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Metagenomic analysis microbial biodiversity of Trojena' the Mountains of Neom

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Abstract

About 80% of the biosphere is constantly exposed to temperatures below 5 °C in cold environments. Microorganisms in cold environments can grow and decompose various organic compounds at sub-zero temperatures despite exposure to conditions that are harmful to their survival, such as sub-zero temperatures and low nutrient and water availability. The present study was designed to investigate metagenomic insights into the microbial diversity in (Al-Lawz Mountains / Trojena Mountains) Saudi Arabia. Metagenomic data sets are obtained by high-throughput sequencing of environmental soil samples and provide an aggregation of all the conceptually genetic materials of the intended area of this project. This study easily overcomes the bottlenecks associated with conventional molecular methods of retrieving genetic information and the unscientific shortage of microbial biodiversity research at Tabuk. High throughput bioinformatic analysis has been highlighted as the accurate exploration of the abundance and diversity of bacterial communities. Environmental DNA can be sequenced to identify the recent presence, relative abundance & distribution of a prokaryotic species or whole communities of bacteria. A total of 333 bacterial metagenomes were sequenced over two seasons, fall and winter. The 16S rRNA genes were quantified during this period. The most significant species regarding the relative abundance and diversity were in the location of sample1 by, *Klebsiella michiganensis* (251), *stenotrophomonass maltophilia* (110), *Escherichia coli* USML2 (88), *Zhongshania aliphaticivorans* (40), *Acidibrevibacterium fodinaquatile* (12) *Calothrix spp.* & *Nibribacter ruber* (10) *Bacillus spp* (10) respectively. On the other hand, the lowest abundances were in sample 4 location with *Pseudomonas fluorescens* (5) and *Corynebacterium glutamicum* (3) with (NA) species. This means these were unidentified yet. All these species have a growing demand for microbial biodiversityevaluations, given the pronounced impact of climate change in this region (Al-Lawz Mountains/TrojenaMountain). Benthic microbial communities are to be considered, given they have a potential role in CO_2 and nitrogen fixation, which is related to plant growth-promoting properties. They can resist salinity, radiation, low-temperature adaptation, and biocontrol properties. Thus, eDNA cold-mountain biodiversity is a fraction of the time it costs to conduct conventional ecological monitoring.

Keywords: Metagenomics, Microbial diversity, Genetic materials

1. Introduction

Bacteria and Archaea represent the two domains of prokaryotes present in almost all conditions on the planet Earth [1]. Therefore, investigations of bacterial diversity at particular locations will help us understand the causes and outcomes of variability in phenotype, genotype, and ecological functions within the microbiome [2]. Such variability is presumed to be the selection outcome, evident in bacterial populations challenged with natural or anthropogenic environmental pressures [3].

Molecular methods have dramatically increased understanding of microbial diversity in recent years both from a phylogenetic and taxonomic perspective and from an ecological level. One such essential tool for cataloging microbial life is the metagenomics technique, which is defined as the study of microbial communities utilizing high-throughput DNA sequencing technologies without needing laboratory culture. It shows promise in reaching the uncultured majority. Estimating the quantity of organisms in a sample based on the frequency of the organism's DNA has been a fundamental challenge in metagenomics. Insights on the population dynamics of microbial communities and the functions performed by specific community members may also be provided through metagenomics[4]. A typical metagenomic sequencing experiment first identifies a community of interest, isolates its complete genomic DNA, and then performs high throughput sequencing on randomly selected DNA fragments in the separated DNA. Shotgun metagenomics or environmental shotgun sequencing are typical names for the process. Sequence readings can be constructed if the sample is simple [4]. Access to the genetic diversity of natural microbial communities has

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been made possiblevia metagenomics. Several techniques have been developed to process and analyze the sequence data, from raw reads to final products like predicted protein sequences or families [5].

It is expected that bacteria can live anywhere on Earth. Temperature is the only variable that might limit their ability to exist or function. Scarce reports of bacterial isolation have been published on isolating bacteria from mountains in Saudi Arabia. Such studies explored the bacterial diversity in the southwestern region of Saudi Arabia (Asir Mountains) and the Al-Ahsa region in eastern Saudi Arabia. They identified several bacterial strains from soil samples, including Bacillus, Micrococcus, Arthrobacter, Pseudomonas, Actinomycetes, and Streptomyces species [6]. The cold-adapted bacteria have developed biochemical and molecular defense mechanisms to tolerate extremely low temperatures [7]. Cold-adapted bacteria alter their lipid composition to preserve cell membrane integrity in low-temperature conditions. According to a recent study, bacteria switch between various metabolic pathways to generate energy in response to low temperatures [8].

It is imperative to start transferring the knowledge regarding small subunit rDNA sequences into information that can be utilized to clarify better and comprehend structure-function interactions within ecosystems, develop new cultivating methods, and discover new products and processes [9]. These studies exploring the microbial world and its diversity would benefit humans and the ecosystems that serve as life-supporting units [10]. Almost all of the time, humans benefit from microbes' activities [11]. According to Bhardwaj and Garg, the direct valueof microbes comes from their use in biotechnology, as unicellular protein products, as bio-fertilizers, and as bioprotectants [12]. In contrast, the indirect value comes from their role as decomposers and participantsin the recycling of plant and animal materials, as indicators of environmental pollution, as bioremediation agents, and in other hidden functions of life [13]. These results underscore the extent of uncharacterized microbial diversity and provide fruitful avenuesfor describing additional phylogenetic lineages [14].

