Antibiotic Susceptibility Profile in Bacteria Isolated from Different Natural Thermal Conditions of Sikkim

A Thesis Submitted

To

Sikkim University

In Partial Fulfilment of the Requirement for the

Degree of Doctor of Philosophy

By

Santosh Kumar

Department of Microbiology
School of Life Sciences

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*"I am deeply grateful to my earlier Ph.D. supervisor, **Late Dr. Hare Krishna Tiwari**, Associate Professor and the then former Head in Department of Microbiology at Sikkim University. I would like to dedicate my doctoral thesis to him. His untimely demise has left an irreparable void in my life. Thank you, sir, for showing me the path of hard work, perseverance and passion towards academics and research."*

You will be remembered always!

- Santosh

6 माइल, सामदुर, तादोंग - 737102
गंगटोक, सिक्किम, भारत
फोन-03592-251212, 251415, 251656
टेलीफैक्स - 251067
वेबसाइट - www.cus.ac.in

6th Mile, Samdur, Tadong-737102
Gangtok, Sikkim, India
Ph. 03592-251212, 251415, 251656
Telefax : 251067
Website : www.cus.ac.in

सिक्किम विश्वविद्यालय
SIKKIM UNIVERSITY

(भारत के संसद के अधिनियम द्वारा वर्ष 2007 में स्थापित और नैक (एनएएसी) द्वारा वर्ष 2015 में प्रत्यायित केंद्रीय विश्वविद्यालय)
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Dr. Nagendra Thakur
Ph.D. Supervisor
Associate Professor
Department of Microbiology
Sikkim University
Gangtok, Sikkim

DR. NAGENDRA THAKUR
Associate Professor & Head
Department of Microbiology
Sikkim University (Central University)
Tadong - 737102, Gangtok, Sikkim

Place: 6th Mile, Tadong, Gangtok
Date: 14/9/23

6 माइल, सामदुर, तादोंग - 737102
गंगटोक, सिक्किम, भारत
फोन-03592-251212, 251415, 251656
टेलीफैक्स - 251067
वेबसाइट - www.cus.ac.in

6th Mile, Samdur, Tadong-737102
Gangtok, Sikkim, India
Ph. 03592-251212, 251415, 251656
Telefax : 251067
Website : www.cus.ac.in

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It is recommended this Ph.D. thesis be placed before the Examiners for evaluation.

15/9/2023

Dr. Nagendra Thakur
Associate Professor
Head of the Department
Department of Microbiology
School of Life Sciences
Sikkim University
Gangtok, Sikkim

अध्यक्ष
Head
सूक्ष्मजीव विज्ञान विभाग
Department of Microbiology
सिक्किम विश्वविद्यालय
Sikkim University

Place: 6th Mile, Tadong, Gangtok
Date: 14/09/2023

6 माइल, सामदुर, तादोंग - 737102
गंगटोक, सिक्किम, भारत
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टेलीफैक्स - 251067
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Submitted by **Mr. Santosh Kumar** under the supervision of **Dr. Nagendra Thakur**, Associate Professor & Head, Department of Microbiology, School of Life Sciences, Sikkim University, Gangtok, Sikkim.

Santosh kumar
(Mr. Santosh Kumar)
Signature of Research Scholar

14/9/2023
(Dr. Nagendra Thakur)
Signature of Supervisor

DR. NAGENDRA THAKUR
Associate Professor & Head
Department of Microbiology
Sikkim University (Central University)
Tadong - 737102, Gangtok, Sikkim

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I declare that the present Ph.D. thesis entitled **"Antibiotic Susceptibility Profile in Bacteria Isolated from Different Natural Thermal Conditions of Sikkim"** submitted by me for the award of the degree of Doctor of Philosophy in Microbiology of Sikkim University under the supervision of **Dr. Nagendra Thakur**, Associate Professor & Head, Department of Microbiology, School of Life Sciences, Sikkim University, Gangtok, Sikkim. This is my original research work solely carried out by me in the Department of Microbiology, School of Life Sciences, Sikkim University, Gangtok, Sikkim. No part thereof has been submitted for any degree or diploma in any University/Institution.

Santosh Kumar

Mr. Santosh Kumar
Ph.D. Scholar
Registration No.: 18/Ph.D./MCB/05
Department of Microbiology
Sikkim University
Gangtok, Sikkim

Place: 6th Mile, Tadong, Gangtok
Date: 15.09.2023

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SANTOSH KUMAR

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ABBREVIATIONS

DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
W.H.O	World Health Organization
^oC	Degree Celsius
USA	United States of America
rRNA	Ribosomal ribonucleic acid
Km	Kilo meter
CO_2	Carbon dioxide
H_2S	Hydrogen sulphide
NH_4	Ammonium
CH_4	Methane
CO	Carbon mono-oxide
µL	Micro liter
mM	Milli-molar
m	Meter
sp.	Species
PCR	Polymerase Chain Reaction
OD	Optical density
EDTA	Ethylenediaminetetraacetic acid
MRGs	Metal Resistance Genes
GPS	Global positioning system
ARGs	Antibiotic Resistance Genes
MIC	Minimum inhibitory concentration

BLAST	Basic Local Alignment Search Tool
PAST	Paleontological Statistics Software
VF	Virulence Factor
COG	Cluster of orthologous group
PCA	Principal Component Analysis
AST	Antibiotic Susceptibility Testing
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
SRA	Sequence Reads Archive
NGS	Next Generation Sequencing
OUT	Operational Taxonomic Unit
ARDB	Antibiotic Resistance Genes Database
ICPMS	Inductively coupled plasma mass Spectroscopy
RAST	Rapid Annotation using Subsystem Technology
NCBI	National Center for Biotechnology Information
KEGG	Kyoto Encyclopedia of Genes and Genomes

Introduction

Chapter 1

1. Introduction

A diverse range of bacteria, archaea, and eukarya inhabits different spheres of the Earth, with each sphere governed by its unique abiotic factors such as pH, temperature, pressure, radiation, acidity, and salinity (Reysenbach and Shock, 2002; Singh, 2017). However, not all environments are suitable for the growth of a wide range of organisms. Some environments, known as extreme environments, are inhospitable for most organisms but some specialized organisms can only thrive in them (Rothschild and Mancinelli, 2001). These extreme environments, which include conditions of extreme heat, cold, salinity, and pressure, are primarily inhabited by microorganisms such as thermophiles, psychrophiles, halophiles, and barophiles (Rothschild and Mancinelli, 2001). In some regions, these extreme growth conditions converge to form Hyper Extremophilic Zones (HEZs).

Geothermal systems found in the lithosphere and hydrosphere are particularly interesting extreme ecosystems. Hot springs, fumaroles, hydrothermal vents, and geysers are examples of geothermal features that represent extreme environments where only hyperthermophilic microorganisms can establish their ecological habitats. These hot spots have been extensively studied to understand the effects of environmental parameters on microbial evolution, diversity, and physiology (Hamilton *et al.*, 2012; Menzel *et al.*, 2015; Alcorta *et al.*, 2018). Researchers have examined various physicochemical parameters to study their influence on the growth and distribution of microorganisms, with extreme temperature and pH levels having the most significant impact on limiting microbial diversity. However, the severity of these impacts depends on the geochemistry and other environmental variables (Power *et al.*, 2018; Tang *et al.*, 2018; Zhang *et al.*, 2018).

Hot springs have been extensively studied to understand the factors and mechanisms that enable microbes to thrive in these environments and form diverse microbial communities

(Martiny *et al.*, 2006; Ward, 2006). Previous research has explored the connection between microbial community composition/diversity and physicochemical variables such as temperature, pH, and water chemistry to gain a better understanding of microbial ecology and their roles in geothermal niches (Wang *et al.*, 2013). Temperature gradients have a significant influence on microbial diversity, as bacterial and archaeal abundance fluctuates significantly with temperature variations (Li *et al.*, 2015). It has been observed that the number of microbial cells is usually lower at high temperatures (90.8 °C). Many thermophilic bacteria that do not produce endospores are unable to disperse over wide geographical areas because they cannot tolerate prolonged heat desiccation (Beblo *et al.*, 2009; Podar *et al.*, 2020).

Similarly, the effect of temperature change on the microbial diversity and community structure has been studied along the chronosequence of sub-arctic glacier fore-fields, revealing that the richness and composition of microbial communities are largely determined by soil surface temperature and exposure period (Mateos-Rivera *et al.*, 2016). The Himalayas stand out as a critically significant cold environment on our planet, drawing parallels in climate to the Polar Regions. This expansive region encompasses diverse ecosystems, ranging from temperate to exceedingly cold environments, primarily found at high altitudes. These areas pose significant challenges due to their exposure to intense solar radiation, limited nutrient availability, and freezing temperatures. Within these harsh conditions, microorganisms that thrive can be categorized as either cold-tolerant (psychrotolerants) or cold-loving (psychrophiles) microorganisms (Dhakar and Pandey, 2020). These microorganisms exhibit a range of structural and functional adaptations that enable them to carry out essential life processes in the face of the extreme cold. Their biological activities play a crucial role in maintaining nutrient cycling within the environment and contribute significantly to global biogeochemical cycles (Dhakar and Pandey, 2020). Studies have shown that liquid water within ice and permafrost remains available for metabolism even below the freezing point,

supporting microbial activity (Price and Sowers, 2004). These studies highlight the significance of temperature in shaping microbial growth and diversity. However, the impact of temperature gradients on microbial diversity has not been extensively studied. A recent study by Podar *et al.*, (2020) examined the consequences of a temperature gradient ranging from warm to boiling and found a significant non-linear reduction in the number of microbial taxa as the temperature increased (Podar *et al.*, 2020). Nonetheless, this study focused on a limited temperature range from warm to hot.

Another comprehensive research conducted by Bendia *et al.*, (2018) on bacteria in sediments from fumaroles and glaciers on Deception Island. This volcanic island offered a wide range of temperatures, spanning from 0 °C to a scorching 98 °C. They examined the survivability of thermophilic and psychrophilic bacteria in harsh conditions of desiccation and UV-C radiation. The study successfully recovered these bacteria from the sediments, indicating their ability to endure extreme environments (Bendia *et al.*, 2018). However, the research focused solely on high and low-temperature ranges, omitting the investigation of bacteria thriving in moderate temperatures.

The isolation and identification of these bacteria from these diverse locations provide crucial insights into their resilience and adaptive mechanisms. It underscores their capacity to survive under harsh conditions, where most other organisms would struggle to thrive (Brooks *et al.*, 2011). Such findings expand our understanding of the remarkable diversity and tenacity exhibited by extremophiles, as they continue to challenge the boundaries of life as we know it. Genomic profiling is crucial due to the immense diversity and significance of microbes across various fields. Improved culture and sequencing methods have enabled the exploration of microbial genomes, ushering in a new era of omics-based research that reveals their unique features and variations (Pal *et al.*, 2022). Until now, no comprehensive research/study has encompassed microbial diversity (culture-dependent and culture-independent) along with the

complete temperature spectrum, specifically from hot to warm to cold regions. Similarly, there is a limited understanding of the connection between antibiotic resistance and these gradient thermal zones. It is crucial to comprehend the microbial communities across various temperature ranges, as temperature greatly influences their composition and dynamics.

