48% bootstrap value. It also formed a separate clade indicating it could be different from the other 3 *Stenotrophomonas* species. *Microbacterium* sp. BNWU2 (Fig 2.3B) had a paraphyletic relationship with other Microbacterium species, and formed a separate clade. On the other hand, *Pseudomonas* sp. BNWU3, BNWU4, and BNWU5 formed separate clades, indicating they are different species, and all of them formed paraphyletic relationship with other *Pseudomonas* species (Fig. 2.3C).

(A)


MN030333.1 Stenotrophomonas pavanii strain SCSB-16 16S ribosomal RNA gene partial sequence
81
ON799341.1 Stenotrophomonas geniculata strain WK16 16S ribosomal RNA gene partial sequence
64
MT605300.1 Stenotrophomonas maltophilia strain JM11 16S ribosomal RNA gene partial sequence
69
Escherichia coli strain:AE1-2|AB269763.1
51
● Stenotrophomonas maltophilia BNWU1
Stenotrophomonas indicatrix strain CCMM B1287 |MW303508.1
Stenotrophomonas tumulicola strain T5916-2-1b |NR 148818.1
97
Stenotrophomonas tumulicola strain gall376 |MW435547.1
84
Stenotrophomonas chelatiphaga|NR 116366.1
92
Stenotrophomonas chelatiphaga strain ATY59|HQ219978.1

(B)


Microbacterium oxydans BNWU2
Escherichia coli strain:AE1-2|AB269763.1
MN624261.1 Microbacterium hydrocarbonoxydans strain SV 98R 16S ribosomal RNA gene partial sequence
PP101514.1 Microbacterium foliorum strain CJB-Su2 16S ribosomal RNA gene partial sequence
OP379574.1 Microbacterium phyllosphaerae strain ZTA2 16S ribosomal RNA gene partial sequence
OR430353.1 Microbacterium phyllosphaerae strain 24688 16S ribosomal RNA gene partial sequence
MK491112.1 Microbacterium oxydans strain BF129 16S ribosomal RNA gene partial sequence
NR 180420.1 Microbacterium algeriense strain G1 16S ribosomal RNA partial sequence
ON810424.1 Microbacterium algeriense strain D98 16S ribosomal RNA gene partial sequence
JQ660100.1 Microbacterium oxydans strain S3-39 16S ribosomal RNA gene partial sequence
ON620321.1 Microbacterium oxydans strain ZZ-57 16S ribosomal RNA gene partial sequence
87
77
98
54
98
51
94
51

(C)

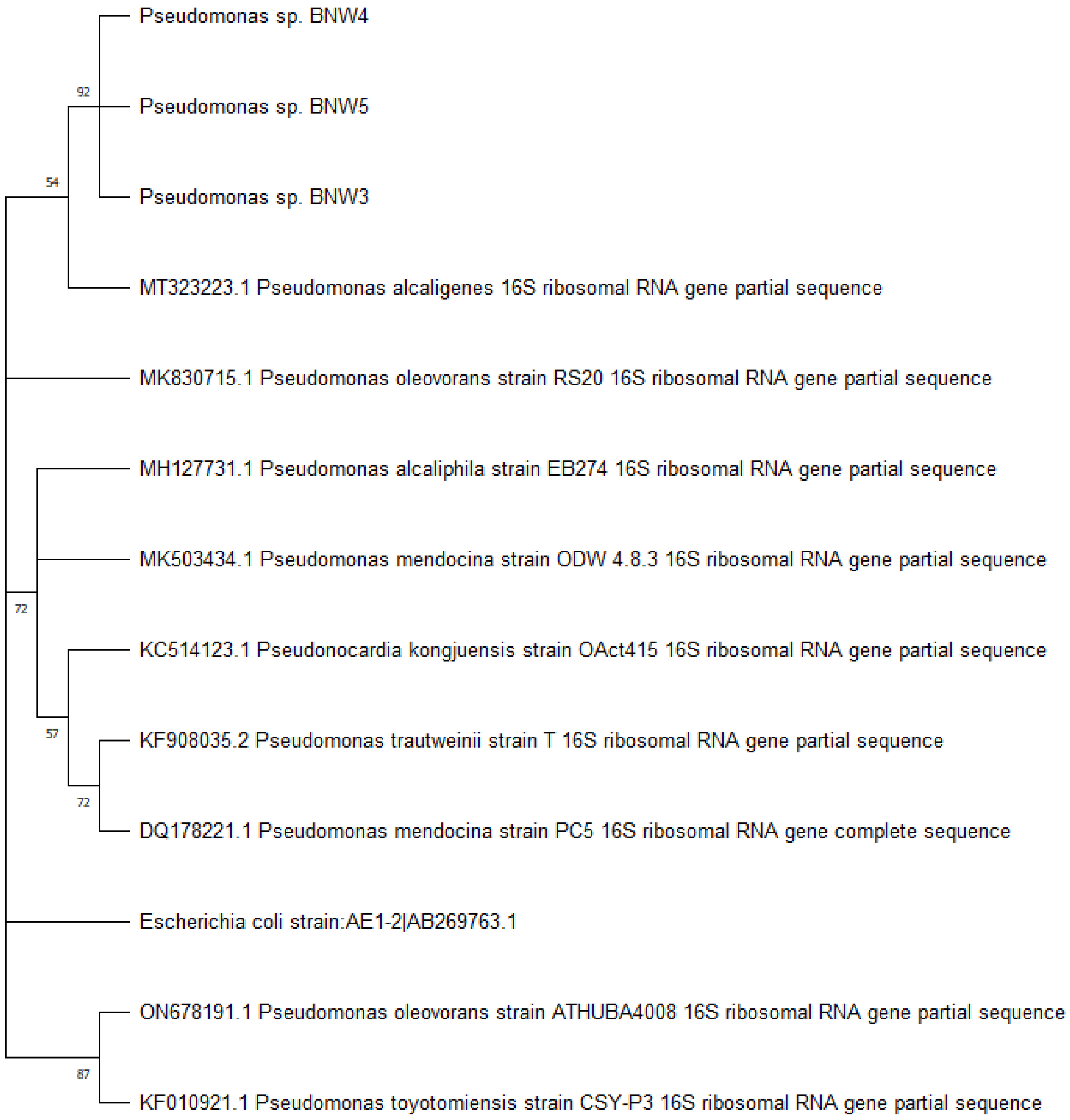


Figure 2: Maximum likelihood phylogenetic tree based on 16S rRNA gene sequence for BNWU1 (A), BNWU2 (B), and BNWU 4 and 5 (C) *versus* selected bacterial species from GenBank. Numbers above or below the nodes indicate bootstrap values generated after 1000 replications.