Amplification by PCR of taxonomy marker genes (sometimes called "DNA barcodes") is frequently used to study the soil microbial population. Typically, these markers are between 100 and 600 base pairs (bp) long and must be sufficiently varied to offer high taxonomic resolution andbe flanked by conserved sections to cover various species [15]. The term "metabarcoding" refers to combining HTS and barcoding [16]. Due to the relatively short length of these markers, a complete differentiation of microbial species often requires alternate methods, such as single-cell genome sequencing or isolation by cultivation. This thorough approach has allowed soil microbiologists to investigate crucial ecological aspects of soil-plant-microbe structures, including the recognition of bacteria organisms that are (i) dominant or low in abundance across various terrestrial ecological systems, (ii) involved in specific processes (such as the breakdown of litter, nitrogen cycling, degradation of toxic compounds, and many more); and (iii) greater sensitivity to abiotic and bioticfactors. DNA metabarcoding also enables soil communities subjected to experimental circumstances or distance from one another to be compared and soil microbiological diversity (in terms of phylogenetic relatedness) to be assessed. As metabarcoding of DNA is more regularly employed for forensics, monitoring agronomic practices, or restoration efforts, it is also a financially advantageous tool for biomonitoring [17]. So, successful microbiological and taxonomic identification depends on having enough technical knowledge and making educated decisions at each phase [18]. However, there are some significant drawbacks to using metabarcoding of DNA for microbial identification, such as the variable number of copies of the chosen gene marker in bacteria genes, the low taxonomic recovery at the level of species for some microbial groups, and the biases in the taxonomic annotations of sequences based on the variable region selected for the analysis [19]. As a result, choosing the proper mode of operation for each step in a metabarcoding workflow is necessary. Using the wrong techniques while studying the microbiota can lead to incomplete and incorrect biological conclusions [20,21]. Significant biases may develop as a result of the accumulation of both systematic and arbitrary errors throughout the workflow, including sampling, DNA extraction, amplicon library building, sequencing, and bioinformatics [22]. Unfortunately, the majority of the mountainous areas remain unexplored. There are scarce studies in the search for isolation of bacteria using the 16s rRNA and this technique, so most of the data comes from the European Alps or the United States, with only a few researchpapers dealing with the Himalayas or the Andes. Other mountain ranges, such as the Karakorum,Urals, Caucasus, and Pamir in Asia, the Alps in New Zealand, the Atlas and Kilimanjaro in Africa, and so on, were rarely considered. These domains provide a broader view of global environmental processes and critical information on the consequences of climate change [23]. It is hypothesized that the antibacterial qualities and the impact of temperature on the generation of secondary metabolites in soil predictions from cold weather will be determined. Furthermore, it is necessary to examine the meaning of psychrophiles and the determination of eDNA to achieve an accurate abundance of data comparable to traditional methodologies. Considering this background information, the current study focused on metagenomic analysis of soil in the mountains of Saudi Arabia to examine the microbial diversity using barcoding techniques from traditional to 16S RNA sequencing.

2. Materials & Methods

2.1. DNA extraction and PCR of the soil samples

2.1.1. DNA Extraction

According to manufacturer protocol, the DNA from the soil samples was extracted using the DNeasy PowerSoil Pro Kit from Qiagen. In addition, two enzymes- RNase A (2 µg/µl and ProteinaseK (25µg/µl) for removing RNA and Protein, respectively, were used.

- **RNase A** (bovine pancreas RNase) is one of the most characterized proteins and is a member of the RNase A protein superfamily. It exhibits transphosphorylation properties and can catalyze the degradation of RNA. RNase A was dissolved in 10 mM Tris-HCl, pH- 7.5,15 mM NaCl, heated to 100 °C for 15 minutes, and stored at -20 °C after cooling [24].
- **Proteinase K** is a serine protease used to digest proteins by cleaving peptide bonds andis termed proteinase K because it digested hair protein ‘Keratin’ [25].

2.1.2. Polymerase Chain Reaction (PCR)

The 16S rRNA from isolated microbial samples were

amplified using PCR, and the master mix, primers, template, and DNA polymerase were mixed and run in a thermocycler. The PCR master mix refers to a pre-made mixture of reagents such as buffer, dNTPs, and divalent ions required for PCR reaction.

The primers used for 16S rRNA amplification are listed below:

16s rRNA	L2513	GCCTGTTTACCAAAAACATCAC
	H2714	CTCCATAGGGTCTTCTCGTCTT

2.1.3. Gel Electrophoresis

Components used – Agarose 1%; Buffer – 1X TAE; 100 bp DNA ladder

2.1.3.1. Agarose is a heteropolysaccharide derived from red seaweed [26]. It is a linear polymer composed of the repeating unit of agarobiose,a disaccharide composed of D-galactose and 3,6-anhydro-L-galactopyranose [27].

2.1.3.2. TAE buffer is a solution containing a Tris base, acetic acid, and EDTA mixture.

2.1.4. Enzymatic Solution

- **Catalase solution:** Hydrogen peroxide (H_2O_2)

It is used to differentiate those bacteria that produce an enzyme catalase from non-catalase-producing bacteria.

- **Phosphate-buffered saline (PBS)** is a balanced salt solution that contains phosphate ions, which help to maintain a stable pH, and sodium and chloride ions, which help to maintain osmotic balance. This buffer was used to make serial dilution.

2.2. Methods

2.2.1 Sample Site

Al-Lawz Mountains/Trojena mountain, is located in the Kingdom of Saudi Arabia in theTabuk region in Neom (Fig 1). Al Lawz Mountains is characterized by its coldness and snowfall in the winter season, which allows some microbes to grow in it or enter a state oflatency. The most famous plant in this region is the wormwood plant.

2.2.2. Samples Collection

Soil Samples from the different regions on Al-Lawz Mountain were collected and analyzed for microbial diversity. Three samples from three different areas were collected, each consisting of a mixture of 5 samples taken from that region.

A) **Sample No. 1-** One of the most popular plants in Al-Lwaz Mountain is *Artemisia* which grows on rocky soil. The soil samples surrounding *Artemisia* plant were collected on Friday, 29-Dec, 2022, when the temperature was 9 ℃ (coordinates 28°43’58.4"N 35°22’26.5"E) (Fig 2).

B) **Sample No. 2 -** Rocky soil samples were collected when there was snow and temperature was around 2-3 ℃. All samples were collected two hours after the snowfall). Soil samples were collected on Friday, February 24, 2023, (Fig 2).