The Sikkim Himalayas offer a unique natural habitat where hot springs, cold deserts, and glaciers exist in close geographical proximity, making it an intriguing environment for simultaneous study of different temperature zones (Najar *et al.*, 2018a; Sherpa *et al.*, 2019a, 2021; Das *et al.*, 2020; Das *et al.*, 2021; Najar *et al.*, 2022a; Najar *et al.*, 2022b). In this study, we employed both traditional media-based cultivation and high-throughput metagenomic sequencing to examine bacterial diversity in these regions, allowing us to compare culture-dependent and culture-independent approaches. Moreover, metagenomic analysis enabled us to investigate the antibiotic resistance patterns of the microbial communities. Previous studies have established links between temperature and community richness, but these studies focused on independent systems without a complete thermal gradient ranging from extremely cold to boiling zones (Cole *et al.*, 2013; Cuecas *et al.*, 2014; Sharp *et al.*, 2014; Power *et al.*, 2018). We also hypothesized that these geographically isolated areas with distinct thermal gradients would have similar chemical compositions but potentially exhibit discrete microbial community compositions at higher taxonomic levels. To correlate the data with temperature and other physicochemical parameters, we analyzed the water chemistry using Inductively Coupled Plasma Mass Spectroscopy (ICP-MS).

Antibiotic resistance is a global concern and a major public health issue (Das *et al.*, 2018, 2020; Najar *et al.*, 2020a; Sherpa *et al.*, 2020; Singh *et al.*, 2020; Kumar *et al.*, 2022a; Kumar *et al.*, 2023a). The emergence of antibiotic-resistant bacteria can occur through mutations or the acquisition of mobile genetic elements containing resistance genes, regardless of the presence of antibacterial drugs (Roca *et al.*, 2015). The overuse and misuse of antibiotics

contribute to the rising rates of resistance (Roca *et al.*, 2015). However, environmental factors may also play significant roles in shaping selective pressures for the emergence and spread of antibiotic resistance (Najar *et al.*, 2022b). Temperature is a crucial factor to consider when studying antibiotic resistance. Several studies have examined the correlation between temperature and microbial diversity (Diehl and LaPara, 2010; Sun *et al.*, 2016; Tang *et al.*, 2018). However, these studies have primarily focused on specific elevated temperatures. Some studies have shown that the quantity of resistance genes decreases with increasing temperature, as observed in sewage sludge and dairy manure (Sun *et al.*, 2016; Tang *et al.*, 2018). However, a recent study has discovered that antibiotic resistance increases with temperature (MacFadden *et al.*, 2018). Nevertheless, the effect of a temperature gradient (from cold to hot environments via an intermediate warm phase) on the occurrence of antibiotic resistance, specifically concerning diversity, has not been studied. Bacterial diversity variation and antibiotic resistance profiles may be correlated with fitness rates, as resistance in bacteria can enhance fitness rates at distinct temperatures compared to their antibiotic-sensitive ancestors (Herren and Baym, 2022). In accordance with the updated classification of bacterial phyla, we have incorporated the most recent nomenclature as well as the old one for bacterial phyla throughout my thesis. Our taxonomic references align with the latest information provided by Panda *et al*. in 2022 and the NCBI database, as outlined in the NCBI Taxonomy update of prokaryote phyla (https://ncbiinsights.ncbi.nlm.nih.gov/2021/12/10/ncbi-taxonomy-prokaryote-phyla-added/).

In this study, we hypothesize that the occurrence of antibiotic resistance among bacteria will be specific to distinct temperature ranges due to varying fitness costs, contact rates, or horizontal gene transfer. The study area we have chosen provides an ideal environment with varying temperatures within a short distance, allowing us to examine the prevalence of antibiotic resistance under changing temperature conditions. This study is one of the first of its kind to explore the effects of temperature on various factors such as fitness costs, contact rates,

or horizontal gene transfer, which may individually or collectively influence the occurrence of antibiotic resistance. High-throughput metagenomic can contribute to this paradigm and open up new research avenues in understanding the occurrence of antibiotic resistance.

Rationale of the study

The ability of certain microbes to thrive in extreme temperatures, either above 100 °C or near the freezing point, challenges our understanding of the limits and constraints of life. Exploring how these resilient microbial communities survive and adapt to such harsh conditions is a fascinating area of study. Additionally, the existence of microorganisms in these extreme environments has opened up possibilities for the search for extraterrestrial life and the potential transfer of life between planets (Rothschild and Mancinelli, 2001).

Recent research has focused on investigating the physical and chemical factors, such as temperature, pH, and nutrient availability that shape the diversity, activity, and structure of microbial communities in geothermal fields and cold habitats. Among these factors, temperature plays the most significant role, leading to distinct microbial communities in different thermal environments. Several studies have examined the microbial community structures of various geothermal springs worldwide, including the Tengchong thermal springs in China (Hou *et al.*, 2013), Nakabusa hot springs in Japan (Kubo *et al.*, 2011), Siloam hot water springs in South Africa (Tekere *et al.*, 2011), Andean Mountain hot water springs in Colombia (Bohorquez *et al.*, 2012), Solfataric Fields in Iceland (Kvist *et al.*, 2007), Great Basin Hot springs (Costa *et al.*, 2009), and Yellowstone National Park in the USA (Meyer-Dombard *et al.*, 2005).

In India, approximately 400 hot springs have been identified in seven geothermal provinces across the country (Chandrasekharam, 2005). Similarly, various psychrotolerant or psychrophilic bacteria have been isolated from different cold habitats, including *Trichococcus patagoniensis*, *Proteocatella sphenisci*, *Pedobacter himalayensis*, *Exiguobacterium indicum*, *Dyadobacter hamtensis*, *Leifsonia pindariensis*, *Bacillus cecembensis*, *Cryobacterium roopkundense*, *Cryobacterium pindariensis*, and *Paenibacillus glacialis* (Chaturvedi *et al.*,

2005; Chaturvedi and Shivaji, 2006; Kishore *et al.*, 2010). While numerous studies have been conducted on mesophilic bacteria, there is a lack of research on microbial diversity within a confined area exhibiting a range of thermal gradients, from hot to warm to cold environments. To date, only one study has investigated the microbial diversity of extreme thermophilic and psychrophilic bacteria (Bendia *et al.*, 2018). However, this study only focused on isolating bacteria under hot and cold conditions, neglecting the warm conditions. Therefore, it would be intriguing to explore the bacterial diversity across all three thermal conditions.

In Sikkim, previous studies have examined the microbial diversity of hot regions (Najar *et al.*, 2018a; Najar *et al.*, 2020a; Najar *et al.*, 2020b; Das *et al.*, 2021; Das *et al.*, 2023a), warm (Panda *et al.*, 2013, 2017; Panneerselvam *et al.*, 2019, 2021), and cold regions (Sherpa *et al.*, 2018, 2019a, 2020, 2021; Mukhia *et al.*, 2021) bacteria using both culture-dependent and culture-independent techniques. However, the distribution and composition of bacterial communities to different thermal conditions remain unknown.

As mentioned earlier, temperature plays a crucial role in shaping microbial diversity and their adaptation to specific habitats. Deviation from the optimal growth temperature for particular bacteria induces stress, leading to differential gene expressions that facilitate their adaptation. In 2019, Cruz-Loya investigated the physiological effects of multiple classes of antibiotics at various temperatures in *E. coli*, suggesting that temperature stress responses may also contribute to coping with antibiotic stress (Cruz-Loya *et al.*, 2019). While this study was conducted under laboratory conditions, the impact of different thermal conditions on the distribution of antibiotic-resistant bacteria and the types of resistance remains unclear. Considering the presence of all three thermal conditions in the Sikkim Himalayas within a short distance provides an opportunity to examine the presence or absence of antibiotic-resistant bacteria in different thermal environments. Previous studies in Sikkim have evaluated the antibiotic susceptibility of bacteria isolated from hot environments (Najar *et al.*, 2020a), warm

(Pandey *et al.*, 2001; Panda *et al.*, 2013; Singh *et al.*, 2019), and cold regions (Sherpa *et al.*, 2019b, 2020). Interestingly, it was observed that hot environments like hot springs generally lack antibiotic-resistance genes and antibiotic-resistant bacteria, whereas warm and cold environments harbor various types of antibiotic-resistant bacteria. However, as these studies were conducted at different sampling sites, it is challenging to speculate how different thermal conditions influence the distribution and types of antibiotic-resistant bacteria.

In the proposed work, we aim to investigate the microbial diversity across three thermal conditions and examine the presence or absence of thermophilic, mesophilic, and psychrophilic bacteria, as well as their antibiotic resistance patterns, in soil samples.

Aims and Objectives

1. Isolation of bacteria from natural thermal zones

2. Biochemical characterization of the bacterial isolates and 16S rRNA gene based partial identification of some of the bacterial isolates

3. To check the antibiotic susceptibility pattern of the selected bacterial isolates

4. To check the minimum inhibitory concentration (MIC) of the selected bacterial isolates

Review of the Literature

Chapter 2

2. Review Literature

2.1. Unique Geothermal Settings in High-Altitude Mountain Regions

High-altitude mountain regions are known for their breathtaking landscapes and diverse geological features. They often harbor unique geothermal settings that vary from hot to warm and even cold regions. These geothermal regions provide the potential for a variety of activities, including geothermal energy generation, natural spas (hot springs), and scientific investigation (Barbier, 2002; Kumar *et al.*, 2023a; Kumar *et al.*, 2023b). The primary natural thermal regions can be categorized as follows:

2.1.1. Hot Geothermal Regions

Some mountain ranges are home to hot geothermal zones at high altitudes that are characterized by ferocious volcanic activity and geothermal manifestations. Geologically active zones and volcanic belts are frequently found in these regions. Hot springs, fumaroles, and geysers are only a few examples of the many geothermal characteristics that exist (Najar, 2018; Cavalazzi *et al.*, 2019).

Hot springs are geothermal springs where hot water emerges from the earth's surface (Cavalazzi *et al.*, 2019). They are connected to a subterranean system and can extend for kilometers. Hot springs are found in regions with young or inactive volcanic activity (Najar, 2018). Magma rises to shallow depths beneath the surface, heating the water that percolates through cracks and fractures. The heated water then reaches the surface through fissures or an underground plumbing system (Najar, 2018). At the surface, hot springs don't erupt but may appear to churn and boil as gases pass through them. The temperature of hot springs can range from tranquil to boiling, with temperatures significantly hotter than the surrounding air (Gartland, 2012). As hot spring water interacts with adjacent rocks, minerals dissolve and are brought to the surface. If volcanic rocks are present, silica is carried to the surface, forming

deposits known as geyserite or sinter. Hot springs can exhibit a variety of colors due to thriving microorganisms in the unique environment (Pirajno, 2020).

In Sikkim, the hot springs are known as "Tatopani", a term derived from the Nepali language, with "Tato" signifying "hot" and "pani" representing "water". Accordingly, Tatopani refers to "hot water", which perfectly captures the character of these springs (Das *et al.*, 2016). Because the Sikkimese culture is so closely entwined with the natural environment, it embraces the significance of these hot zones and values them as places of solace, healing, and spiritual significance.