The bacterial isolates were subjected to Gram staining for morphological characteristics and from this Gram stain reaction, all the isolates were observed to be rod-shaped/bacilli and Gram-negative apart from EER6, which was found to be Gram positive (Table 2.3).

Table 2-3: Morphological characteristics of bacterial endophytes.

| Isolates | Phyla | Gram stain | Shape |
|---|---|---|---|
| EE-R1 | Proteobacteria | Gram-negative | Rod-shaped |
| EE-R6 | Actinobacteria | Gram-positive | Rod shaped |
| EE-R7 | Proteobacteria | Gram-negative | Rod-shaped |
| EE-R9 | Proteobacteria | Gram-negative | Rod-shaped |
| EE-R10 | Proteobacteria | Gram-negative | Rod-shaped |

## 2.4 Discussion

The result of 16S rRNA sequencing were reported as sequencing 'reads' which were analysed using several basic bioinformatics tools such as DADA2, Mothur, and qiime2, which are referred to as 'pipelines' when they are integrated. These pipelines function by allowing a linear series of data transforms to be linked to a microbial genomic database thus accurately identifying and profiling the bacteria that are present in the specimens (Callahan *et al*., 2016). In this study, it was observed that the leaves (2459 read counts) exhibited significantly higher total read counts compared to the rhizome (693 read counts), suggesting a greater abundance of microbes in the leaves as opposed to the rhizome. The metagenomics analysis was conducted at both the phylum and genus levels.

The bacterial diversity of the leaves and rhizome consisted of phyla Proteobacteria, Bacteroidota, Gemmatimonadota, Actinobacteriota, Verrucomicrobiota, Dependentiae, Firmicutes, and Armatimonodata. The most prevalent bacterial phylum identified in both the rhizome and leaves was Proteobacteria, with Actinobacteria being the second most abundant phylum, as depicted in Figure 2.1. This finding aligns with several other studies conducted on various plant species, such as Bt maize (Niu *et al*., 2017), *Oryza sativa* L (Kunda *et al*., 2018), and *Arabidopsis thaliana* (Lundberg *et al*., 2012). The phylum Proteobacteria made up most of the bacterial diversity accounting for more than 70% of the bacterial population in both the leaves and rhizome.

Proteobacteria has consistently emerged as the most prevalent bacterial phylum in numerous plant species and various environmental samples, including water and soil, a trend that was consistent with the findings of this study (Yaish *et al*., 2016, Moronta-

Barrios *et al*., 2017). Within this phylum, there are several bacteria known to play pivotal roles within the plant endosphere. Notable examples include *Acidovorax faecalis*, known for its plant growth-promoting properties, *Rhizobium*, which is involved in nitrogen fixation, and *Pseudomonas geniculata*, a biocontrol agent (Gopalakrishnan *et al*., 2015; Koskey *et al*., 2018). Furthermore, we observed a difference in diversity of the phyla Actinobacteriota and Firmicutes between leaves and rhizome, this could have been due to the different functions and environmental factors in the two plant parts (Bulgarelli *et al*., 2013; Niu *et al*., 2017). In our analysis, we also noticed a substantial number of unidentified bacteria, as illustrated in Figure 2.1. It is important to note that these could potentially represent novel bacterial species that have not yet been explored or cultured in laboratory settings. The identification of microbial taxa through 16S rRNA sequencing heavily relies on reference databases, and as a result, it is constrained to previously studied microorganisms (Peterson *et al*., 2021). These unknown bacteria may offer exciting prospects for future research and discovery in the field of microbiology.

Distinct bacterial genera fulfil various roles within plant systems; nonetheless, it is essential to recognize that the functions of bacterial endophytes are not solely determined by taxonomic categorizations. Instead, they are influenced by environmental factors and the specific host plant (Hardoim *et al*., 2015). Consequently, bacterial genera do not disperse randomly throughout plant tissues but seem to exhibit a tissue- or organ-specific distribution pattern. Numerous studies involving diverse plant tissues have highlighted noteworthy distinctions in the composition of the plant endophytic microbiota (Bodenhausen *et al*., 2013; Yang *et al*., 2017). At the genera level for both the rhizome and leaves, *Novosphingobium*, *Mesorhizobium*, *Methylobacterium*, and *Ralstonia*, made up most of the microbiota (Figure 2.2). Several studies have shown that members of these genera play important roles such as nitrogen fixation, pathogen protection, aiding plants against biotic and abiotic stresses, and production of growth hormones in plant growth promotion in different plant species including potato, maize, *O. sativa*, *Spinacia oleracea*, *Lactuca sativa*, and *A. thaliana* (Mano and Morisaki, 2008; Bulgarelli *et al*., 2013; Niu *et al*., 2017; Narayanan and Glick, 2022).

Several notable genera including *Mesorhizobium*, *Microbacterium*, *Sphingobium*, *Pseudomonas*, *Methylobacterium*, *Novosphingobium*, *Ralstonia,* and *Bradyrhizobium* identified in this study, have previously been reported to produce diverse secondary metabolites such as alkaloids, flavonoids, terpenes, steroids, saponins and phenolic compounds (Jalgaonwala and Mahajan, 2011; Trapp *et al*., 2015; Photolo *et al*., 2020; Narayanan and Glick, 2022). These secondary metabolites have many biological activities and can be potentially used in pharmaceuticals and crop production (Gouda *et al*., 2016). Therefore, there is a need to further isolate and identify these bioactive metabolites producing endophytic bacteria and study the metabolite they produced.

In this study, five endophytic bacteria were isolated from the rhizome of *E. elephantina.* The surface sterilization technique was found to be adequate as the control plate had shown no microbial growth*.* Therefore, these isolates obtained in the study can be considered endophytic bacteria of *E. elephantina*. To the best of our knowledge, this is the first report on isolation of bacterial endophytes from *E. elephantina.* In this study, the plant samples were collected in March 2022 during the autumn season. It is noteworthy that we employed LB agar for the cultivation and isolation of bacterial endophytes. This choice of growth medium and conditions might have led to a potential limitation in the diversity of identified bacterial endophytes. It is possible that the selected growth conditions could have introduced bias, favouring the proliferation of Proteobacteria. Further investigations using varied growth media and conditions might offer a more comprehensive understanding of the endophytic microbial community associated with these plants. The isolated strains were further identified and characterized using molecular techniques such as Gram staining and 16S rRNA sequencing. The 16S rRNA gene sequence is widely endorsed for bacterial identification, representing a potent tool for promptly identifying bacterial species (Clarridge, 2004). This preference is attributed to several factors: the 16S rRNA gene's ubiquity in prokaryotes, encompassing approximately 1500 bases, providing sufficient material for species analysis; its lower susceptibility to horizontal gene transfer; and the presence of variable regions within the 16S rRNA genes that facilitate the classification of species across diverse taxonomic groups (Kitahara and Miyazaki, 2013).