C) **Sample No.3 and Sample No.4 -** Soil samples (*Alrteem*) were collected from very solid soil, which was difficult to excavate (Fig 2). Soil samples were collected on Friday, 29-Dec 2022, when the temperature was 9℃ (coordinates 28°43’57.8"N 35°22’26.2"E).

Genotypic Methods

2.2.3.1. DNA Extraction from Soil Samples

The initial stage in the metabarcoding method is to extract genetic material from ambient samples. Whole genomic DNA extraction is a critical stage where potential biases must be reduced using proper laboratory techniques [28]. Several commercial kits andsoil, seeds, and plant tissue techniques are available to isolate complete genomic DNA from terrestrial environments (soil and plant material). As soil samples contain all types of microorganisms including bacteria, archaea, fungi and protists and each has different cell disruption method [29]. Therefore, before lysis, the prokaryotic and eukaryoticorganisms were first separated from the soil matrix using density gradient centrifugation [30]. Afterwards, the isolated cell populations were sorted at the single-cell level using flow cytometry or microfluidic devices before DNA extraction and metabarcoding [31].

DNA was extracted from 250 mg soil sample using a Qiagen PowerSoil DNA Extractionkit (Qiagen, Hilden, Germany), and the concentration of DNA was quantified with a Nanodrop.

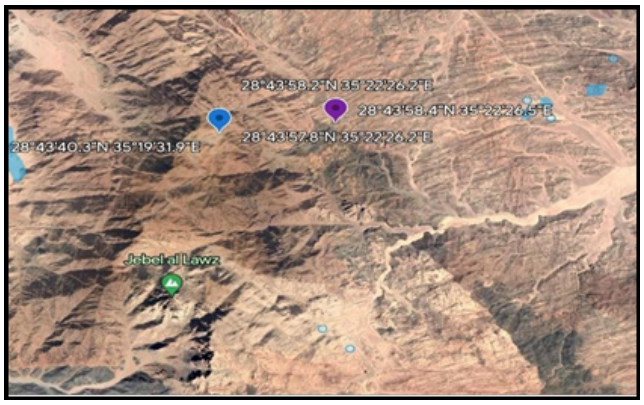

Fig. 1. Sample collection site of Al-Lawz / Trojena Mountain of Neom, Saudi Arabia.

Fig. 2. The sites of the samples collected from soil in Al-Lawz / Trojena Mountain, Neom.

2.2.3.2. DNA Extraction

The Power Bead Pro Tube was spun to ensure the beads had settled at the bottom.250 mgof soil and 800 µl of Solution CD1 was added. The Power Bead Pro Tube was secured horizontally on a Vortex Adapter and vortexed for 10 min at maximum speed. The sample was then incubated in a heat block for 10 min. After incubation, the sample was then centrifuged at15,000 x g for 1 min and the supernatant was then transferred to a clean 2 ml Microcentrifuge Tube. 200 µl of Solution CD2 and 5.0 µl of Proteinase K were added to the supernatant and vortex for 5 sec. The mixture was then centrifuged at 15,000 x g for 1 min at room temperature and the supernatant (~700 µl) was transferred to a clean 2 ml microcentrifuge tube carefully without disturbing the pellet. Next, 600 µL of CD3 solution was added to supernatant and vortexed for 5 sec. Further, 650 µL of lysate was loaded onto an MB Spin column and centrifuged at 15,000 x g for 1 min. The flow was discarded and the process was repeated with remaining lysate. The MB Spin Column was carefully placed into aclean 2 ml Collection Tube and any splashing of flow-through was avoided. Further, 500 µl of Solution EA was added to the MB Spin Column and centrifuged at 15,000 x g for 1 min. The flow-through was discarded and the MB Spin column was placed back into same 2 ml Collection Tube. After that, 500 µl of SolutionC5 was added to the MB Spin Column and centrifuged at 15,000 x g for 1 min. The flow-through was discarded and MB Spin Column was placed into a new 2 ml Collection Tube and centrifuged at up to 16,000 x g for 2 min. The MB column was then placed carefully into a new 1.5 ml Elution Tube. Next, 20 µl of distilled water was added to the center of the white filter membrane and centrifuged at 15,000 x g for 1 min. Lastly, discard the column and check the DNA quality using a Nanodrop device.

2.2.3.3. Gel Electrophoresis Methods

Ethanol-precipitated DNA (2 to 25 µl) was electrophoresed in 0.7% agarose (Seakem, Marine Colloids, Inc.) dissolved in Trisborate buffer (89 mM Tris base, 2.5 mM disodium EDTA, and 8.9 mM boric acid) from cleared lysates. Prior to electrophoresis, DNA samples were treated with a dye solution containing bromophenol blue (0.07%), SDS (7%), and glycerol (33%). The electrophoresis was performed in a vertical lucite slab gel device [32]. The gel's dimensions were 9.6 by 14.2 by 0.6 cm. A Lucite comb with 14 teeth, each 0.508 cm wide and spaced by 0.478 cm, was used to make sample wells. The power supply was provided by Heath kit-regulated high-voltage power supply, type 1P-17, and the electrophoresiswas performed at 60 mA, 120 V, for 2 hours, or until the dye reached the bottom of the gel. Thegel was then stained for 15 minutes in a solution of ethidium bromide in water (0.4 ug/ml) [32].

2.2.3.4. Metagenomic Sequencing

The DNA extracted from soil samples was subjected to metagenomic analysis to catalog microbial life using ribosomal RNA sequencing. 16S rRNA region from the DNA sample was amplified using specific primer via PCR.