Tshachu, meanwhile, is a phrase from the Tibetan language, where "Tsha" means "hot" and "Chu" means "water". The name Tshachu is a reflection of this legacy and highlights the substantial Tibetan impact on the local culture. The hot springs have great cultural and therapeutic significance for the Tibetan people and provide a feeling of harmony with nature and well-being (Das *et al.*, 2016; Das and Thakur, 2021). Research has indicated that the utilization of hot spring water can bring about various benefits, including alleviating arthritis, muscular strains, joint pains, and inflammatory skin diseases (Fioravanti *et al.*, 2010; Kamioka *et al.*, 2010).

The dissolved minerals found in hot springs, such as sulfur, carbonates, and additional trace minerals, have proven effective in treating several conditions, including rheumatoid arthritis, osteosis, dermatosis, and inflammatory bowel diseases, among others (Kamioka *et al.*, 2010). Besides their medicinal properties, hot springs serve various purposes, including religious and social activities. Additionally, they are utilized for generating electricity, providing heating and hot water for domestic and other purposes, as well as driving industrial processes like drying and concentration (Lund *et al.*, 2011). Sikkim boasts over 10 hot springs situated in different regions that signify the geothermal active zones and thermophilic habitats

(Najar *et al.*, 2018a). These hot springs are located at an impressive altitude of 4,500 meters above sea level in Sikkim. They create a fascinating ecosystem that experiences contrasting thermal conditions throughout the year. While the winter season brings freezing temperatures to these hot springs, the summer and monsoon climates transform them into diverse thermic environments (Najar *et al.*, 2018a).

2.1.2. Warm Region (Plain field)

In certain high-altitude mountain ranges, there are prevalent warm geothermal regions. These areas display geothermal activity with temperatures lower than those found in hot geothermal regions, but still significant enough to support various geothermal features. Warm geothermal settings often foster the development of unique ecosystems. These warm regions provide favorable conditions for microbial diversity that thrive in warm temperatures around 37 °C. At our sampling sites, Yumesamdong and New Yumesamdong, the hot springs are located at slightly higher elevations. The water from these hot springs flows into colder regions. In the transitional area between the hot and cold regions, a plain field forms, known as the warm region.

2.1.3. Cold region (Semi freeze zone)

Microorganisms that have adapted to cold environments are becoming increasingly significant due to their capacity to inhabit extremely low-temperature habitats. These microorganisms are now emerging as a promising reservoir of bioactive compounds. The Himalayas are renowned as one of the 34 internationally acknowledged biodiversity hotspots, owing to their exceptional and diverse range of life forms (Pandey *et al.*, 2019). In the region of Sikkim, there exist approximately 84 glaciers, varying in size from small to large, covering an extensive area of 440 sq. km. (Bahuguna *et al.*, 2001). These glaciers are predominantly located in the Mt. Kanchenjunga range of North and West Sikkim, which is renowned as a biological hotspot in

the Eastern Himalaya and has been recognized as a World Heritage Site by UNESCO in 2016. The overall permanent snowfields in Sikkim have been estimated to occupy an area of 251 square kilometers, and when combined with the glaciers, the entire snow-covered area reaches approximately 691 square kilometers (Luitel *et al.*, 2012). It has also been reported that the glacial and snow cover areas in Sikkim hold a significant water volume of around 145 km^3 (Pradhan *et al.*, 2004). The discharge water from these glaciers contributes to the two primary river systems of Sikkim: the Teesta and Rangit rivers. The Teesta River is primarily fed by glaciers such as Teesta Khangsa, Tent peak, Changme Khangpu, Changme Khang, South Lhonak, North Lhonak, Langbu, Zemu, Hidden, Chungsang, Talung, Nepal gap, and Zumthal Phuk glaciers. Likewise, the main feeder glacier for the Rangit River is the Rathong glacier (Pradhan *et al.*, 2004).

2.2. Microbial Diversity and Niches

Microbes are ubiquitous in Nature and present the three domains of life on Earth (Gupta *et al.*, 2017). Extremophiles are organisms that can live and flourish in hostile environments. The biocatalysts, known as extremozymes, are produced by these extremophiles and have exceptional tolerance to salt, thermostability, and cold adaptivity. Extremozymes provide new opportunities for biocatalysis, biotransformations, and other research because of their robustness. According to preferred development environments, microorganisms are categorized as thermophiles (heat lovers), psychrophiles (cold lovers), halophiles (Love high salt concentrations), and barophiles (lovers of high pressure) (Rothschild and Mancinelli, 2001).

2.2.1. Thermophilic Bacteria

Thermophilic microorganisms, which thrive at temperatures of 50 °C or higher, have garnered significant attention due to their ability to produce thermostable enzymes (Pikuta *et al.*, 2007).

These thermophiles have been found in various ecological zones such as hot springs and the deep sea (Mohammad *et al.*, 2017; Oztas Gulmus and Gormez, 2020; Das *et al.*, 2023b). They can exhibit diverse characteristics, being either Gram-positive or Gram-negative or Gram-variable, aerobic or anaerobic, spore or non-spore-forming, and motile or non-motile prokaryotes (Gupta *et al.*, 2021). The discovery of thermophilic bacteria dates back to Miquel in 1888, but it was the identification of *Thermus aquaticus* that sparked the modern era of studying thermophilic microorganisms (Pikuta *et al.*, 2007).

Among thermophiles, the genera *Pyrobaculum*, *Pyrodictium*, *Pyrococcus*, and *Melanopyrus* in the archaea domain, as well as certain Ascomycete and Zygomycete fungi, demonstrate the highest growth temperatures (103-110 °C). In the bacterial domain, *Thermotoga maritime* and *Aquifex pyrophilus* exhibit the highest growth temperatures of 90 °C and 95 °C, respectively. These microorganisms possess enzymes with exceptional thermostability and resistance to chemical denaturants, making them valuable for various industries such as detergent, food, feed, starch, textile, leather, pulp and paper, and pharmaceuticals (Najar *et al.*, 2020a). They also serve as models for understanding the factors contributing to thermostability and thermoactivity, offering insights into protein engineering (Pikuta *et al.*, 2007).

Temperature plays a crucial role in the bioavailability and solubility of various polymeric compounds, leading to accelerated reaction rates, enhanced bioremediation, and reduced risks of microbial contamination and complications (van den Burg, 2003; Sinha *et al.*, 2012). Over time, a variety of polymer-degrading enzymes have been discovered and studied in extremely thermophilic and hyper-thermophilic microorganisms. Among these enzymes are amylases, cellulases, lipases, chitinases, pectinases, pullulanases, proteases, xylanases, isomerases, esterases, phytases, dehydrogenases, and DNA-modifying enzymes (deMiguel Bouzas *et al.*, 2006; Verma and Kanwar, 2012; Dumorné *et al.*, 2017).

Thermophilic microorganisms can be classified into five groups:

(a) Facultative thermophiles: These are thermophiles that have a temperature range that extends mostly within the mesophilic range, below 45 °C.

(b) Thermo-tolerant microorganisms: These are microorganisms with an optimum temperature of ≤45 °C but can still grow at temperatures above 45 °C.

(c) Moderate thermophiles (optimum growth temperature of 50-60 °C): This group includes thermophilic microorganisms such as *Bacillus caldolyticus*, *Geobacillus stearothermophilus*, *Thermoactinomyces vulgaris*, *Clostridium thermohydrosulfuricum*, *Thermoanaerobacter ethanolicus*, and *Thermoplasma acidophilum*.

(d) Extreme thermophiles (optimum temperature higher than 70 °C): Examples of thermophilic microorganisms belonging to this group are *Bacillus caldolyticus*, *Geobacillus stearothermophilus*, *Thermoactinomyces vulgaris*, *Clostridium thermohydrosulfuricum*, *Thermoanaerobacter ethanolicus*, and *Thermoplasma acidophilum*.

(e) Hyperthermophiles (optimum temperature higher than 90 °C): Among bacteria, there are only a few species that can be classified as hyperthermophiles, such as *Thermotoga* and *Aquifex*, with an optimum temperature range of 90 to 95 °C (Huber *et al.*, 2000). In environments with temperatures above 90 °C, archaea dominate, with examples including *Pyrolobus fumarii*, which has an optimum temperature of 106 °C and can survive at temperatures up to 113 °C. The first hyperthermophilic archaea discovered in extremely hot and acidic hot springs was *Sulfolobus acidocaldarius* (Madigan *et al.*, 2008). Other hyperthermophiles include *Methanoccus jannaschii*, *Acidianus infernos*, *Archaeoglobus profundus*, *Methanopyrus kandleri*, *Pyrobaculum islandicum*, *Pyrococcus furiosus*, *Pyrodictium occultum*, *Pyrolobus fumarii*, *Thermococcus littoralis*, and *Ignicoccus islandicum* (Reysenbach and Shock, 2002; Ghosh *et al.*, 2003).

2.2.2. Mesophilic Bacteria

Microorganisms known as mesophiles may grow at temperatures between 20 °C and 45 °C, with a typical optimum growth range of 30 °C to 39 °C. They are members of the Bacteria, Eukarya, and Archaea domains and may be found in a variety of habitats, including soil and water (Schiraldi and Rosa, 2014). Many mesophilic bacterial species are involved in biodegradation processes, playing a role in the digestion and decomposition of organic matter (Kumar *et al.*, 2022a). On the other side, certain mesophiles are dangerous since they may infect both humans and animals with illnesses. *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Salmonella spp*., *Proteus vulgaris*, and certain strains of *Escherichia coli* are examples of harmful mesophiles (Al-Allaf *et al.*, 2009).

However, mesophiles may also be useful, especially in the food sector. Mesophilic lactic acid bacteria are frequently employed in manufacturing milk and dairy products such as cheese and yogurt and in dietary supplements and nutraceutical goods for improving gastrointestinal health (Shiby and Mishra, 2013). Nonetheless, there are mesophilic bacteria like *Listeria monocytogenes* that pose a threat to food safety, spoiling meat, salami, and soft cheese, leading to contamination and decay (Davidson and Critzer, 2011). Under appropriate conditions of temperature, pH, and salinity, mesophiles can undergo cell division through mitosis and exhibit rapid reproduction, with their population doubling in as little as 20 to 30 minutes. Alternatively, *Escherichia coli*, a well-known mesophiles, is frequently employed as a bacterial platform for the expression of heterologous proteins and extremozymes, facilitating greater enzyme output. Since the early 1970s, culture methods and the modification of *E. coli*'s physiology have been studied and modified for optimal output (Shiloach and Fass, 2005).