Based on the molecular identification (16S rRNA gene sequencing), the isolated endophytic bacteria were classified into 3 genera (*Stenotrophomonas, Microbacterium,* and *Pseudomonas*) from 2 phyla (Proteobacteria and Actinobacteria). The Gram staining results further confirmed the 16S rRNA identities of the endophytic bacteria as *Stenotrophomonas* and *Pseudomonas* that are known to be gram-negative and

*Microbacterium* Gram-positive. The findings from this investigation align with various studies that have identified different genera of Actinobacteria and Proteobacteria, including *Pseudomonas* (Tamošiūnė *et al*., 2018), *Enterobacteria* (Szilagyi-Zecchin *et al*., 2014), *Stenotrophomonas* (Zhu *et al*., 2012), *Microbacterium* (Gao *et al*., 2017), and *Streptomyces* (Worsley *et al*., 2020). Consequently, bacterial species from these genera appear to be prevalent within the bacterial endophyte communities of numerous plants. Additionally, it is worth noting that the genera *Microbacterium* and *Pseudomonas*, which were isolated in our study, were also identified through the 16S rRNA gene-based metagenomic analysis. An interesting observation is that the isolated species of *Microbacterium* and *Pseudomonas* in this study, displayed a polyphyletic relationship with related strains. Given these findings, we recommend the use of additional molecular marker genes, such as *rpo*B genes, for more comprehensive species description and to enhance the phylogenetic delineation of these microorganisms. This approach may provide a deeper understanding of their genetic diversity and evolutionary relationships.

## 2.5 Conclusion

This study provided initial insights into the overall bacterial community present in the leaves and rhizome of *E. elephantina*. It serves as a case study exploring the culturability of bacteria associated with this medicinal plant, utilizing a combination of high-throughput sequencing technology and cultivation on LB growth media. The microbial diversity of the leaves and rhizome consisted of phyla Proteobacteria, Bacteroidota, Gemmatimonadota, Actinobacteriota, Verrucomicrobiota, Dependentiae, Firmicutes, and Armatimonodata with Proteobacteria being the most dominant phylum. The five endophytes were identified as: *Stenotrophomonas maltophilia*, *Microbacterium oxydans*, and the other three as *Pseudomonas species*. The *Pseudomonas* species were identified up to genus level using 16S rRNA gene analysis. This research offers foundational data that could serve as a starting point for future investigations dedicated to the exploration and study of new endophytes linked with medicinal plants, with potential applications in agricultural, medical, and industrial sectors. In the next chapter, we present the investigation of the growth, secondary metabolite production profiles and the identification of the metabolites produced.

# Chapter 3: Bioactive secondary metabolites from bacterial endophytes isolated from *Elephantorrhiza elephantina*

## 3.1 Introduction

According to the International Union for Conservation of Nature and the World Wildlife Fund, over 15,000 flowering medicinal plants face a threat of extinction, primarily as a result of over harvesting and habitat destruction (Chen *et al*., 2016). All this is driven by the growing global population and increased demand for plant-based resources (Ross, 2005; Chen *et al*., 2016). Consequently, there is a growing interest in the quest for new sources of bioactive compounds, driven by the need for sustainable approaches to discover novel drugs (Chen *et al*., 2016). Microbial endophytes, extracted from medicinal plants, have emerged as promising alternative sources of bioactive metabolites. These microbial endophytes have been reported to produce bioactive compounds that are similar to those produced by their host plants (Zhao *et al*., 2011; Alvin *et al*., 2014). As a result, most endophytic isolates share certain medicinal properties with the host plant (Alvin *et al*., 2014).

Bacterial bioactive metabolites are characterized by their relatively small molecular weight, typically ranging from 100 to 1000 Da. These metabolites exhibit an immense chemical diversity, leading to a wide range of biological activities (Chevrette *et al*., 2020). The enzymes responsible for catalysing the assembly of these molecules are encoded within biosynthetic gene clusters (BGCs). Next-generation sequencing (NGS) techniques have unveiled an unparalleled potential for bioactive compound production in bacteria, highlighting the importance of studying these genetic pathways (Petersen *et al*., 2020). Bioactive compounds, including alkaloids, steroids, terpenoids, peptides, polyketones, flavonoids, quinols, phenols, and natural insecticides have been reported from endophytic bacteria (Li *et al*., 2008; Kusari *et al*., 2012; Morina *et al*., 2012). These bioactive compounds have significant value in agricultural, industrial, and medical applications (Kobayashi and Palumbo 2000; Zinniel *et al*., 2002).

Utilizing endophytic bacteria as a source of biological activity presents numerous advantages. These include their straightforward isolation, culturing, and genetic manipulation, as well as the ability to easily scale up the production of bioactive metabolites in laboratory settings (John, 2010; Newman and Cragg, 2012). These factors collectively position endophytic bacteria as a highly promising resource for a wide range of biotechnological applications (Xu *et al*., 2008; Newman and Cragg, 2012). In this chapter, we conducted a comparative analysis of the growth and secondary metabolite production in bacterial isolates obtained from *Elephantorrhiza elephantina*. The study also includes a comparison of the antioxidant and antimicrobial activities of the crude extracts and the identification of the secondary metabolites produced.

## 3.2 Materials and methods

### 3.2.1 Materials

The bacteria endophytes used in this chapter were isolated from *E. elephantina* as described in Chapter 2. Luria-Bertani broth, ethyl acetate, chloroform, ascorbic acid, methanol, 2, 2- diphenyl-1-picrylhydrazyl (DPPH), dimethyl sulfoxide (DMSO), Tryptic Soy broth, streptomycin, ampicillin, and iodonitrotetrazolium chloride were bought from Merck Life Science (Pty) Ltd (Modderfontein, South Africa). Formic acid and methanol both of HPLC grade were purchased from Merck Life Science (Pty) (Darmstadt, Germany). The pathogenic test strain used for the antimicrobial testing was donated by Professor Collins Njie Ateba (Antibiotic Resistance and Phage Biocontrol Research Group, North-West University).

### 3.2.2 Bacterial growth analysis and extraction of secondary metabolites

A starter culture was prepared by inoculating a single colony of the bacterial sample into 5 mL of LB broth and incubating it overnight at 37°C with continuous shaking at 120 rpm. Subsequently, 1 mL of the starter culture was transferred into 50 mL of LB broth (g/L; tryptone 10, yeast extract 5, and sodium chloride 10, pH 7.0) and cultivated at 37°C with coupled with shaking at 120 rpm. Optical density readings were recorded in triplicate every 2 hours over a 48-hour period using the ONDA (UV/VIS) spectrophotometer (Lasec® Group, Cape town, South Africa) at a wavelength of 600 nm.