2.2.4. Bioinformatics Analysis

The sequenced 16SrRNA region was analyzed and identified using the National Center for Biotechnology Information (NCBI) gene bank, which helps in science and health advancementby providing access to biomedical and genomic information. (https://www.ncbi.nlm.nih.gov/).

a) Bioinformatics analysis workflow - Raw data are filtered to obtain high-quality cleandata, after which clean reads that can overlap with each other are merged into tags and further clustered into Operational Taxonomic Units (OTU). Taxonomic classifications are assigned to OTU representative sequences using the Ribosomal Database Project database. Analysis like alpha diversity, beta diversity, differential species analysis, and network and model prediction are carried out based on the OTU profile table and taxonomic annotation results.
b) Data Filtering: Raw data was filtered to generate high-quality clean reads. Firstly, truncate reads with average Phred quality values lower than 20 over a 25 bp sliding window were truncated. Reads whose length was 75% of their original lengths after truncation were removed. After that, the reads that are contaminated by adapter sequences are removed. Followed by removal of reads with ambiguous base (N base) and low complexity. Clean readswere assigned to corresponding samples through alignments (0 base mismatch) against barcode sequences (16s rDNA) by in-house scripts to ensure the removal of barcode sequences from pooling libraries.
c) OTU is a unified marker for analyzing a taxon unit in the research of phylogeny or population genetics. To quantify the abundance of bacteria on every level in eachsample, the sequences were clustered into OTU with 97% similarity. OTU taxonomy annotation and representative sequences are aligned against the database for taxonomic annotation.
d) Databases: 16S (including bacteria and archaea) & 18S fungus annotation results are filtered by the following steps. The readers who are not annotated and whose that does not match the project's research background were removed.
e)

2.2.5. Phenotypic study

2.2.5.1. Serial dilution

To analyze soil sample's bacterial diversity, 5 ml of Phosphate-buffered saline (PBS)was taken in 15 ml centrifuge tube. This 2 g of soil sample was added and vortexed for 30 min. After 30 min, 200 µl of undiluted soil mixture was taken and spread on a nutrient agar plate.Further, 900µl of Phosphate-buffered saline (PBS) was added in five Eppendorf (EP) tubes andmarked 1 to 5. In the first tube, 100 µl from the unadulterated soil mixture was added and vortexed for 5 sec. From the first tube, 100 µl of soil-PBS mixture was again taken and added to 2nd tube containing 900 µl of PBS and vortexed for 5 sec. Again, take 100 µl of mixture from the 2nd tube and add to the 3rd tube of PBS. Repeated this process for the 4th and 5th PBS tubes. Then, 200µl of each diluted soil sample was added to the nutrient agar and incubated for two weeks at 4 °C.

2.2.5.2. Culture-based methods

The bacteria that grew after the serial dilution process was purified and selected, then cultured on nutrient agar and incubated at 4 °C for two weeks in the laboratory.

2.2.5.3. Gram – Staining

Gram staining is critical for characterizing prokaryotes and classifying them as gram positive and gram negative depending on colour of bacteria when seen under microscope. The Gram stain easily distinguishes bacteria into Gram-positive and Gram-negative groups based on the cell wall and membrane permeability. The bacteria are first spread and fixed on clean grease-free slide. The bacteria is first treated with crystal violet which stains the bacteria blue. The bacteria smear is then treated with iodine. Afterwards, smear is washed with alcohol and then stained with safranin at last and observed under microscope. The gram-positive bacteria appear blue due to presence of thick walls and low lipids whereas alcohol removes crystal violet from gram negative cells allowing them to stain as pink.

3. Results

3.1.1. Cell Enumeration of Bacterial Isolates

Bacterial cell enumeration determines the quantity of bacteria existing in the given sample under specific conditions. Depending on the method used for counting, enumeration process is categorized into four categories - direct, indirect, viable, and total cell count. In this study, indirect method was used to determine the number of bacteria present in the soilsample. Serial dilutions of soil sample in PBS buffer were made (till 10-5) and 100 µl from eachdilution and undiluted sample were spread on nutrient agar plate and incubated for two weeks for bacterial growth. The isolated bacterial colonies were observed on 10-1 and 10-2 dilution (Fig 3). Very limited colonies were observed on plates on which 10-3 and 10-4 dilutions were spread and no bacterial colonies were observed on 10-5.

No bacterial colonies were observed on 10^{-5}. Details regarding the soil samples, including location, temperature at the time of collection, and other characteristics, are summarized in Table 1.

A detailed breakdown of the colony forming units (CFUs) observed at each dilution is presented in Table 2.

3.1.2. Estimation of DNA Quality and Quantity

In this study, we used Qiagen PowerSoil DNA Extraction kit based in Hilden, Germany. This kit was specifically designed for extracting DNAfrom the soil samples, using a manufacturing protocol that allows for efficient isolation of DNA from various soil types, overcoming the challenges posedby the complex nature of soil matrices. The isolated DNA samples were further confirmed using a Nanodrop device to measure the DNA concentration in the soil samples. The concentration and purity of DNA samplesare listed in Table 3.

3.2. Metagenomics Sequencing

3.2.1. Alpha Diversity of Soil Samples

Alpha diversity is a measure of species diversity within sample or community. Metagenomics sequencing revealed that it refers to the diversity of microbial species in

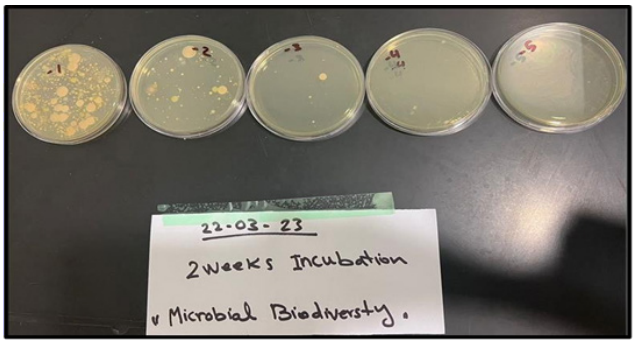

Fig. 3. Bacterial colonies obtained from serial dilution from 10^{-1} to 10^{-5} (A to E) cultivated on nutrient agar.