2.2.3. Psychrophilic Bacteria

A psychrophilic microbe thrives best at 10 °C, and it demonstrates limited growth at 0 °C or lower temperatures (Kashyap *et al.*, 2022; Rekadwad *et al.*, 2023). While psychrotolerant microorganisms are defined as microorganisms capable of flourishing at 0 °C, although their prime growth occurs typically within the temperature span of 20–40 °C (Bölter, 2004; Rekadwad *et al.*, 2023). These microorganisms are commonly encountered in diverse low-temperature habitats across the Earth. These environments include Polar Regions, glaciers, ocean depths, shallow subterranean regions, the upper atmosphere, refrigerated appliances, and plants and animals inhabiting cold regions (Pikuta *et al.*, 2007). The majority of these microorganisms belong to the bacterial family, including species such as *Pseudomonas*, *Pseudoalteromonas*, *Vibrio*, *Arthrobacter*, and *Bacillus* (Pikuta *et al.*, 2007; Sherpa *et al.*, 2018; Ali *et al.*, 2023), as well as yeasts like *Candida and Crystococcus* (Pikuta *et al.*, 2007).

The existence of psychrophilic microbes in glacial ecosystems has been known for a long time, and research conducted on Lake Vostok in Antarctica during the late 1980s sparked further interest in studying cryosphere microbes (Miteva, 2008). Examination of ice core samples retrieved from depths of 3,000 meters has uncovered the presence of viable bacterial cells at minimal concentrations, as determined through the utilization of microscopy and cultivation methodologies (Miteva, 2008). Additionally, research on ice core samples from 20,000 years ago revealed the presence of many bacterial communities. Various bacterial phyla, including Actinomycetota (Actinobacteria), Bacillota (Firmicutes), and Pseudomonadota (Proteobacteria), have been isolated from glaciers, with dominant genera such as *Acinetobacter*, *Arthrobacter*, *Chryseobacterium*, *Exiguobacterium*, *Frigoribacterium*, *Janthinobacter*, *Methylobacterium*, *Rhodococcus*, *Sphingomonas*, and *Pseudomonas* (Sherpa, 2018; Kumar *et al.*, 2022b; Nawaz *et al.*, 2023). Recent research has shown abundant biodiversity in polar habitats, including those in Antarctica, the Arctic, and nearby Polar

Regions (Makhalanyane *et al.*, 2015). In contrast to other frigid ecosystems across the world, the wide array of microbes of Himalayan glaciers has not been as thoroughly studied. Pseudomonadota, Actinomycetota, Bacillota, and Deinococcus are present in Himalayan glaciers, according to a few studies of research that have looked at the variety of bacteria there (Shivaji *et al.*, 2011; Sherpa, 2018).

Psychrophiles can be categorized into two groups based on their optimal growth temperature:

a) Psychrophiles

Psychrophiles are microorganisms that are adapted to thrive and grow at extremely low temperatures. They belong to various groups such as bacteria, archaea, and fungi. These cold-loving organisms have evolved unique biochemical and physiological adaptations to survive and function effectively in cold environments (Rekadwad *et al.*, 2023). Psychrophiles are of significant interest to researchers as they offer insights into the adaptation strategies of life in extreme conditions. Their study provides valuable knowledge about the biochemical processes and cellular mechanisms that enable organisms to survive and reproduce in cold habitats. Furthermore, the study of psychrophiles has potential applications in biotechnological processes that require enzyme activity and stability at low temperatures (Gupta *et al.*, 2023). These cold-adapted microorganisms are commonly found in Polar Regions, deep-sea environments, glaciers, and other cold ecosystems (Gupta *et al.*, 2023). Understanding their unique features and adaptations not only expands our understanding of extremophiles but also has implications for astrobiology and environmental research.

b) Psychro-tolerant

The organisms can grow optimally above 20 °C and tolerate temperatures below 5 °C (Canganella and Wiegel, 2011). Psychrotolerant bacteria isolated from various cold habitats

include *Trichococcus patagoniensis*, *Proteocatella sphenisci*, *Pedobacter himalayensis*, *Exiguobacterium mindicum*, *Dyadobacter hamtensis*, *Leifsonia pindariensis*, *Bacillus cecembensis*, *Cryobacterium roopkundense*, *Cryobacterium pindariensis*, and *Paenibacillus glacialis* (Chaturvedi *et al.*, 2005; Shivaji *et al.*, 2005; Chaturvedi and Shivaji, 2006; Kishore *et al.*, 2010).

To survive in extremely cold conditions, psychrophilic and psychrotolerant bacteria employ various adaptation strategies for optimal functioning (Kumar *et al.*, 2023a). The production of psychrophilic enzymes by these cold-adapted microorganisms exhibits high catalytic efficiency at low temperatures, making them valuable in industries such as detergents, textiles, food processing, pharmaceuticals, leather production, brewing, winemaking, paper, and pulp, as well as in bioremediation of soils and wastewater (Kumar *et al.*, 2011; Singh, 2017). The adaptation of enzymes to low temperatures is attributed to their flexible structures, achieved through a combination of structural features such as reduced hydrophobicity in the core, decreased ionic interactions, increased surface charge, and longer surface loops. These modifications result in increased structural flexibility for psychrophilic proteins. Psychrophiles are particularly important due to the flexible structure of their enzymes, which offer tremendous potential for use in low-temperature processes (Gupta *et al.*, 2023).

2.3. Heat and Cold Shock Proteins: Functions and Significance in Microbial Adaptation

2.3.1. Heat Sock Protein

Every organism and cell has heat-shock proteins (HSPs), also known as stress proteins (Sharp *et al.*, 1999; Li and Srivastava, 2003; Hu *et al.*, 2022) HSPs have a high degree of stability. HSP protein families are categorized based on their molecular weights, encompassing several major groups such as large HSPs, HSP40, HSP60, HSP70, HSP90, and small HSPs. These families serve as molecular chaperones within cells, forming an interconnected network that

contributes to various essential processes. They assist in the folding of newly synthesized polypeptides, facilitate the refolding of unstable proteins, aid in the assembly of protein complexes, disassemble protein aggregates, and participate in the degradation of misfolded proteins (Li and Srivastava, 2003; Hu *et al.*, 2022). Apart from their chaperone activities, HSPs also exert a significant influence on cell signaling transduction, cell cycle regulation, and apoptosis control. Consequently, the dysfunction of HSPs is associated with numerous diseases, including cancer, neurodegenerative disorders, and various other ailments (Hu *et al.*, 2022).

The majority of HSPs typically express themselves within cells and work intracellularly. However, several HSPs are capable of being released to the cell surface, especially in anticipation of physiologic stress (Zininga *et al.*, 2018). Furthermore, it has been shown that the circulatory system of the host contains HSPs produced by parasites and antibodies that recognize them (Zininga *et al.*, 2018). Therefore, when HSPs enter the circulatory system of the host, they can connect with the immune system's cells and affect the way they function. HSPs are thought to have a role in the progression of autoimmune diseases because of their extensive existence and conservation, together with their role in influencing the host's immunological response (Dreiza *et al.*, 2010). The HSP peptide complexes (PC) have been used as vaccines to trigger antigen-specific Cytotoxic Lymphocytes (CTL) to produce targeted immunological responses (Srivastava, 2000; Manjili *et al.*, 2002; Gong *et al.*, 2010; Murshid *et al.*, 2012). Antigen Presenting Cells (APCs) must internalize the polypeptides that bind to HSPs and incorporate them into their antigen presentation pathways for adaptive immunity to be triggered. This process enables the associated antigens to trigger an immune response (Murshid *et al.*, 2012).

2.3.2. Cold Sock Protein

A class of highly conserved proteins known as cold shock proteins (CSPs) is present in a broad variety of species, including bacteria, archaea, and eukaryotes (Cavicchioli *et al.*, 2000). They are essential to the cellular response to external stressors like cold temperatures. However, CSPs are also present in species that are not acclimated to cold settings and are engaged in several physiological processes in addition to temperature regulation. CSP's main job is to safeguard the cell against the damaging effects of cold stress by stabilizing RNA molecules and controlling gene expression (Gualerzi *et al.*, 2003). The fluidity of the cell membrane reduces at low temperatures, which compromises the stability of RNA structures. Single-stranded RNA is bound by CSPs, which prevent secondary structures from forming and promote correct folding. CSPs ensure that crucial cellular activities, such as translation and transcription, may proceed successfully even under cold environments by serving as RNA chaperones (Barria *et al.*, 2013).

To survive in extremely cold conditions, psychrophilic and psychrotolerant bacteria employ various adaptation strategies to ensure optimal functioning. It is well known that the composition of phospholipids in bacteria changes with growth temperature (Chakravarty and Banerjee, 2023), resulting in different absorption mechanisms compared to mesophilic species. Cryoprotectant proteins enable psychophilic bacteria to preserve their metabolic stability in cryo-habitats (Feller, 2017). Psychrophilic enzymes cryostability has demonstrated their relevance as biocatalysts for a variety of biotechnological fields and industrial uses. Due to their capacity to create enzymes that help to operate at low temperatures, psychrophiles are particularly intriguing organisms from a biotechnological perspective (Hamid *et al.*, 2022). Enzymes that break down extracellular polymers have many uses in biotechnology-based companies (Radhakrishnan, 2014).

2.4. Function and Significance in Microbial Adaptation

The unique ability of microbes to change and survive in various settings by changing their physiological, morphological, or behavioral traits is referred to as microbial adaptation. Microorganisms need this adaptability to survive in a variety of environments and respond to the numerous biotic and abiotic elements they come into contact with (Kumar *et al.*, 2023a; Kumar *et al.*, 2023b). Abiotic variables are those that are not influenced by biological processes, such as temperature, salinity, pH the availability of nutrients, and physical conditions. Biotic factors comprise relationships with other living organisms, such as competition, predation, and symbiosis (Kumar *et al.*, 2023a). Microbes adapt to changing environmental circumstances using an intricate network of cellular and molecular systems in a complicated and heterogeneous ecosystem as well as amid environmental stress. Their habitats range from desert to marine, very alkaline zones to severely acidic places, including the icy microcosms of Antarctica and geothermal volcanic spots (Wani *et al.*, 2022).

2.5. Microbial adaptation purposes

2.5.1. Survival and Growth

Microorganisms adapt to certain environments to maximize their chances of survival and development. They may effectively utilize all the resources present in a particular environment by modifying their metabolic pathways (Kumar *et al.*, 2023a; Kumar *et al.*, 2023b). For instance, depending on the available carbon sources, certain bacteria can shift between them.

2.5.2. Nutrient Acquisition

Microbes use a variety of methods to get necessary nutrients from their environment (Gupta *et al.*, 2017). They may develop enzymes that may degrade complex organic substances, giving them access to nutrients that would otherwise be inaccessible to them. To obtain nutrients indirectly, certain microbes develop mutualistic interactions with other species. For instance,

nitrogen-fixing bacteria associate with leguminous plants, in mutually beneficial relationships where they exchange carbohydrates for fixed nitrogen (Mahmud *et al.*, 2020).

2.5.3. Resistance to Stress

Microbes encounter a variety of stresses in their natural surroundings, such as extreme temperatures, pH changes, high levels of salinity, and being exposed to toxins. Microorganisms build defenses against and endure these pressures through adaptation. To prevent denaturation of their proteins, they may develop HSPs/CSPs, change the composition of their membranes to preserve fluidity in harsh environments, or create defense mechanisms like osmoprotectants and anti-oxidants (Delmas *et al.*, 2001).