Extraction of metabolites from endophytic bacteria was conducted following the procedure outlined in Balachandran *et al*., (2012), with slight adjustments. In summary, LB broth (1 L) was prepared in 2 L Erlenmeyer flasks and sterilized at 121°C for 20 minutes. Each of the 2 L flasks was then inoculated with the isolates and incubated at 37°C for 7 days with continuous shaking at 120 rpm. Cell harvesting was achieved through centrifugation at 10,000 rpm for 15 minutes to remove biomass, and equal volumes of ethyl acetate and chloroform (1:1 v/v) were added to the supernatant, followed by vigorous shaking. The organic solvent layer was collected in a boiling flask and subsequently concentrated using a vacuum rotary evaporator (Lasec® Group, Cape Town, South Africa) at 40°C.

### ***3.2.3*** **Antioxidant assay**

The examination of the antioxidant capabilities of the extracts from endophytic bacterial isolates was conducted through the DPPH free radical scavenging assay. The methodology employed was adapted from Takao *et al*., (2015) with slight adjustments. Briefly, the crude extracts from the bacterial isolates (experiment) and the positive control (vitamin C) were dissolved in methanol to obtain an initial concentration of 1 000 µg/mL and mixed with a 200 µM of methanolic DPPH to a final extract and ascorbic acid concentrations of 500; 250; 125; 62.5; 31. 25; 15. 625; 7. 813; 3. 906 and 1. 953 µg/mL in a 96 well plate (Greiner Bio-One North America Inc, North Carolina USA). Methanol served as a solvent control (negative control). The plates were incubated for 30 min at 25°C and the absorbance read in a Multiskan GO microplate reader (Thermo Fisher Scientific Inc, Waltham, USA) at a wavelength of 517 nm. All experiments were conducted in triplicate, and the $IC_{50}$ was determined through graphical calculation, with the percentage inhibition computed as outlined below:

$$\%\text{Inhibition} = \frac{(\text{absorbance of control} - \text{absorbance of the sample})}{\text{absorbance of control}} \text{ x } 100$$

### 3.2.4 Antimicrobial assay

The Minimum Inhibitory Concentration (MIC) investigations of the crude extracts from the isolates were carried out following the methodology outlined by Andrews (2001), with slight adjustments. In summary, initial stock solutions were prepared by dissolving 1 mg of the extract in 1 mL of 1 M DMSO, resulting in a final concentration of 1 mg/mL. These stock solutions were then subjected to serial dilution with fresh media, resulting in final concentrations of 500, 250, 125, 62.5, 31.25, 15.625, 7.813, 3.906, and 1.953 µg/mL using tryptic soy broth (g/L; casein peptone (pancreatic) 17, dipotassium hydrogen phosphate 2.5, glucose 2.5, sodium chloride 5, and soy peptone 3. The pathogenic test strains used included Gram-negative bacteria *Escherichia coli* (ATCC25922), *Escherichia coli* 0.157 (environmental strain), *Salmonella* sp, and *Vibrio cholerae* and Gram-positive bacteria, *Staphylococcus aureus* (ATCC26923), *Bacillus sp.,* and *Enterococcus durans*. Utilizing the McFarland 0.5 standard, 50 µL of each pathogen was inoculated into 15 mL of tryptic soy broth and then cultured at 37°C for 24 hours. In a 96 well microtitre plate, 100 *µ*L of the pathogenic test strains were added horizontally and 100 µL of the different dilutions of the crude extracts were also added vertically, starting from the highest to the lowest concentrations. Similarly, positive controls of ampicillin and streptomycin, as well as a negative control using DMSO, were subjected to the same arrangement. The microtiter plate was sealed using parafilm and placed in an incubator at 37°C for a duration of 16-20 hours. Following incubation, in each well; a 10 µL of iodonitrotetrazolium chloride solution at a concentration of 4 mg/mL was added. The Minimum Inhibitory Concentration (MIC) was determined as the lowest concentration exhibiting clear wells, indicating the absence of microbial growth. Replicates of the antimicrobial experiments were carried out in triplicate (n = 3).

### 3.2.5 Metabolite identification using liquid chromatography mass spectrometry

The crude extracts were analysed utilizing a liquid chromatography–quadrupole time-of-flight tandem MS (LC-QToF-MS) instrument (LCMS-9030 qTOF, Shimadzu Corporation, Kyoto, Japan), following the procedure outlined by Ramabulana *et al*., (2021), with slight adjustments. Chromatographic separation was executed on a Shim-pack Velox C18 column (100 mm × 2.1 mm with a particle size of 2.7 µm) from Shimadzu Corporation, Kyoto, Japan, maintained at 55 °C. A 3 µL injection volume was employed for all samples, utilizing a binary mobile phase gradient. Solvent A consisted of 0.1% formic acid in Milli-Q water, while solvent B was composed of methanol with 0.1% (v/v) formic acid. The flow rate remained constant at 0.3 mL/min over the 20-minute gradient. The separation conditions included maintaining 5% B for 3 min, transitioning to 5–40% B over 3–5 min, 40–95% B over 5–12 min, sustaining 95% B from 12–16 min, adjusting to 5% B between 16–18 min, maintaining 5% B for 2 min, and finally returning to initial conditions between 18–20 min, followed by a 3-minute column requilibration period.

The chromatographic effluents underwent additional analysis employing the qTOF high-definition mass spectrometer configured to capture negative electrospray ionization data. The specified parameters included an interface voltage of 4.0 kV, an interface temperature of 300 °C, nebulization and dry gas flow set at 3 L/min, a heat block temperature of 400 °C, DL temperature at 280 °C, a detector voltage of 1.8 kV, and the flight tube temperature maintained at 42 °C. Sodium iodide (NaI) served as a calibration solution to ensure high mass accuracy. Simultaneous generation of MS1 and MS2 (via data-dependent acquisition) was conducted for all ions within an m/z range of 100–1000, surpassing an intensity threshold of 5000. Fragmentation experiments were executed using argon as a collision gas at a collision energy of 30 eV with a spread of 5 eV.

For quality control (QC), pooled samples were utilized to condition the LC-MS system and implement non-linear signal correction. These QC samples were injected at the start and conclusion of the batch to ensure system equilibration. Additionally, sample acquisition was randomized, and QC samples were analyzed every 10 injections to monitor and rectify changes in the instrument response.