Table 1. Phenotypic Study of Bacterial Colonies observed on 10^{-1} dilution.

Characteristics	Colonies code				
	IS-1	IS-2	IS-3	IS-4	IS-5
Number	121	114	20	11	21
Shape	Circular	Circular	Circular	Circular	Circular
Elevation	Flat	Flat	Flat	Flat	Flat
Size	Small	Medium to small	Small	Small	Large
Opacity	Opaque	Opaque	Opaque	Opaque	Opaque
Color	Orange	Light Yellow	Red	White	Greyish white
Surface	Soft	Soft	Soft	Soft	Soft

Table 2. Phenotypic Study of Bacterial Colonies observed on 10^{-2} dilution.

Characteristics	Colonies Code				
	IS-1	IS-2	IS-3	IS-4	IS-5
Number	9	19	13	18	11
Shape	Circular	Circular	Circular	Circular	Circular
Elevation	Flat	Flat	Flat	Flat	Flat
Size	Small	Small	Small	Small	Large
Opacity	Opaque	Opaque	Opaque	Opaque	Opaque
Color	Orange	Light Yellow	Red	White	Greyish white
Surface	Soft	Soft	Soft	Soft	Soft

Table 3. The concentration of DNA from the Collected Soil Samples.

Samples & Control ID	Concentration	260/280	260/230
S1	86.9	2.04	0.49
S2	11.2	2.55	0.34
S3	11.7	1.58	0.37
C3	17.1	2.32	0.24

a soil sample. Alpha diversity results from metagenomics sequencing involve assessing various metrics that provide insights into the richness and evenness of microbial species in the sample. Some standard metrics were used to assess alpha diversity inmetagenomics data.

Sample 1 exhibits a considerable variety of microbial species. However, the numberof observed species (represented by the red dot) is lower than the estimated species richness measured by the Chao1 index (represented by the green dot). This suggests that the sequencing effort may not have captured the full extent of the microbial diversity present in the sample. Additional sequencing runs might be necessary to obtaina more comprehensive picture of the microbial community in this sample. Sample 2 indicated that diversity in this sample was relatively acceptable. The observed species (reddot) show a favorable level of diversity, and the estimated species richness (green dot) is similar. This indicates that the sequencing effort has successfully captured the sample's representative range of microbial species. In sample 3, we observed diversity (red dot), which is very close to the estimated species richness (green dot) measured by the Chao1 index. This suggests that the inferred alpha diversity in thissample is relatively low, meaning that the sequencing effort has likely captured most of the microbial species diversity present. The small difference between observed and estimated diversity indicates a relatively comprehensive representation of the microbial community in this sample. Sample 4 exhibits, no diversity is observed in this sample basedon the measurements taken. This suggests a lack of microbial species diversity in the control sample, potentially due to experimental conditions or other factors. The absence of diversity indicates a more uniform microbial community in this particular sample. Figure 4 visually represents these findings, illustrating the differences and distances between the observed and estimated diversity measures (red and green dots). This visualization helps assess the adequacy of

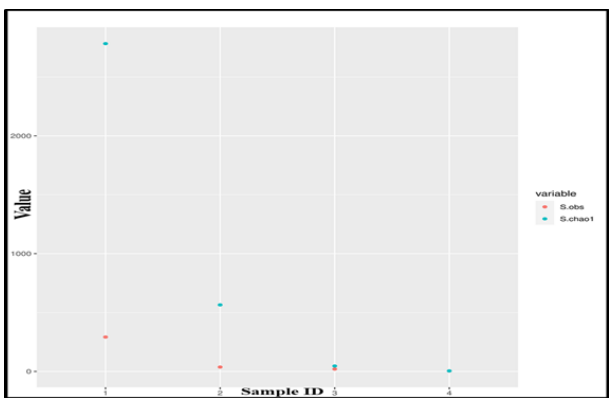

Fig. 4. Alpha diversity in collected soil samples from Al-Lawz/ Trojena Mountain. Sample 1 shows the variety of microbial species and the numberof observed species (represented by the red dot), estimated species richness measured by the Chao1 index (represented by the green dot).

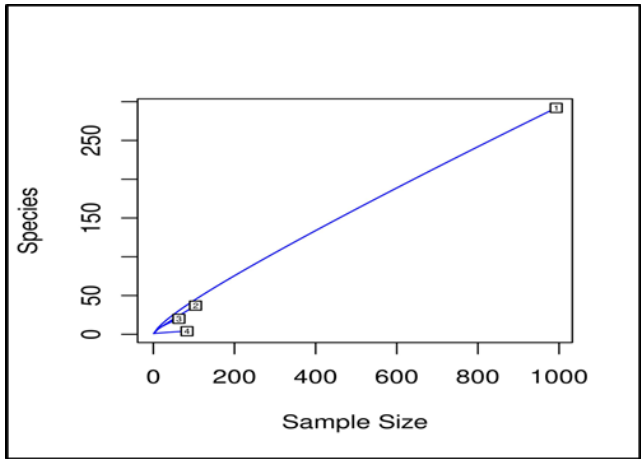

Fig. 5. Refraction analysis of microbial diversity to characterize collected soil sample.

the sequencing efforts and providesinsights into the overall diversity observed in the soil samples.

3.2.2. Refraction Analysis of Metagenomic data to assess Bacterial Diversity in Soil Samples

In the context of a metagenomic study of soil samples, the term "diversity" refers to thevariety and abundance of DNA sequences derived from different microbial organisms present in the sample.

Based on the results obtained from Sample 1, it can be concluded that this particular sample exhibits a significantly higher diversity level than the other samples (Samples 2, 3, and 4). This means that Sample 1 contains more distinct DNA sequences and a wider range of bacterial species than the other samples. As depicted in Figure 5, the metagenomic sequencing data supports this finding by showing a greater dispersion and spread of DNA sequences in Sample 1 compared to theother samples. This suggests a rich and diverse microbial community within Sample 1, which can be further explored and characterized through additional sequencing efforts.