2.6. Antibiotic Resistance: Environmental Impacts, and Public Health Consequences

Antibiotic resistance is on the rise, which poses a serious threat to the global community at present (Aslam *et al.*, 2018). It causes a rise in death rates, the introduction of incurable illnesses, and rising healthcare expenses. Bacteria, in particular, have evolved and built-up defense mechanisms to withstand the effects of antibiotics, which were formerly successful in treating bacterial diseases. This phenomenon presents a threat to world health since it lowers the efficacy of medicines and breeds multidrug-resistant bacteria, called "superbugs" (Medina and Pieper, 2016). According to research, drug-resistant bacterial infections are responsible for worrying mortality rates in many nations, including a total of 23,000 deaths annually in the US, 25,000 in the EU, and 58,000 in India (Gelband *et al.*, 2015). The emergence of antibiotic resistance is significantly influenced by microbial adaptability. Through the horizontal gene transfer mechanism, microscopic organisms may acquire antibiotic-resistance genes that enable them to endure in the presence of drugs. This adaptation presents a substantial difficulty in healthcare settings because it reduces the efficacy of therapeutic drugs and promotes the growth of multidrug-resistant bacteria (MDR) (Tsakou *et al.*, 2020).

Temperature can significantly influence the effectiveness of antibiotics, as demonstrated in various studies. For instance, certain antibiotics have been found to have increased or reduced effectiveness in patients with fever or hypothermia. Previous research has shown that *Francisella tularensis*, *Listeria monocytogenes*, and *Klebsiella pneumoniae* exhibit increased resistance to gentamicin (GEN) at 26 °C compared to 37 °C. On the other hand, *Pseudomonas aeruginosa* has shown increased effectiveness of streptomycin (STR), tetracycline (TET), ampicillin (AMP), and cefoxitin (FOX) at 46 °C compared to 37 °C. A study by Cruz-Loya et al. (2019) also reported the synergistic effects of aminoglycosides (GEN, STR, TOB) with high temperatures and antagonistic effects with low temperatures. Beta-lactams (AMP and FOX) were found to have synergistic effects with high temperatures, while TET exhibited synergy at both 46 °C and 22 °C. Other antibiotics such as LVX, TMP, and NTR showed a pattern of being synergistic with extreme temperatures and either additive or slightly antagonistic with less stressful temperatures (Cruz-Loya *et al.*, 2019).

In the United States alone, at least 2 million people are infected with antibiotic-resistant bacteria each year, resulting in approximately 23,000 deaths annually (CDC, 2018). In Sikkim, around 90 % of children were found to carry resistance to at least one commonly used antibiotic, and multidrug resistance (MDR) was observed in 41 % of individuals under the age of 14 (Singh *et al.*, 2019; Singh *et al.*, 2020). Antibiotic residues, antibiotic resistance genes (ARGs), and their bacterial hosts can enter the river system through various waste streams, raising concerns about their impact on the environment (Vikesland *et al.*, 2017). In China, it has been estimated that approximately 24,748 tons of antibiotics and 9.47 x 1013 copies/day/person of ARGs are released into rivers and related waterways (Zhang *et al.*, 2015; Su *et al.*, 2017). While reservoir systems may be less susceptible to antibiotic and ARG pollution compared to river systems, antibiotics can still accumulate and persist in natural aquatic ecosystems, exerting selective pressure on bacterial communities. This can increase the

likelihood of spontaneous mutation leading to resistance and horizontal gene transfer (HGT) among bacterial communities through mobile genetic elements (MGEs), facilitating the emergence and spread of ARGs (Grenni *et al.*, 2018).

2.7. An overview of antibiotic classes and their mechanisms of action

The term "antibiotic" refers to a class of chemotherapeutic agents that either inhibit or kill microbes (pathogens) through specific interactions with bacterial targets (Dafale *et al.*, 2016). Bactericidal antibiotics can kill bacteria, while bacteriostatic antibiotics only inhibit bacterial growth (Etebu and Arikekpar, 2016). Antibiotics play a crucial role in defending against infectious diseases caused by microorganisms. However, the increasing rate of antibiotic resistance in microorganisms poses a critical and threatening risk to human health (Dafale *et al.*, 2015).

There are several classes of antibiotics:

2.7.1. β-lactams

This class of antibiotics contains a 3-carbon and 1-nitrogen ring structure (Etebu and Arikekpar, 2016). The most important members of the beta-lactam class include penicillins, cephalosporins, carbapenems, and monobactams (Tsakou *et al.*, 2020). These antibiotics are bactericidal, as they kill bacteria by inhibiting the production of the cell wall. They achieve this by targeting an enzyme called penicillin-binding protein (PBP), which helps in the cross-linking of peptide units. However, bacteria have developed resistance to beta-lactams through various mechanisms, such as restricted permeability of the bacterial cell wall to antibiotics, alteration of penicillin-binding proteins, and production of bacterial enzymes called β-lactamases, which inactivate the antibiotics (Soares *et al.*, 2012).

2.7.2. Monobactam

Antibiotics of the monobactam family, which includes aztreonam, have a unique chemical structure that includes a beta-lactam ring lacking fused rings or extra side chains. They are mainly employed to treat bacterial illnesses triggered by Gram-negative organisms. Compared to other antibacterial agents like cephalosporins and penicillins, monobactams inhibit the formation of the bacterial cell wall. However, monobactams concentrate primarily on preventing the function of a certain class of enzymes known as penicillin-binding proteins (PBPs) (Sharifzadeh *et al.*, 2020).

2.7.3. Aminoglycosides

Aminoglycoside antibiotics are effective against both Gram-positive and Gram-negative bacteria (Garneau-Tsodikova and Labby, 2016). Their structure consists of a 2-deoxystreptamine ring linked to two or more amino-modified sugars through glycosidic bonds (Becker and Cooper, 2013). Aminoglycosides bind to the bacterial ribosome (30S subunit) and impair bacterial protein synthesis (Mingeot-Leclercq *et al.*, 1999). Streptomycin was the first aminoglycoside discovered and was effective against Mycobacterium tuberculosis (Mahajan and Balachandran, 2012). However, bacterial resistance to aminoglycosides has developed through mechanisms such as ribosomal mutations, ribosomal modification by methyltransferases, modification of aminoglycosides by aminoglycoside-modifying enzymes (AMEs), and active transport of aminoglycosides out of the cell through efflux pumps (Becker and Cooper, 2013; Etebu and Arikekpar, 2016).

2.7.4. Macrolides

Macrolides are broad-spectrum antibiotics with diverse activities, including antibacterial, antifungal, prokinetic, and immunosuppressant properties (Kanoh and Rubin, 2010). They consist of macrocyclic lactose rings with 14, 15, or 16 members, linked to rare deoxy sugars such as L-cladinose and D-desosamine (Zuckerman *et al.*, 2011). Erythromycin was the first

macrolide discovered, isolated from the fungus *Saccharopolyspora erythraea* (Park and Yoon, 2019). Macrolides are effective against Gram-positive bacteria and spirochetes and are often used as an alternative for patients allergic to penicillin. Bacterial resistance mechanisms against macrolides include drug efflux, target alteration through ribosomal methylation, and antibiotics (Wilson, 2014).

2.7.5. Cephalosporin

The beta-lactam family of antibiotics includes the cephalosporins (Holten and Onusko, 2000). They are often employed to treat different bacterial illnesses. Because they both have a beta-lactam ring within the chemical makeup, cephalosporins, and penicillins are chemically similar. Cephalosporins, on the other hand, feature an extra dihydro thiazine ring, which improves their stability and increases their sensitivity to certain bacterial enzymes (Lima *et al.*, 2020). The microorganisms targeted by cephalosporin therapy encompass a range of species, including both commensal organisms like coagulase-negative staphylococci (CNS), *Pseudomonas aeruginosa*, enterococci, and *Candida albicans*, as well as more well-known pathogens like *Clostridium difficile*, penicillin-resistant pneumococci, multiply-resistant coliforms, and methicillin-resistant *Staphylococcus aureus* (MRSA) (Dancer, 2001). Cephalosporins are essential in the fight against bacterial infections because they interfere with the formation of cell walls, which ultimately causes vulnerable bacteria to die.

2.7.6. Rifamycin

A group of antibiotics known as rifamycins is effective against several bacteria, notably Mycobacterium tuberculosis, which is the cause of tuberculosis. The most well-known rifamycin is rifampin, which is frequently used to treat illnesses brought on by susceptible bacteria, including tuberculosis (TB) (Ducati *et al.*, 2006). Rifamycins target bacterial RNA polymerase, a crucial enzyme involved in the conversion of bacterial DNA into RNA, as part

of their mode of action. Rifamycins specifically attach to the bacterial RNA polymerase's component, reducing its activity and obstructing bacterial RNA synthesis (Campbell *et al.*, 2001).

2.7.7. Oxazolidnodes

The class of antibiotics known as oxazolidinones is effective against a variety of Gram-positive bacteria, including vancomycin-resistant *Enterococcus faecium* (VRE) and methicillin-resistant *Staphylococcus aureus* (MRSA) (Chien *et al.*, 2000). Linezolid is the most popular oxazolidinone drug and has been used extensively in healthcare (Rybak and Roberts, 2015).

Oxazolidinones attract the bacterial ribosome, especially the 50S subunit, which is in charge of protein synthesis, as their method of action. Oxazolidinones hinder the establishment of functional ribosomal complexes and the start of protein synthesis (Shinabarger, 1999). As a result, susceptible bacteria are eliminated and bacterial development is finally inhibited.

2.7.8. Aminocoumarin

A group of antibiotics known as aminocoumarins is effective against some gram-positive microbes, such as Mycobacterium tuberculosis, which is the cause of tuberculosis (TB). Novobiocin (NV) is a particularly widely recognized antibiotic containing an aminocoumarin (Genilloud, 2017).

Aminocoumarins target the bacterial DNA gyrase, known as topoisomerase II. DNA gyrase is involved in the replication, transcription, and recombination of DNA. The DNA replication process has interfered, when aminocoumarins attach to DNA gyrase's ATP-binding site and limiting its action (van Eijk *et al.*, 2017).

2.7.9. Diaminopyrimidines

Diaminopyrimidines are a class of antibiotics that play a crucial role in the treatment of urinary tract infections caused by Gram-negative bacteria, with *Escherichia coli* being a primary culprit. Among diaminopyrimidines, trimethoprim is the most frequently prescribed antibiotic in this class (Prescott, 2013).

The mode of action of diaminopyrimidines centers around their ability to selectively target and inhibit an enzyme called dihydrofolate reductase (DHFR). DHFR is a vital enzyme involved in the synthesis of tetrahydrofolate (THF), which serves as a critical coenzyme required for various essential cellular processes, including the production of nucleic acids and amino acids (Ju *et al.*, 2020). When diaminopyrimidines like trimethoprim (TR) enter the bacterial cell, they bind tightly to the active site of DHFR, impeding its enzymatic activity. By doing so, they hinder the conversion of dihydrofolate (DHF) into tetrahydrofolate THF. As a consequence, the bacterial cell experiences a significant depletion of THF levels (Ju *et al.*, 2020).