The identification of metabolites was conducted using the SIRIUS software as outlined by Dührkop *et al*., (2019). SIRIUS software version 4.9.12 was obtained from the Lehrstuhl Bioinformatik Jena website (https://bio.informatik.uni-jena.de/software/sirius/). Raw data acquired from the Shimadzu LCMS-9030 qTOF was converted to an open-source format (.mzML) before being imported into the SIRIUS software. To compute molecular formulas, the instrument type was specified as qTOF, mass accuracy was set at 10 ppm, and possible ionization was chosen as $[M–H]^+$. Element searches focused on C, H, N, and O, with the number of candidates set to 10. For structure elucidation through

CSI: FingerID, searches spanned all databases with $[M–H]^+$ as the sole adduct, following the approach by Dührkop *et al*., (2015). Canopus Class Prediction was also activated, following the methodology outlined by Hoffmann *et al*., (2021).

### 3.2.6 Statistical analysis

The data was analysed using analysis of variance (ANOVA). The antimicrobial and DPPH data were described as mean ± standard deviations (SD). This analysis was done using GraphPad prism 9 (Belayneh *et al*., 2022) and *p* values less than 0.05 were statistically different.

## 3.3 Results

### 3.3.1 Bacterial growth and metabolite extraction

The isolates were grown in LB broth for 48 hours for monitoring bacterial growth over time. Figure 3.1 shows the growth curves for the five endophytic bacterial isolates and similar growth profiles were recorded for the *Pseudomonas* isolates (EER7, EER9, EER10). Furthermore, the *Pseudomonas* isolates were observed to reach the stationary phase of growth within the first 12 hours post inoculation while EER1 and EER6 reached this phase in 24 hours. The stationary growth phase was selected for the extraction of the crude extract as secondary metabolites are formed in a fermentation medium after the microbial growth is completed.

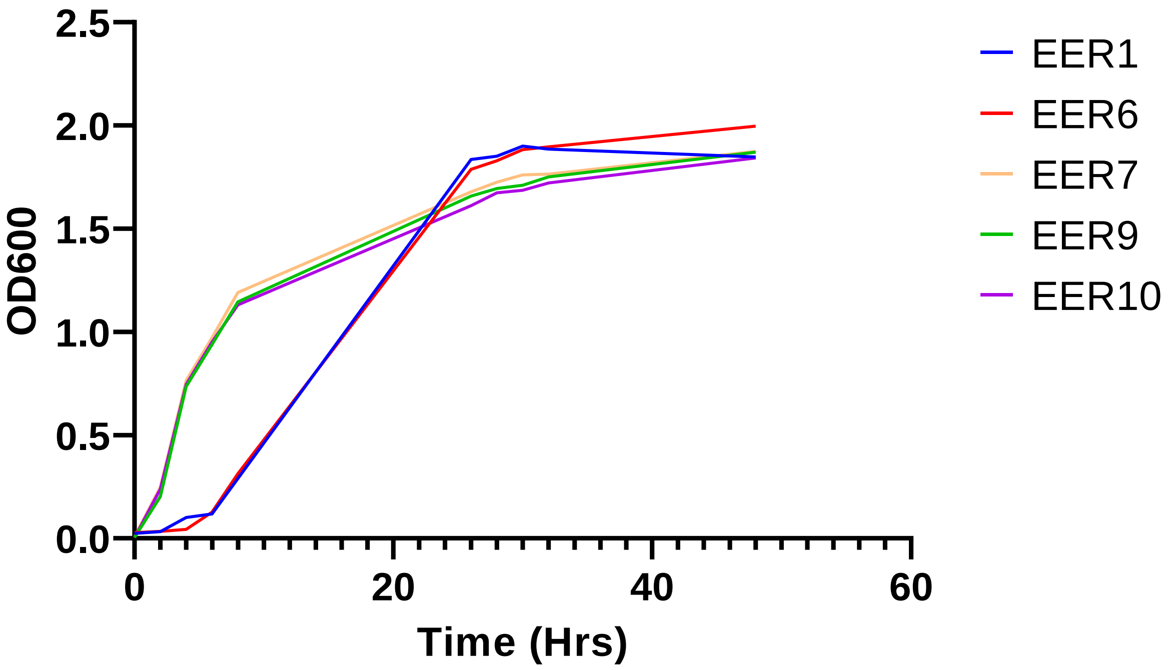


Figure 3-1: The growth analysis of the bacterial isolates (2 hours interval for 48 hours).

### 3.3.2 Antioxidant activity assay

The DPPH assay with the crude extracts revealed that all the bacterial isolates were positive for free radical scavenging activity. Isolates showed antioxidant activity on all tested concentration range (3-500 µg/mL) with inhibition percentages ranging from 33% to 76% (Figure 3.2). The scavenging activity of crude extracts is concentration dependent. At the lowest concentration (3.90625 µg/mL) EER6 and EER9 had a higher percentage inhibition than the positive control ascorbic acid (Figure 3.2). This showed that the crude extracts were good antioxidant agent even at lower concentrations. The endophytic extracts showed higher $IC_{50}$ values ranging between 15.65-42.12 µg/mL compared to vitamin C, which had an $IC_{50}$ value of 8.33 µg/mL. Vitamin C showed a higher antioxidant potential than the endophytic extracts. EER6 showed high antioxidant activity amongst the endophytic crude extracts as it had the lowest $IC_{50}$ value of 15.65 µg/mL (Table 3.1).