This extended sequencing effort can provide a more comprehensive understanding of the soil sample's microbial composition and functional potential, revealing valuable insights into its ecological dynamics and potential applications. Overall, the metagenomic study of Sample 1 highlights its high diversity, indicating the presence of awide array of bacterial species. Further sequencing and analysis can unlock a deeper understanding of the microbial community and its functional attributes, contributing to our knowledge of soil microbiology and its impact on various ecological processes.

3.2.3. Exploring Dominant Genera in Soil Samples: Insights from Metagenomic bar-plot Analysis

The dominant genera in soil samples were analyzed using metagenomic bar plot analysis. The results revea-

led distinct patterns of genus concentrations among the samples. In Sample 1, all genera showed significant concentrations (Fig. 6). A total of 333 bacterialmetagenomes were sequenced over two seasons, fall and winter.

The 16S rDNA genes were quantified during this time period. The most significant species regarding the relative abundance and diversity in the location of sample 1 were *Klebsiella michiganwns* is by (251), stenotrophomonass (110), *Escherichia coli* USML2 (88), *Zhongshania aliphaticivorans* (40), *Acidibrevibacterium fodinaquatile* (12) *Calothrix sp* & *Nibribacter ruber* (10) *Bacillus spp* (10) respectively. On the other hand, the lowest abundant were in sample 4 location with *Pseudomonas fluorescens* (5), *Corynebacterium glutamicum* (3) with (NA) species, this means, these were unidentified yet. All these species have There is a growing demand for microbial biodiversity evaluations given the pronounced impact of climate change in this region (Al-Lawz Mountains/Trojena Mountain). Benthic microbial communities are important to consider given their potential role in Co2 and Nitrogen fixation, linking with the plant growth-promoting properties. They have the ability to resist salinity, radiation, low-temperature adaptation and biocontrol properties.

CIP-10, which was approximately equal to the dominance observed in Sample 1. Thesefindings highlight the variation in genus concentrations across the soil samples, indicating distinct microbial compositions and potential ecological differences. Further investigation into these dominant genera's functional roles and interactions can provide valuable insights into the soil microbiome and its impact on ecosystem dynamics.

3.2.3.1. Escherichia coli (E. coli) plays a significant role in promoting Soil health due to its various beneficial activities

Nutrient cycling: E. coli contributes to the cycling of essential nutrients in the soil, particularly nitrogen. By fixing atmospheric nitrogen into forms that plants can utilize, E. coli enhances nitrogen availability for plant growth. This reduces the reliance on synthetic nitrogen fertilizers, which can lead to nutrient imbalances and environmental pollution. Soil fertility improvement: E. coli aids in the decomposition of organic matter in the soil.As it breaks down organic materials, it releases nutrients such as phosphorus and potassium, vital for plant growth. This process increases the nutrient content and availability in the soil, improving its fertility and supporting healthier plant development. Soil structure enhancement: The presence of E. coli and its activities contribute to improving soil structure. As the bacteria break down organic matter, they help to create a crumbly soil texture with better aggregation. This enhanced soil structure promotes good aeration, water infiltration, and root penetration, leading to healthier plantroot systems and improved overall soil health. Disease suppression: E. coli can compete with harmful soil-borne pathogens for resources and space. By occupying niches and producing antimicrobial compounds, E. coli creates an environment that is less favorable for the growth and proliferation of pathogenic organisms. This competitive exclusion mechanism helps suppress plant disease incidence and maintain a healthier soil ecosystem.

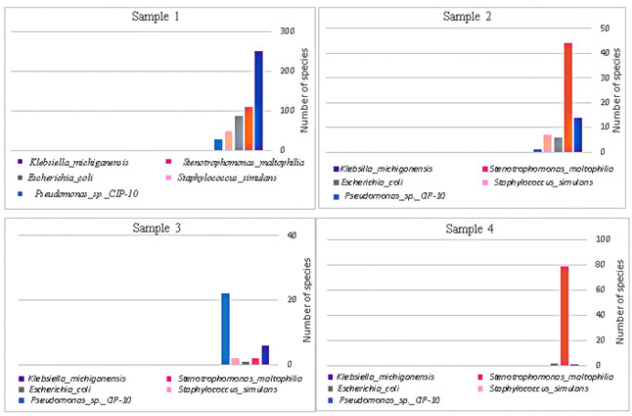

Fig. 6. Relative abundance of microbial diversity in collected soil samples from Al-Lawz / Trojena Mountain of Neom.

Microbial diversity and ecosystem balance: E. coli is a part of the diverse microbial community in the soil. Its presence contributes to the overall microbial diversity, which is crucial for the functioning and resilience of soil ecosystems. A balanced microbial community helps maintain nutrient cycling, decomposition of organic matter, and other important soil processes. E. coli positively impacts soil health through its activities in nutrient cycling, soil fertility improvement, soil structure enhancement, disease suppression, and maintenance of microbial diversity. It contributes to the availability of nutrients, improves soil structure, suppresses pathogens, and supports a balanced soil ecosystem. However, precautions must be taken to prevent the spread of harmful strains. Proper hygiene and responsible use of manure or compost can mitigate associated risks.

3.2.3.2. Benefits of Pseudomonas for Soil Health

Pseudomonas is a genus of bacteria that is widely distributed in soil and water. Many species of Pseudomonas are known to have beneficial effects on soil health and plant growth [33]. One of the key benefits of Pseudomonas in soil is its ability to promote plant growth. The bacteria produce a range of plant growth-promoting substances, including auxins, cytokinins, and gibberellins, which can stimulate root and shoot growth and improve plant nutrient uptake [34]. Pseudomonas can also solubilize phosphorus, making it more available to plants [35]. In addition, Pseudomonas can help to protect plants from harmful pathogens. The bacteria produce a range of antimicrobial compounds that can inhibit the growth of plant pathogens such as fungi and bacteria [36]. Pseudomonas can also induce systemic resistance in plants, making them more tolerant to disease [37]. Research has also shown that Pseudomonas can positively impact soil structure. The bacteria produce exopolysaccharides, which can help to improve soil aggregation and reduce erosion [38]. Pseudomonas can also help break down organic matter in soil, releasing nutrients such as nitrogen and phosphorus that plants can take up [39]. Overall, the benefits of Pseudomonas in soil are numerous and diverse. The bacteria play an important role insupporting healthy plant growth and maintaining soil fertility.