THF is indispensable for the synthesis of purines, pyrimidines, and certain amino acids, which are vital building blocks for DNA, RNA, and proteins. With insufficient THF, the bacterial cell faces significant obstacles in its ability to replicate DNA, produce essential proteins, and carry out other essential cellular functions (Pranzini *et al.*, 2021). In addition, diaminopyrimidines, when used in combination with sulfonamides, exhibit a synergistic effect. Sulfonamides, such as sulfamethoxazole, act by inhibiting an earlier step in the folate synthesis pathway, preventing the formation of dihydropteroic acid. When combined with diaminopyrimidines, it blocks the conversion of DHF to THF (Irvine, 2020). The disruption of the folate metabolism pathway is intensified, leading to a more potent antimicrobial effect against susceptible bacteria. It is important to note that bacterial resistance to diaminopyrimidines can emerge through various

mechanisms, including alterations in the structure of DHFR or the acquisition of resistant genes (Irvine, 2020).

2.7.10. Glycopeptide

Antibiotics known as glycopeptides, such as vancomycin and teicoplanin, are essential for treating infections triggered by Gram-positive bacteria, particularly MRSA and VRE (Kulkarni *et al.*, 2019). By attaching to peptidoglycan precursors and preventing transpeptidases from cross-linking them, they specifically target the bacterial cell wall. The integrity of the cell wall is compromised by this inhibition, which hinders the production of peptidoglycan. As a result, the compromised cell wall gives way to internal osmotic pressure, causing lysis of cells and the death of bacterial cells (Salamaga *et al.*, 2021). In addition, glycopeptides have a bactericidal capacity that kills pathogens as soon as they appear. However, the rise of vancomycin-resistant forms raises serious concerns. Reduced affinity caused by alterations in the D-Ala-D-Ala binding site is the cause of resistance (Ge *et al.*, 1999).

2.7.11. Chloramphenicol

The drug chloramphenicol a broad-spectrum antibiotic, was initially identified in the late 1940s (Hawkey, 2008). It is a product of the soil microorganism *Streptomyces venezuelae* and has been used extensively in healthcare settings to treat a wide range of bacterial illnesses. In addition to some anaerobic species, chloramphenicol acts efficiently against a variety of bacteria, including gram-positive and gram-negative (Kasten, 1999).

Chloramphenicol works by preventing the creation of bacterial proteins. It particularly binds to the 23S rRNA that is intended for the bacterial ribosome's 50S subunit (Tereshchenkov *et al.*, 2018). This binding stops peptide bonds from establishing between amino acids throughout protein synthesis, which hinders the lengthening of the expanding polypeptide chain (Tereshchenkov *et al.*, 2018).

2.7.12. Lincosamide

The primary usage of lincosamides is for the treatment of various bacterial infections. Lincosamides originate from a naturally occurring substance called lincomycin, which was initially identified from the bacterium *Streptomyces lincolnensis*. Since lincosamides are bacteriostatic agents rather than bacteriophages, they prevent bacterial growth and reproduction (Zhang and Cheng, 2022).

Lincosamides work by preventing bacteria from synthesizing proteins. They specifically target the building of new proteins by the bacterial ribosome's 50S subunit (Jiao *et al.*, 2023). Lincosamides block the insertion of fresh amino acids into the expanding protein chain by interacting with this component, hence obstructing the production of bacterial proteins (Roberts, 2002).

2.7.13. Fluoroquinolone

Wide-spectrum antibiotics known as fluoroquinolones are used to treat a variety of bacterial ailments (Mateu and Martin, 2001). They are distinguished by the fluorine atom located in the 6-position that makes up the quinolone ring of their molecular structure. In comparison to other antibiotics in the quinolone family, this structural change increases their effectiveness and range of action. They specifically target topoisomerase IV and DNA gyrase, two crucial enzymes that regulate the replication of DNA, transcription, and repair functions in bacteria (Levine *et al.*, 1998). Fluoroquinolones interfere with the normal operation of bacterial DNA by blocking these enzymes, which causes the fragmentation of DNA and ultimately results in bacterial cell death (Fàbrega *et al.*, 2009).

2.7.14. Sulfonamide

Sulfonamides, usually referred to as sulfa medicines, are a type of antibiotic that works by chemically imitating para-aminobenzoic acid (PABA), a chemical precursor to folic acid

(Bourne, 2014). To make the fundamental components of DNA, RNA, and proteins, microorganisms must synthesize folic acid. Dihydropteroate (DHPA), an essential intermediary in the manufacture of folic acid, is produced by the bacterial enzyme DHPS by converting PABA and dihydropteridine pyrophosphate (DHPPP) with each other. Since PABA is unable to attach to the active site of DHPS, sulfonamides impede the synthesis of DHPA by binding competitively to the site. Folic acid synthesis is then interfered with, which inhibits bacterial development and growth. Furthermore, sulfonamides have demonstrated a wide range of pharmacological effects, including anti-diabetic properties, anticancer activity, and antiviral potential (Azzam *et al.*, 2020).

2.7.15. Tetracycline

Since the 1950s, Tetracycline has been extensively utilized to treat a broad range of bacterial infections caused by both gram-positive and gram-negative bacteria (Eliopoulos *et al.*, 2003). Apart from their efficacy against conventional bacterial strains, tetracycline has also demonstrated effectiveness in combating infections caused by intracellular chlamydia, mycoplasmas, rickettsia, protozoan parasites, as well as various noninfectious conditions. Their significance extends to the treatment and prevention of infections involving bacteria that have the potential for use in biological weapons (Eliopoulos *et al.*, 2003). Tetracycline is an example of an antibiotic with a broad spectrum that functions by preventing protein production. This is accomplished by blocking aminoacyl-tRNAs from interacting with the ribosome's acceptor site (Stepanek *et al.*, 2016).

2.7.16. Carbapenem

Carbapenem antibiotics are widely recognized as the primary choice for treating infections caused by highly resistant bacteria. These bacteria include *Acinetobacter baumannii*, Enterobacteriaceae (such as *Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter sp.*,

Serratia sp., and *Proteus sp*.), *Shigella sp.*, *Streptococcus pneumonia*, *Pseudomonas aeruginosa*, *Enterococcus faecium*, *Haemophilus influenzae*, and *Mycobacterium tuberculosis* (Aurilio *et al.*, 2022). Carbapenems are a class of β-lactam antimicrobials that exhibit bactericidal activity and have been successfully used to treat severe infections caused by bacteria producing extended-spectrum β-lactamases (ESBLs). These potent antibiotics include well-known examples such as imipenem, meropenem, doripenem, ertapenem, panipenem, and biapenem, which are widely employed worldwide (Codjoe and Donkor, 2017). Carbapenems work by binding permanently to PBPs, preventing the cross-linking of peptidoglycans, and causing bacterial cell wall disintegration and lysis. They are extremely useful in treating serious infections brought on by bacteria that are multidrug-resistant because of their broad-spectrum action and stability against beta-lactamases.

2.8. Metagenomic Approaches: Unveiling Microbial Diversity and Antibiotic Resistance

Two primary techniques, namely high throughput amplicon sequencing and shotgun metagenomic, have enabled in-depth characterizations of microbial communities, their components, and functions. Amplicon sequencing is a cost-effective method that provides taxonomic compositional profiles of microbiota. This technique allows even small research groups to conduct large-scale analysis projects focused on bacterial microbiota. On the other hand, metagenomic offers a more comprehensive analysis of the entire viral, bacterial, and eukaryotic microbiota, encompassing both taxonomic and functional aspects. However, metagenomic is generally more expensive compared to amplicon sequencing. Both methods have been successfully employed in large-scale studies involving thousands of samples. With the advancement of DNA sequencing and bio-computational tools, the genetic diversity of the uncultivable microbial communities can be explored to characterize microbial diversity. Metagenomic refers to the study of genomic and genetic data derived from environmental and

clinical samples. Based on the type of data used, metagenomic can be divided into two categories: amplicon or targeted gene data and shotgun or untargeted gene data, inferred from amplicon and shotgun sequencing, respectively. Amplicon metagenomic data are amplified sequences of marker genes such as 16S/18S/26S rRNA and intragenic transcribed spacers (ITS). Meanwhile, shotgun metagenomic data includes all DNA sequences in the samples (Nam *et al.*, 2023). Amplicon sequencing is the most often used technology where the community (e.g., water, soil) is sampled, and the total DNA is extracted from the sample. PCR is then used to target and amplify a taxonomically informative genomic marker that is shared by almost all species of interest. The resulting amplicons are sequenced and bio-informatically characterized to establish which microorganisms are present and in what relative abundance in the sample. Amplicon sequencing investigations in bacteria and archaea typically target the small-subunit ribosomal RNA (16S) locus, which is a taxonomically and phylogenetically informative marker (Sharpton, 2014). Comprehensive literature by Kuczynski *et al.*, (2012) provides a thorough understanding of the use of 16S rRNA amplicon sequencing in investigating microbiome studies (Kuczynski *et al.*, 2012). Several studies have been conducted concerning the 16S rRNA locus, to decipher the microbial diversity on various ecosystems. A study conducted by Saxena *et al.*, (2017) on large-scale metagenomic analysis of hot springs namely Badi Anhoni, Chhoti Anhoni, and Tattapani, revealed the presence of unique residual thermophilic microbial community and crucial metabolic pathways (Saxena *et al.*, 2017). Novel microbes and numerous crucial pathways and genes essential for survival in thermophilic conditions were revealed.

Amplicon sequencing is associated with various limitations. First, given the many biases associated with PCR, it may fail to resolve a significant portion of the diversity in a community. Second, amplicon sequencing can yield wildly disparate estimates of diversity. Different genetic loci, for example, have varying ability to resolve taxa. Furthermore,

sequencing errors and improperly constructed amplicons (i.e., chimeras) can result in fake sequences that are frequently difficult to recognize. Third, amplicon sequencing often only offers information about the microbial community's taxonomic composition. Using this method, it is impossible to directly resolve the biological roles associated with these taxa. In some circumstances, phylogenetic reconstruction can infer biological activities encoded in a genome containing a specific 16S sequence (Logares *et al.*, 2014; Sharpton, 2014). However, the accuracy with which these methods estimate a community's genuine functional diversity is linked to how well the community's genetic diversity is represented by genomes available in sequence databases. Finally, amplicon sequencing can only be used to analyze taxa that have known and amplified taxonomically informative genetic markers. Examining novel or highly divergent microorganisms, particularly viruses, is challenging using this method. Furthermore, because the 16S locus can be transferred across distantly related taxa (i.e., horizontal gene transfer), 16S sequence analysis can lead to an overestimation of community diversity (Sharpton, 2014).

Shotgun metagenomic sequencing is a method for studying uncultured microbiota that bypasses these limitations. DNA is retrieved from all cells in a community once again. Instead of a single genomic area being targeted for amplification, all DNA is sheared into tiny fragments that are independently sequenced. This produces DNA sequences (i.e., reads) that align to numerous genomic sites in the sample's diverse genomes, including non-microbes. Some of these reads will come from taxonomically informative genomic loci (e.g., 16S), while others will come from coding sequences that provide information about the biological functions encoded in the genome. As a result, metagenomic data allows researchers to investigate two features of a microbial community at the same time: who is there and who is absent? (Sharpton, 2014).