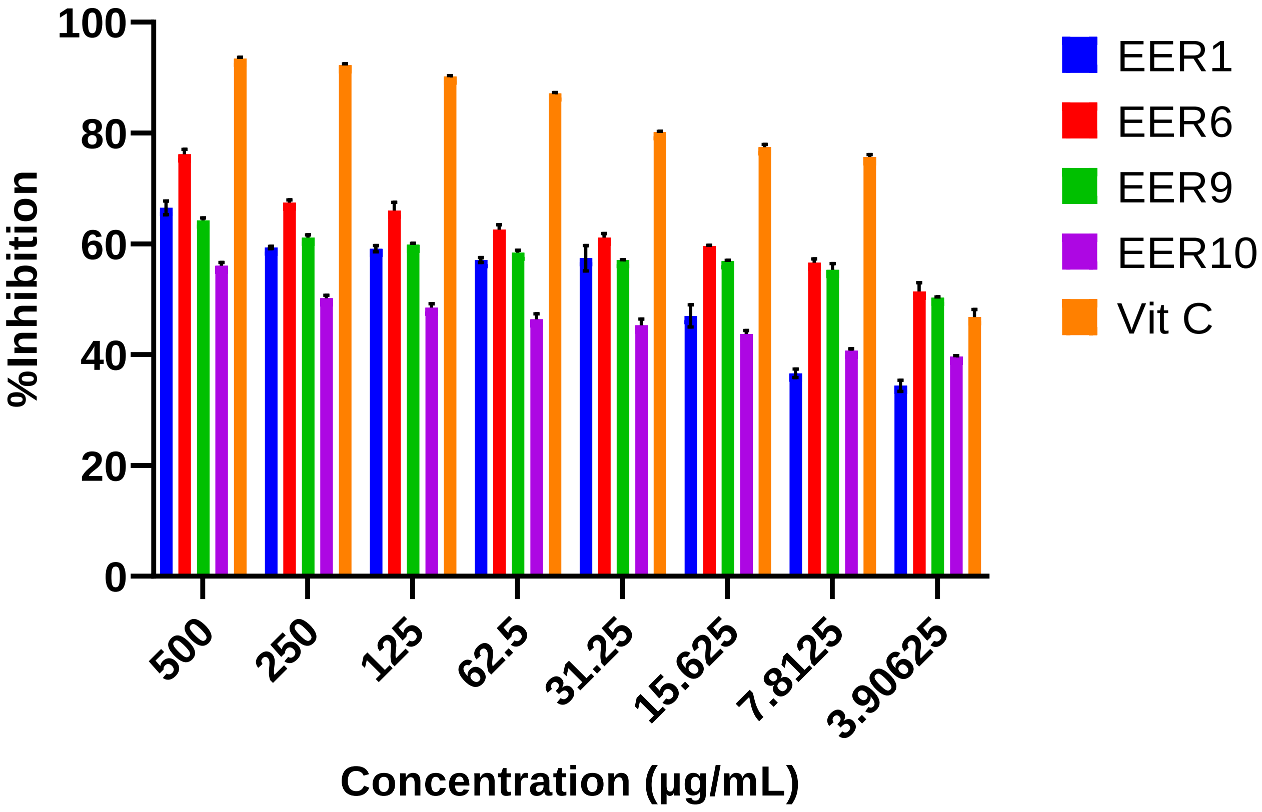


Figure 3-2: DPPH free radical scavenging activity of endophytic bacteria crude extracts (n=3), *p* values less than 0.05 were statistically different. Vitamin C was used as a positive control.

Table 3-1: Half-maximal inhibitory concentration values of vitamin C and bacterial endophytes' crude extracts for the DPPH assay.

| **Isolate crude extract** | **IC50 values (µg/mL)** |
|---|---|
| **EER1** | 25.77 |
| **EER6** | 15.65 |
| **EER9** | 18.32 |
| **EER10** | 42.12 |
| **Vit C (Positive control)** | 8.33 |

### 3.3.3 Antimicrobial activity of crude extracts

The antibacterial activity of endophytic bacteria's crude extracts was determined against seven pathogenic strains: Gram-negative bacteria *Escherichia coli* (ATCC25922), *E. coli* 0.157 (ON645905), *Salmonella* sp, and *Vibrio cholerae*; Gram-positive bacteria

*Staphylococcus aureus* (ATCC26923), *Bacillus sp.,* and *Enterococcus durans.* The MIC activity exhibited variability based on the specific pathogen employed, revealing noteworthy outcomes particularly in terms of antimicrobial effectiveness. The range of the minimum inhibition concentration for the extracted secondary metabolites from the crude extracts of bacterial endophytes was observed to be between 62.5 and 250 µg/mL. EER10 recorded the lowest MIC values amongst all the extracts, which shows high antibacterial activity. When the endophytic bacteria's crude extracts were tested against *Salmonella* sp, the endophytic bacterial crude extracts showed lower MIC values (250 µg/mL) than the positive controls (ampicillin). The lowest MIC value was 62.5 µg/mL, which was observed for EER10 against the pathogenic strain *Bacillus* sp. (Table 3.2).

Table 3-2: The MIC values of antimicrobial activities of endophytic bacterial extracts.

| **Bacterial Strain** | **BNWU1 MIC (µg/mL)** | **BNWU2 MIC (µg/mL)** | **BNWU4 MIC (µg/mL)** | **BNWU5 MIC (µg/mL)** | **Ampicillin MIC (µg/mL)** | **Streptomycin MIC (µg/mL)** |
|---|---|---|---|---|---|---|
| ***Staphylococcus aureus* (ATCC26923)** | 250 | 250 | 250 | 125 | 62.5 | Not tested |
| ***Escherichia coli* 0.157 (ON645905)** | 250 | 250 | 125 | 125 | 15.625 | Not tested |
| ***E. coli* (ATCC25922)** | 250 | 250 | 250 | 125 | 15.625 | Not tested |
| ***Salmonella* sp** | 250 | 250 | 250 | 250 | No activity | 500 |
| ***Bacillus* sp.** | 250 | 125 | 125 | 62.5 | 125 | Not tested |
| ***Enterococcus durans*** | 250 | 125 | 250 | 125 | 15.625 | Not tested |
| ***Vibrio cholerae*** | 125 | 125 | 250 | 125 | No activity | 62.5 |

### 3.3.4 Metabolite identification

To determine the compounds responsible for the observed antioxidant activities (Table 3.3) and antimicrobial effects (Table 3.4), LC-qTOF-MS analysis was conducted. The identification of compounds relied on their retention times (RT), parent masses, and molecular formulas, as depicted in Tables 3.3-4. The LC-qTOF-MS examination of bioactive compounds within the bacterial endophyte extracts revealed the presence of fatty acids, alkaloids, flavonoids, peptides, and various phenolic compounds. These identified compounds were associated with the noteworthy antimicrobial and antioxidant properties observed in the bacterial endophyte extracts (Table 3.3-4).

Table 3-3: Metabolites associated with antioxidant activity.

| RT(min) | Parent mass | Molecular formula | Name of the compound | Biological activity | Bacterial endophyte | References |
|---|---|---|---|---|---|---|
| **Alkaloids** | | | | | | |
| 2.02 | 251.15 | $C_{12}H_{18}N_4O_2$ | Cyclo (histidyl-leucyl) | Antioxidant activity | EER1,EER10 | Furukawa *et al*., 2012 |
| 4.95 | 188.07 | $C_{11}H_9NO_2$ | Indoleacrylic acid | Antioxidants, anti-inflammatory properties | All | Wlodarska *et al*., 2017 |
| 5.57 | 217.10 | $C_{12}H_{12}N_2O_2$ | L-Abrine | Anticancer, antioxidant properties | EER9 | Laskar *et al*., 2019 |
| **Flavonoids** | | | | | | |
| 12.82 | 311.16 | $C_{20}H_{22}O_3$ | Avobenzone | Antioxidants, Anti-aging, Anti-radiation activity | All | Gamit *et al.,* 2023; Gholap *et al*., 2023 |
| 12.50 | 379.24 | $C_{24}H_{27}NO_2$ | Octocrylene | Antioxidant activity, Anti-aging, Anti-radiation activity | All | Li *et al*., 2018; Saboon *et al*., 2023 |
| **Lipid** | | | | | | |
| 14.15 | 473.40 | $C_{31}H_{52}O_3$ | Alpha-Tocopherol Acetate (Vitamin E acetate) | Antioxidant, neuroprotective, antiviral, immunomodulatory, and anti-proliferative | EER6 | Yang and McClements, 2013 |
| **Phenolic compounds** | | | | | | |
| 11.24 | 279.16 | $C_{16}H_{22}O_4$ | Dibutyl Phthalate | Antifungal, antibacterial, antiviral, and antioxidant activities | All | Khatiwora *et al*., 2012; Shobi and Viswanathan, 2018 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 5.76 | 261.12 | $C_{14}H_{16}N_2O_3$ | Maculosin | antioxidant, anti-cancer and non-toxicity | All | Paudel *et al*., 2021 |