3.2.3.3. Benefits of Klebsiella michiganensis on Soil

Klebsiella michiganensis, a species of Klebsiella, has been found to have potential benefits for soil health and plant growth. While research on its specific effects is limited, studies on other Klebsiella species suggest seve-

ral potential mechanisms for plant growthpromotion and soil improvement. One hypothesis for the plant growth promotion capacity of Klebsiella spp. is its ability to fix atmospheric nitrogen, particularly when associated with gramineous species. This can enhance plant growth by providing a readily available nitrogen source [40]. Klebsiella spp. also produces phytohormones such as indoleacetic acid (IAA) and tryptophol, which can stimulate plant growth. They can solubilize phosphate through the expression of acidphosphatases, aiding in nutrient availability for plants. Additionally, the production of catechol and hydroxamate types of siderophores by Klebsiella spp. can help in nutrient uptake and plant growth promotion [41]. Furthermore, Klebsiella spp. has the ability to form biofilms, which can aid in effective colonization of plant roots and compete with indigenous microflora. This colonization can contribute to improved plant growth promotion through enhanced nitrogen fixation, mineral uptake, phosphorus solubilization, and protection against abiotic stress and pathogens [42]. It is importantto note that these potential benefits of Klebsiella spp. on soil and plant health are based on studies conducted on various species within the Klebsiella genus.

3.2.3.4. Benefits of *Stenotrophomonas maltophilia* on Soil

Stenotrophomonas maltophilia, a species within the Stenotrophomonas genus, has beenfound to have various benefits for soil and plant health [43].

a) Plant growth promotion: Stenotrophomonas spp., including *S. maltophilia*, can enhance plant growth and development. They produce plant growth hormones like indole-3- acetic acid (IAA), contribute to nitrogen fixation, and metabolize organic compounds inthe rhizosphere, providing plants with essential nutrients and protection against phytotoxic compounds.
b) Bioremediation and phytoremediation: Stenotrophomonas spp. has the ability to degradea wide range of organic pollutants, including phenolic compounds, polycyclicaromatic hydrocarbons, and xenobiotics. This metabolic capability makes them valuable in bioremediation and phytoremediation strategies, as they can help detoxify contaminatedsoils.
c) Rhizosphere colonization: Stenotrophomonas spp., including *S. maltophilia*, are commonly found in association with plants, both in the rhizosphere (soil surrounding theroots) and internal plant tissues. They can form beneficial endophytic relationships with plants, contributing to plant health and nutrient acquisition.
d) Influence on leaf surface properties: *Stenotrophomonas* spp. can alter the properties of the leaf surface to which they attach. This can increase water permeability of the cuticles, enhancing water availability and dissolved compound uptake in the phyllo sphere (above-ground plant parts).

It is important to note that while *Stenotrophomonas* spp. offer potential benefits, they can also pose risks, especially in clinical settings where they can exhibit multidrug resistance.

3.3. s-rDNA Gel Electrophoresis Analysis

Gel electrophoresis is a technique used to separate and visualize DNA fragments based on their size. In this case, it was used to assess PCR product quality for 16s-rDNA. A 2% agarose gel stained with Ethidium bromide was prepared, and PCR products were loaded into wells. An electric current was applied, causing DNA fragments to migrate through the gel. The gel was then visualized using UV light to confirm fragment size andamplification success. 16s-rDNA gel electrophoresis analysis of soil samples is a molecular technique used to study the microbial diversity present in soil. It involves extracting DNA from soil samples, amplifying the 16s-rDNA gene region using PCR (polymerase chain reaction), and then visualizing the amplified DNA fragments using gel electrophoresis. The process begins with soil sample collection, followed by DNA extraction to isolate the genetic material from the microorganisms present in the soil. Various DNA extraction methods can be used, depending on the specific study objectivesand the nature of the soil. Once the DNA is extracted, the 16s-rDNA gene region, whichis a conserved region found in the bacterial and archaeal genomes, is amplified using PCR. PCR primers specific to the 16s-rDNA gene region are used to selectively amplifythe target DNA. The PCR reaction includes the extracted DNA, primers, DNA polymerase, nucleotides, and buffer. After the PCR amplification is complete, the resulting DNA fragments are separated by size using gel electrophoresis. A small portionof the PCR reaction mixture is loaded into wells created in an agarose gel. The gel is submerged in a buffer solution and an electric current is applied.

The DNA fragments migrate through the gel matrix based on their size, with smaller fragments traveling faster and farther than larger ones. To visualize the DNA fragments, the gel is stained with a fluorescent dye, such as Ethidium bromide or a more modern alternative like SYBR Green, that binds to the DNA. The gel is then placedunder UV light, which causes the stained DNA bands to fluoresce. A UV fluorescence documentation system or gel imaging system is used to capture an image of the gel, allowing the visualization and analysis of the DNA fragments. The resulting gel image (Figure 7) provides information about the microbial diversity present in the soil sample. Each distinct band represents a different DNA fragment, indicating the presence of specific microbial species or taxa in the soil. By comparing the band patterns among different soil samples or experimental conditions, researchers can assess differences in microbial community composition and identify potential shifts or relationships. Gel electrophoresis analysis ofsoil samples provides valuable insights into the microbial populations and diversity within the soil ecosystem, aiding in the understanding of

Fig. 7. PCR amplification of 16S rDNA hypervariable regions by using universal primers. Lane L: 100 bP molecular weight DNA marker; Bounded Lanes:1-4 samples.

soil health, nutrient cycling, and ecological interactions. It is often used as an initial screening method before more advanced sequencing techniques, such as metagenomics or amplicon sequencing, are employed for more detailed analysis.