The metagenomic approaches serve the purpose of identifying novel microbes across different environments. The data from short-gun metagenomic implies information on all the DNA sequences in a sample, rather than a specific portion of a genome as utilized by amplicon sequencing. Thus, a variety of workflows have been described to reconstruct a complete genome derived from metagenomic data. For instance, by producing metagenome-assembled genomes (MAGs), metagenomic sequencing provides a culture-independent method for investigating complex microbial populations. A MAG is a microbial genome represented by a set of sequences from genome assembly having comparable properties. It allows us to detect new species and comprehend their potential roles in a changing ecosystem (Setubal, 2021; Yang *et al.*, 2021; Goussarov *et al.*, 2022; Chivian *et al.*, 2023). For instance, a report by Li *et al.*, (2023) describes the annotation of metagenome-assembled genomes from horse-gut microbiome into 4015 novel species (Li *et al.*, 2023). Besides the bacterial genomes, viral and fungal genomes have also been assembled from the short-gun metagenomics data (Stajich, 2017; Roux *et al.*, 2019; Peng *et al.*, 2021; Bassi *et al.*, 2022; Johansen *et al.*, 2022).

Soil, an important component of the Earth comprises both organic and inorganic components and serves as a niche for various species (Sabale *et al.*, 2019). Various studies reveal the microbial community structure analyzed from hot spring soil (Kaur *et al.*, 2018; Rawat and Joshi, 2019; Sousa *et al.*, 2022; Goma-Tchimbakala *et al.*, 2023). With the advent of Metagenomics, it is possible to decipher microbial diversity in various soil types. One investigation on soil microbial communities in various environments, such as deserts, woods, grasslands, and tundra, found a link between bacterial composition, functional genes, and the environment (Nam *et al.*, 2023). Deserts, for example, were discovered to have limited taxonomic and functional diversity when compared to other investigated areas. Furthermore, osmoregulation and dormancy genes were prominent in the desert samples.

In contrast, the desert bacterial community had a low number of antibiotic-resistant genes, indicating limited competition in the desert ecosystem. In a similar study made by, microbiomes in tropical rainforest soil were observed to be less diverse than those found in grassland (Karthikeyan *et al.*, 2021). Similarly, Arctic microbiome studies at various depths reveal a significant decrease in microbial biodiversity structure and changes in function genes with increasing depth (Wu *et al.*, 2021). Besides the soil microbial studies, metagenomic analysis of hot spring water samples has also revealed unique microbial community structures. A study made by Vora *et al.*, (2023) revealed the presence of untapped functional potential of microbial community found in hot springs from Gujarat, for various biotechnological and environmental applications (Vora *et al.*, 2023). Another study on the high-altitude volcanic hot spring of China deciphers the untapped fungal and bacterial diversity, and the study was conducted to carry out a comprehensive understanding of the diversity, ecological interactions between microbes, and the effect of pH on shaping the community structure. A prominent change in microbial diversity was observed, where the diversity was dependent on varying pH under alkaline conditions (Wang and Pecoraro, 2021).

Materials

Chapter 3

3. Materials

3.1. Materials used for collection of geographic location data

Global Positioning System (GPSMAP78S), Garmin, Olathe, Kanas, USA.

Q-GIS Software for creating a sampling site map

3.2. Materials used for the collection of samples

- Sterile zip-lock bag
- Sterile shovel
- pH strip
- Thermometer
- Nitrile gloves
- 70 % ethanol
- Marker pen
- Icebox

3.3. Materials for the cultivation of the microorganisms

Luria Bertani agar	Hi-Media, Mumbai, India	(The composition is detailed in Appendix)
R2A broth	Hi-Media, Mumbai, India	(The composition is detailed in Appendix)
Carbohydrate fermentation broth	Hi-Media, Mumbai, India	(The composition is detailed in Appendix)
Mueller Hinton Agar (MHA)	Hi-Media, Mumbai, India	(The composition is detailed in Appendix)
SOC Broth	Hi-Media, Mumbai, India	(The composition is detailed in Appendix)
Nutrient Broth	Hi-Media, Mumbai, India	(The composition is detailed in Appendix)
Plate Count Agar	Hi-Media, Mumbai, India	(The composition is detailed in Appendix)
Thermus Agar	Hi-Media, Mumbai, India	(The composition is detailed in Appendix)
Starch agar	Hi-Media, Mumbai, India	(The composition is detailed in Appendix)
Urea Agar	Hi-Media, Mumbai, India	(The composition is detailed in Appendix)
Simmons Citrate agar	Hi-Media, Mumbai, India	

Agar Agar Type-1	Hi-Media, Mumbai, India
Bacteriological peptone	Hi-Media, Mumbai, India
Trypticase	Hi-Media, Mumbai, India
Yeast extract	Hi-Media, Mumbai, India
Beef extract	Hi-Media, Mumbai, India

3.4. Materials utilized for the staining of the microorganisms.

A gram stain kit (HI Media K001-1KT) was used to stain bacterial isolates.

3.5. Chemicals for Biochemical Tests

Ethyl Alcohol	Changshu Yangyuan Chemical, China
Magnesium Chloride	Hi-Media, Mumbai, India
Sodium Chloride	Hi-Media, Mumbai, India
Potassium Chloride	Hi-Media, Mumbai, India
Phenol Red	Hi-Media, Mumbai, India
Iodine Solution	Hi-Media, Mumbai, India
Urea	Hi-Media, Mumbai, India
Nitrate	Hi-Media, Mumbai, India
Hydrogen Peroxide	Hi-Media, Mumbai, India
Dipotassium hydrogen phosphate	Hi-Media, Mumbai, India
Potassium dihydrogen phosphate	Hi-Media, Mumbai, India
Disodium hydrogen phosphate	Hi-Media, Mumbai, India
Zinc Chloride ($ZnCl_2$)	Hi-Media, Mumbai, India
Manganese Chloride ($MgCl_2$)	Hi-Media, Mumbai, India
Phenol	Hi-Media, Mumbai, India
Sulfanilic acid	Hi-Media, Mumbai, India
Acetic acid	Hi-Media, Mumbai, India
α-naphthylamine	Hi-Media, Mumbai, India

3.6. Materials utilized for carbohydrate fermentation tests

D (-) Arabinose	Hi-Media, Mumbai, India
D (+) Dextrose	Hi-Media, Mumbai, India
D (+) Galactose	Hi-Media, Mumbai, India
D (+) Lactose	Hi-Media, Mumbai, India
D (+) Rhamnose	Hi-Media, Mumbai, India
D (-) Sorbitol	Hi-Media, Mumbai, India
D (-) Mannitol	Hi-Media, Mumbai, India
Inositol	Hi-Media, Mumbai, India
Lactose	Hi-Media, Mumbai, India
D (+) Arabitol	Hi-Media, Mumbai, India
D (-) Fructose	Hi-Media, Mumbai, India
D (+) Sucrose	Hi-Media, Mumbai, India

3.7. Antibiotics utilized for Antibiotic Susceptibility Testing (AST)

Antibiotic disks	**Short form**	**Concentration**	**Manufacturer**
Gentamicin	GEN	10mcg	Hi-Media, Mumbai, India
Streptomycin	S	10mcg	Hi-Media, Mumbai, India
Kanamycin	K	30mcg	Hi-Media, Mumbai, India
Ampicillin	AMP	10mcg	Hi-Media, Mumbai, India
Methicillin	MET	10mcg	Hi-Media, Mumbai, India
Amoxycillin	AMC	30mcg	Hi-Media, Mumbai, India
Vancomycin	VA	5mcg	Hi-Media, Mumbai, India
Erythromycin	E	15mcg	Hi-Media, Mumbai, India
Azithromycin	AZM	15mcg	Hi-Media, Mumbai, India
Linezolid	LZ	30mcg	Hi-Media, Mumbai, India
Ciprofloxacin	CIP	5mcg	Hi-Media, Mumbai, India
Ofloxacin	OF	5mcg	Hi-Media, Mumbai, India
Norfloxacin	NX	10mcg	Hi-Media, Mumbai, India
Tetracycline	TE	30mcg	Hi-Media, Mumbai, India
Chloramphenicol	C	30mcg	Hi-Media, Mumbai, India
Sulfafurazole	SF	300mcg	Hi-Media, Mumbai, India
Imipenem	IPM	10mcg	Hi-Media, Mumbai, India

Ceftazidime	CAZ	30mcg	Hi-Media, Mumbai, India
Cefixime	CFM	5mcg	Hi-Media, Mumbai, India
Cefuroxime	CXM	30mcg	Hi-Media, Mumbai, India
Ceftriaxone	CTR	30mcg	Hi-Media, Mumbai, India
Cephalothin	CEP	30mcg	Hi-Media, Mumbai, India
Cefotaxime	CTX	30mcg	Hi-Media, Mumbai, India
Aztreonam	AT	30mcg	Hi-Media, Mumbai, India
Rifampin	RIF	5mcg	Hi-Media, Mumbai, India
Novobiocin	NV	30mcg	Hi-Media, Mumbai, India
Clindamycin	CD	2mcg	Hi-Media, Mumbai, India
Trimethoprim	TR	10mcg	Hi-Media, Mumbai, India

3.8. Antibiotics powder utilized for Minimum Inhibitory Concentration (MIC) assay

Antibiotic Powder	**Catalog Number**	**Manufacturer**
Streptomycin sulfate	PCT1120	Hi-Media, Mumbai, India
Ampicillin salt	CMS645	Hi-Media, Mumbai, India
Vancomycin hydrochloride	PCT1114	Hi-Media, Mumbai, India
Erythromycin	PCT1123	Hi-Media, Mumbai, India
Ciprofloxacin hydrochloride monohydrate	CMS1891	Hi-Media, Mumbai, India
Tetracycline hydrochloride	PCT1126	Hi-Media, Mumbai, India
Sulfametaoxale	PCT1135	Hi-Media, Mumbai, India
Ceftazidime pentahydrate	CMS1194	Hi-Media, Mumbai, India
Rifampicin	RM1889	Hi-Media, Mumbai, India
Novobiocin sodium salt	CMS643	Hi-Media, Mumbai, India
Clindamycin hydrochloride	CMS9386	Hi-Media, Mumbai, India
Trimethoprim	RM216	Hi-Media, Mumbai, India

3.9. Material used for genotypic characterization (DNA isolation, PCR amplification, and sequencing)

- The genomic DNA was extracted using Qiagen QIAamp DNA Mini Kit (Qiagen, USA)
- PCR amplification was done using GoTaq Green Master Mix (Promega)

- Gel electrophoresis was used to analyze the amplified PCR products
- The PCR product was purified using a QIAquick PCR purification Kit (Qiagen-28106, USA)
- Sequencing was done with BigDye™ Terminator v3.1 cycle sequencing Kit (Applied Biosystem)
- 16S rRNA gene amplifications were done by using two universal primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′).