Table 3-4: Metabolic identification of antimicrobial compounds from bacterial endophytes' crude extracts associated with *E. elephantina.*

| **RT(min)** | **Parent mass** | **Molecular formula** | **Name of the compound** | **Biological activity** | **Bacterial endophyte** | **References** |
|---|---|---|---|---|---|---|
| **Alkaloids** | | | | | | |
| 12.51 | 250.09 | $C_{16}H_{13}NO_3$ | 3-Hydroxy-3-phenacyloxindole | Antimicrobial, anticancer, and anti-inflammatory properties. | All | Barupal and Fiehn, 2019 |
| 12.50 | 250.09 | $C_{16}H_{11}NO_2$ | Cinchophen | Antimicrobial | EER1,<br>EER6,<br>EER9 | Ezelarab *et al*., 2023 |
| **Lipids** | | | | | | |
| 13.02 | 563.56 | $C18H35NO_2$ | 9-Octadecenamide | Antimicrobial | All | Dos Reis *et al*., 2019 |
| 13.59 | 310.31 | $C_{20}H_{39}NO$ | cis-11-Eicosenamide | Antimicrobial activity, Biocontrol | All | Qi *et al*., 2022 |
| **Peptides** | | | | | | |
| 0.343 | 756.39 | $C_{43}H_{53}N_3O_9$ | Beauvericin G2 | Anticancer, Anti-inflammatory, Antimicrobial activity | ERR10 | Xu *et al*., 2007, Wu *et al*., 2018 |
| 4.73 | 279.13 | $C_{14}H_{18}N_2O_4$ | Tyrosylproline | Antibacterial | EER9 & EER10 | Wattana-Amorn *et al*., 2016 |
| 2.57 | 369.11 | $C_{16}H_{20}N_2O_6S$ | Phenoxomethylpenicilloyl (penicilloyl V) | Antimicrobial activity | All | (WHO *et al*., 2009) |

### 3.4 Discussion

In this study, the isolated bacterial endophytes were observed to reach the stationary growth phase post the 20-hours and this growth phase was selected for the extraction of secondary metabolites. During the stationary phase, bacteria are prone to producing secondary metabolites, or metabolites produced after active growth, such as antibiotics and siderophores (Ruiz *et al*., 2010). A similar growth profile was observed for *Pseudomonas* species (EER7, EER9, and EER10) when cultured in LB broth. This possibly indicates a genus-specific phenotype in this context and demonstrates that there was a close relationship between the isolates. After growth analysis, secondary metabolites were extracted and assessed for antioxidant and antimicrobial properties using the broth dilution method and DPPH assay, respectively.

The DPPH assay is a popular, quick, easy, and affordable tool for the measurement of antioxidant properties of natural products based on the quenching of stable-colored radicals (Narkhede *et al*., 2016; Baliyan *et al*., 2022). Ascorbic acid was used as a positive control because of its known antioxidant activities. Antioxidant activity is considered as follows: very strong (IC50 < 50 µg/mL), strong (50≤ IC50 < 100 µg/mL), moderate (100≤ IC50 < 150 µg/mL), and low (IC50 > 150 µg/mL) (Saptarini and Wardati, 2020). The IC50 values for the bacterial endophyte extracts ranged between 15.65 - 42.12 *µ*g/mL, therefore, on this basis, all bacterial endophyte extracts showed very strong antioxidant activities.

EER6 had the highest free radical scavenging among all the other isolates with an IC50 value of 15.65 µg/mL, this may be because it was the only isolate that produces alpha-tocopherol acetate (vitamin E acetate), which is known for its capability to neutralize endogenous free radicals (Jiang, 2014). EER1 also showed high free radical scavenging activities value of 18.32 µg/mL and this is also supported by Rahman *et al*., (2017), who reported that endophyte *S. maltophilia* isolated from *Fagonia indica* showed high DPPH scavenging activity. The pseudomonas species (EER9 and EER10) showed potential antioxidant activities. This observation is a correlation study to the one conducted by Nxumalo *et al*., (2020), who reported that *Pseudomonas species* produced secondary metabolites with antibacterial and antioxidant activities. The notable antioxidant activity demonstrated by the crude extracts in scavenging free radicals strongly suggests that endophytes hold promise as a valuable reservoir of bioactive compounds. These compounds could be harnessed for the formulation of innovative therapeutic drugs and antioxidant supplements.

The high antioxidant and antimicrobial properties showed in this study may be associated with flavonoids, alkaloids and phenolic compounds quantities in crude extracts, as shown in our LC/MS results (Table 3.3-4), wherein a number of flavonoids and phenolic compound were detected and identified. Phenolic compounds and flavonoids are known to have an ideal chemical structure for free radical scavenging activity (Kannan *et al*., 2016). Dibutyl phthalate was a phenolic compound identified in this study, it has been reported to possess antifungal, antibacterial, antiviral, and antioxidant activities (Khatiwora *et al*., 2012; Shobi and Viswanathan, 2018). This observation corresponds to that of Shobi and Viswanathan (2018), who reported on the antibacterial activity of dibutyl phthalate isolated and purified from *Begonia malabarica*. Furthermore, alkaloids and flavonoids including octocrylene, cyclo(histidyl-leucyl), avobenzone, indoleacrylic acid, and L-abrine were also detected in this study, which were previously reported to possess antimicrobial and antioxidant properties of the endophytic bacteria's extracts observed. Avobenzone and octocrylene, which are known antioxidant agents, are used as ingredients in skin care products and cosmetics (Jesus *et al*., 2023). Indoleacrylic acid, which is a promising compound in drug discovery, possesses antibacterial, antioxidant, anti-inflammatory, and antidiabetic properties (Wlodarska *et al*., 2017; Liu *et al*., 2023). Flavonoids and phenolic compounds function as both primary and secondary antioxidants, demonstrating a significant role in mitigating lipid peroxidation and thereby serving as effective antioxidants (Kaur *et al*., 2020).