4. Discussion

One possible way to address important problems in contemporary agriculture is to use beneficial microorganisms as soil fertilizers and pest control tools [44]. This research revealed a wide range of advantages connected with these bacteria, stressing their possible uses in soil restoration, enhancement of plant development, and long-term insect control. Beneficial microorganisms, including some forms of fungus and bacteria, have remarkable qualities that might greatly affect the condition of the soil. Promoting ideal plant development depends on their capacity to release vital nutrients by breaking down organic components, hence increasing nutrient availability. Emphasising their possible roles in soil remediation, improved plant development, and sustainable insect control, this study demonstrated a broad spectrum of benefits linked with beneficial bacteria [45].

Beneficial microorganisms including several types of bacteria and fungi show special physiological and biochemical capacities that greatly enhance soil fertility and condition. For example, phytohormones and enzymes produced by bacterial strains including Bacillus and Pseudomonas species can boost plant development and maximise nutrient cycling (e.g., indole-3-acetic acid and gibberellin[45]. Moreover, the capacity of these bacteria to fix atmospheric nitrogen is essential in enhancing the soil with this essential component, thereby reducing the demand for synthetic nitrogen fertilizers [44]. Also, an Enhanced understanding of decomposition components elucidates a crucial pathway by which plant species diversity affects decomposition by altering microenvironmental conditions, including soil, microclimate, and throughfall [46].

Furthermore, by means of aggregation and water retention, these bacteria enhance soil structure, therefore helping to reduce soil erosion and runoff a necessary condition for maintaining agricultural output. Especially via mycorrhizal linkages, the development of symbiotic interactions between helpful bacteria and plants enhances plant resilience and general health [47]. Particularly phosphorous, these interactions improve nutrient absorption and give more resistance to drought, disease, and different environmental challenges. Using cooperation of bacteria as soil fertilizers enhances crop yields, plant health, and agricultural sustainability [48].

Products made from helpful bacteria provide a workable substitute for traditional chemical pesticides in pest control. While having little negative impact on beneficial organisms and the environment, Lacey,[49] showed that bacteria including *Bacillus thuringiensis* (Bt), *Beauveria bassiana*, and *Metarhizium anisopliae* can efficiently target and manage particular pests including caterpillars, aphids, whiteflies, and thrips. By reducing reliance on harmful chemical pesticides, these highly selective and environmentally friendly microbial insecticides assist in creating a more sustainable and balanced pest control method.

Identification and addressing of the constraints discovered during this research is crucial to guarantee the efficient application of these microbial-based treatments. The difficulties in obtaining sufficient DNA yields, especially from silt soils may influence the accuracy and sensitivity of molecular analyses [50]. One can circumvent this limitation by consulting experts in the field and investigating other DNA extraction methods catered for challenging soil conditions. This might also help the results to be more reliable. The arduous character of culture-based techniques in cold conditions allows one to lengthen the microbial isolation and identification process. Using molecular techniques like PCR or qPCR, which provide quick and accurate microbe detection, can help greatly reduce the incubation period and speed up the identification procedure [51].

Logistical challenges also arise from postponement in obtaining the necessary permits for accessing specific research sites. Early initiation of the permit approach process, establishing effective communication channels with related authorities, and collaborating with local stakeholders and experts can help mitigate these delays and ensure timely access to desired research locations. Additionally, metagenomic techniques, while powerful for comprehensive understanding of the genetic diversity of microbial communities, require labor-intensive DNA extraction and advanced bioinformatics analyses. Dealing with bioinformatics experts can optimize the data processing pipeline and facilitate the interpretation of metagenomic results, reducing the time and resources required for analysis [52].

5. Conclusion

In summary, this study has highlighted the significant potential of beneficial microbes in addressing key challenges in agriculture. By addressing the identified limitations and leveraging innovative approaches and collaborations, researchers can further unlock the benefits of these microbial-based strategies. The integration of beneficial microbes intoagricultural practices offers a pathway towards sustainable agriculture, with improved soil fertility, enhanced plant health, and reduced reliance on chemical inputs. Embracing these microbial solutions paves the way for a more resilient and environmentally friendly future for global food production. Based on our study, we recommend the following points for consideration.

i. Exploration of microbial biodiversity will help us utilize beneficial microbes as fertilizer for the soil, which can be highly advantageous for rehabilitating the soil and promoting plant growth. Products derived from beneficial microbes can also be utilized to control existing pests.
ii. Concentration of DNA: Obtaining sufficient DNA yields, particularly from clay samples, can be challenging. Clay soils have a high affinity for DNA, making it difficult to extract and concentrate DNA from these samples. The low DNA concentration can limit theaccuracy and sensitivity of downstream molecular analyses.
iii. Culture-Based Method and Cold Conditions: Using culture-based methods under cold conditions can be time-consuming. The incubation of samples at low temperatures, typically required for the growth of specific microorganisms, can extend the incubation duration to two weeks to obtain visible colonies. This prolonged incubation period can significantly impact microbial isolation and identification efficiency and speed.

iv. Delay in Permit Issuance: There may be delays in obtaining necessary permits to access and conduct research in specific locations, such as the Al-Lawz Mountains. These delays can result from bureaucratic processes, logistical challenges, or regulatory requirements. The inability to access particular sites within the desired timeframe can affect the collection of samples and subsequent data acquisition.

v. Metagenomic Techniques: Metagenomic techniques, which involve extracting and analyzing genetic material directly from environmental samples, require additional time and resources. The extraction of DNA from complex environmental samples can be labor-intensive and time-consuming. Furthermore, interpreting metagenomic results involves advanced bioinformatics analyses, which necessitate expertise and may require extended data processing and interpretation time.

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Conflict of interest

All the authors mentioned in the manuscript have no conflict in the research work and compilation.

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