The antimicrobial activity of the crude extracts from the bacterial isolates was determined against seven pathogenic strains: *Escherichia coli* (test strain), *E. coli* 0.157 (environmental strain), *Salmonella* sp, *Vibrio cholera*, *Staphylococcus aureus* (ATCC26923), *Bacillus sp.,* and *Enterococcus durans.* The MIC activity differed depending on the test strain utilised, the extracts showed interesting results as they were effective against all the test strains with MIC ranging between 62.5-250 µg/mL. Crude extracts exhibiting a minimum inhibitory concentration of 1000 µg/mL or lower are considered significantly active (Van Vuuren, 2008). Therefore, all crude extracts were strong antimicrobial agents. All the strains outperformed the positive control in terms of MIC against *Salmonella* sp. EER10 had high activity against bacillus when compared to the control. EER6 and EER9 recorded the same MIC as the control against *Bacillus* sp. This observation underscores the substantial potential of endophytic bacteria derived from *E. elephantina* for the synthesis of bioactive compounds with demonstrated efficacy against pathogenic microbes, as demonstrated in Table 3.3. The obtained results agreed with other previous studies, that have proven that endophytic bacteria are potential sources of novel antimicrobial agents (Makuwa and Serepa-Dlamini, 2021). Furthermore, the alkaloids, flavonoids, and phenolic compounds that were detected in this study had been previously reported to possess antimicrobial activities and exert antioxidant properties both *in vitro* and *in vivo* (Martins *et al*., 2016; Ademović *et al*., 2017; Yin *et al*., 2016; Rehman and Khan, 2017).

Bioactive compounds including cinchophen, 9-octadecenamide, tyrosylproline, beauvericin, and phenoxomethylpenicilloyl (penicilloyl V) (Table 3.4) that are known for antimicrobial activities were detected in this study. Beauvericin, which was only detected in EER10, belongs to the enniatins antibiotic family, and is a cyclic hexadepsipeptide identified by the alternate presence of three d-hydroxy-isovaleryl and three N-methyl-phenylalanyl groups (Hamill *et al*., 1969). Caloni *et al*., (2020) reported that beauvericin has many biological activities such as antibacterial, antiviral, antifungal, antiparasitic, insecticidal and anticarcinogenic activities, and this explains the high antimicrobial activity of this endophytic bacterium. Phenoxomethylpenicilloyl is presently employed as a prescribed antimicrobial agent for the treatment of specific bacterial infections, including upper respiratory tract infections caused by *Streptococcal* and *Pneumococcal* strains, as well as for the prevention of rheumatic fever and chorea (WHO *et al*., 2009; Rawson *et al*., 2021).

Epicatechin-(4beta->8)-4'-O-methylgallocatechin, a flavonoid identified in this study, has been reported to have wound healing activity (Schmidt *et al*., 2011; Ramalingam *et al*., 2022). Interestingly, similar compounds that include epigallocatechin gallate, epicatechin gallate, and epicatechin, have been reported in *E. elephantina* (Maroyi, 2017). Therefore, this supports the reports on endophytes that synthesize related bioactive compounds as their host plant and therefore, can be further explored as alternative sources for the sought-after bioactivities.

## 3.5 Conclusion

The secondary metabolite extracts of EER1, EER6, EER9, and EER10 showed potential antioxidant bioactivities that were comparable to the positive control of ascorbic acid. This showed that the isolated endophytes have a great potential to be used as antioxidant agents that reduce free radicals. Through this discovery, the isolated endophytes might help to solve the issues of aging, cancer, chronic diseases, and cardiovascular diseases. Additionally, the bioactive secondary metabolites showed interesting antimicrobial activities with EER10 showing the highest antimicrobial activity. Bioactive compounds such as alpha-tocopherol acetate, dibutyl phthalate, cinchophen, 9-octadecenamide, tyrosylproline, beauvericin, and phenoxomethylpenicilloyl were detected in this study. These compounds have been previously reported to possess antimicrobial and antioxidant activities. Therefore, it would be interesting to investigate other biological activities such as antioxidant, antidiabetic, and anticancer of the endophytes' crude extracts using *in-vivo* assays.

# Chapter 4: Summary and concluding remarks

This study gave first insights into the endophytic bacterial community found in the leaves and roots of the medicinal plant, *Elephantorrhiza elephantina*. The leaves and rhizome were found to consists of the following phyla: Proteobacteria, Bacteroidota, Gemmatimonadota, Actinobacteriota, Verrucomicrobiota, Dependentiae, Firmicutes, and Armatimonodata, with Proteobacteria being the dominant phylum. Furthermore, 4.51% and 17.60% bacteria in leaves and rhizome respectively were novel as they did not show homologies to previously studied bacteria phyla in the database. Therefore, this means that there was a potential to isolate previously unidentified bacteria. The study thus provides a foundation for future research on focused isolation, characterization, and application of metabolite-producing endophytic bacteria from *E. elephantina*.

In this study 5 endophytic bacteria were isolated from the surface-sterilized rhizome of *E. elephantina* and identified as S*tenotrophomonas maltophilia*, *Microbacterium oxydans*, and other three as *Pseudomonas* species using the 16S rRNA gene analysis. The diversity and abundance of endophytic bacteria isolated from *E. elephantina* was lower as compared to metagenomics analysis. Therefore, for future studies, we recommend using various culture conditions including modified culture media and adjusted temperatures. The morphological identification of endophytic bacteria was extended through the application of the Gram staining technique, resulting in their classification into two groups: Gram-positive bacteria (comprising 4 isolates) and Gram-negative bacteria (comprising 1 isolate).

To the best of our knowledge, this is the first study to report on the antimicrobial and antioxidant bioactivities of endophytic bacteria from *E. elephantina*. All the crude extracts of the selected endophytic bacteria showed potential antimicrobial and antioxidant bioactivities. The demonstrated antibacterial effects of the crude extracts from the bacterial endophytes acquired in this investigation offer support for the idea that endophytic bacteria may possess the potential to outcompete pathogenic bacteria and this also suggests their potential utility in drug discovery. While the endophytic bacteria linked to *E. elephantina* did not synthesize identical bioactive secondary metabolites as their host, they were related to the classes of nature of compounds identified, which possessed the same biological activities. The analysis of the extract from bacterial endophytes revealed the existence of diverse bioactive compounds previously documented for their prospective applications in pharmaceuticals and agriculture. Consequently, further investigations into bacterial endophytes hold promise for the innovation of novel therapeutic drugs. Such studies, focused on the screening and identification of the secondary metabolites they produce, have the potential to contribute to an enhanced comprehension of the biochemical pathways governing the synthesis of these biologically active compounds.

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