TAE buffer	EDTA
Agarose Powder	TE buffer
Ethidium bromide	Tris Base
Milli-Q water	Sodium acetate
Isoamyl alcohol	Chloroform
Sodium dodecyl sulfate (SDS)	Glacial acetic acid

3.10. A list of laboratory equipment and instruments was used

Autoclave	Instrumentation, India
Microscope	Olympus, Model: 808209
Water Distillation Unit	Riviera, Mumbai, India
Shaking Incubator	Remi, Kolkata, India
Incubator	Remi, Kolkata
Laminar Air Flow	Thermo Scientific
Cooling Incubator	Remi, Kolkata
Weighing Balance	MettlernToledo, Switzerland
Hot Air Oven	Remi, Kolkata
Microwave	Samsung, India
Freezer (4 °C)	Samsung, India
Freezer (-20 °C)	Voltas, India
Freezer (-80 °C)	Thermo Scientific

Spectrophotometer	Perkin Elmer, USA
Thermo Cycler	BIORAD-thermal cycler (MJ Mini)
Nanodrop	BIORAD
DNA sequencer (Automated)	Applied Biosystem
ICP-MS	Agilent 7800 ICP-MS
Microwave Digestion System	MARS 6, One Touch, Technology

3.11. Utilized glassware list

Microbiological Petri plates	Borosil, Gujrat, India
Test tubes	Borosil, Gujrat, India
Glass Spreader	Borosil, Gujrat, India
Glass Pipettes	Borosil, Gujrat, India
Glass slides	Borosil, Gujrat, India
Reagent Bottles	Borosil, Gujrat, India
Conical Flasks	Borosil, Gujrat, India
Cover Slip	Borosil, Gujrat, India
Durham tubes	Borosil, Gujrat, India
Droppers	Tarsons, Kolkata, India

3.12. Additional materials that were employed

Cryo Vials	Tarsons, Kolkata, India
Test tubes rack	Tarsons, Kolkata, India
Micro Pipettes	Tarsons, Kolkata, India
Inoculation Loop	Hi-Media, Mumbai, India
Spirit Lamp	Hi-Media, Mumbai, India
Absorbent Cotton	Bengal Surgicals Ltd, Kolkata, India
Non-Absorbent Cotton	Bengal Surgicals Ltd, Kolkata, India
Aluminum Foil	Hi-Media, Mumbai, India
PCR Tubes	Tarsons, Kolkata, India
Floating Rack	Tarsons, Kolkata, India
Micro Tips	Tarsons, Kolkata, India

Cryo Vial Stand	Tarsons, Kolkata, India
pH paper strip	Hi-Media, Mumbai, India
Syringe Filter (0.2 µm)	Hi-Media, Mumbai, India
Dispovan Clinical Syringe (5, 10 ml)	Hindustan Syringes & Medical Devices Ltd., Haryana, India
High Dispo Bag	Hi-Media, Mumbai, India

Methodology

Chapter 4

4. Methodology

4.1. Sampling and Site Description

The present study was designed to work simultaneously on the three temperature zones ranging from 4 °C to 60 °C. The samples were collected from the upper Yumesamdong Valley of North Sikkim, India. The sampling site was chosen as it contains the three distinct natural thermal regions i.e., hot, warm, and cold regions within a short-range area of ~20 m. The hot springs (New Yumesamdong Hot Spring) having temperatures from 61-65 °C form the main geothermal zone whereas the semi-frigid river Lachung Chuu has a temperature of 4 - 8 °C (Das *et al.*, 2021). The geographical stretch lying in between the frigid and fervid zone acts as an intermediate buffer temperate zone that showcases its graded thermos with a decreasing gradient of temperature on reaching the river **Fig 1**. During the heavy winters i.e., end of December to February fortnight, the annual average temperature at (Zero Point) upper Yumesamdong Valley is -12 °C to -7 °C, and hence this river lying in the closest proximity of the hot springs gets frigid at the surface layer whereas the aquifer maintains its tepid state (Das *et al.*, 2023b).

The geographical coordinates and the elevation of the sampling sites were checked by GPSMAP 78S (Garmin, India). The sampling area is constituted of three major sample pooling sites – (a) hot region - the hot spring (b) warm region – the geographical stretch from where the hot water of the aquifer dissipates and (c) cold region – the semi-frigid river basin. Two geographically different and distinctly separate sampling sites (originating from two different hot springs) were taken and the soil samples were collected from all these three zones corresponding to specific thermal degrees **Fig1.** The geographic locations of both sampling sites; Yumesamdong (Site-I) and New Yumesamdong (Site-II) were shown in **Fig. 2**. From site 1, six soil samples, of 100g each in triplicates (100g × 6 × 3 = 1800g) from each zone were taken and similarly, from site 2 six soil samples, of 100g each in triplicates (100g × 6 × 3 =

1800g) from each zone were taken. Thus, a total of 12 soil samples were taken from these two sites. The samples were collected using a sterile shovel and spatula into sterile Ziplock polythene bags and were transported to the laboratory (Department of Microbiology, Sikkim University) in a cooling ice box and kept at -20 °C. Physical parameters such as temperature and pH were measured in situ at the sampling sites using a thermometer and pH strip following the methods described by Das *et al.*, (2021) (Das *et al.*, 2021).

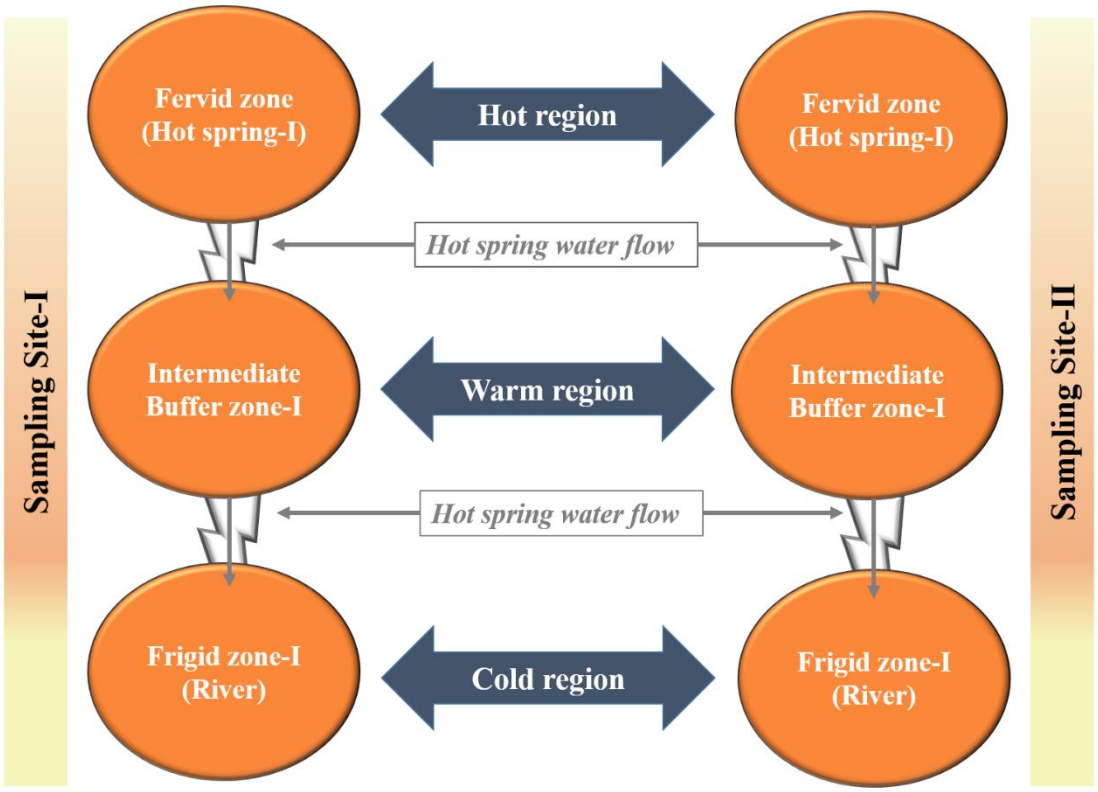


Fig. 1. Showing the distinct natural thermal zones (sampling sites- I and II)

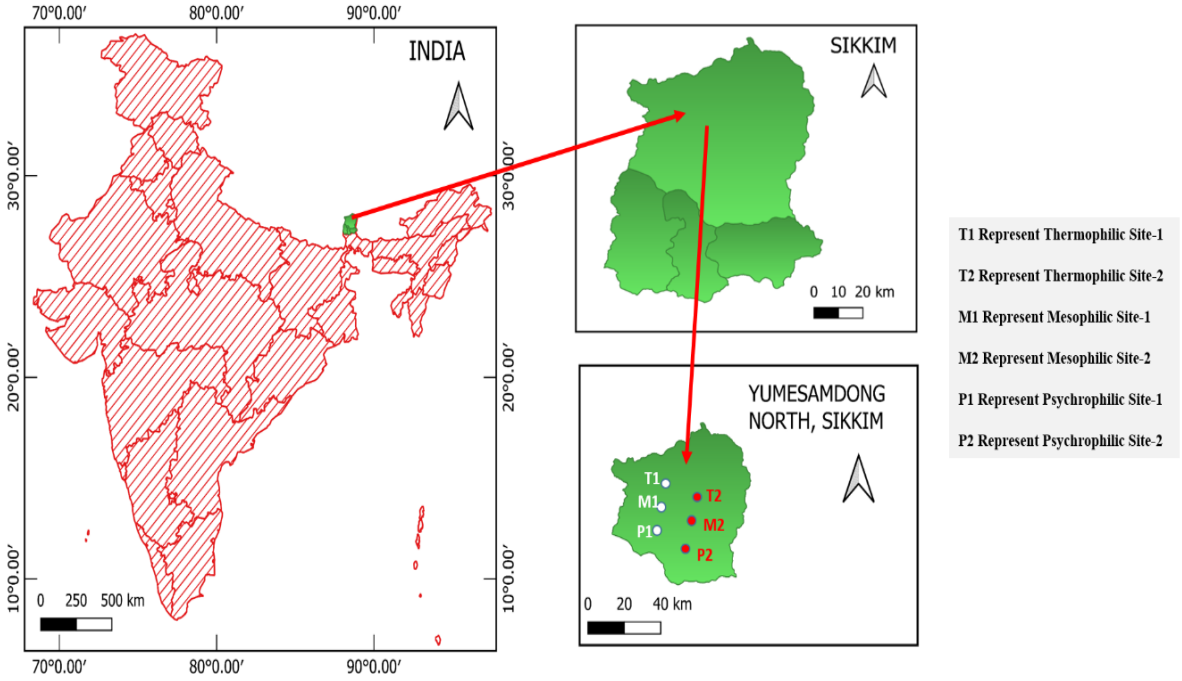


Fig. 2. Showing the geographic locations of both sampling sites (Yumesamdong and New Yumesamdong, North Sikkim, India)

4.2. Culture-dependent study

4.2.1. Isolation and characterization of bacterial isolates

The soil samples were enriched in different enrichment broths like - Super Optimal broth with Catabolite repression (SOC) medium, Reasoner's 2A (R2A) broth, Luria-Bertani (LB) broth, and Nutrient broth medium. Each of the enrichment broths was incubated at three different temperatures and for thermophilic isolation the broths were placed at 60 °C (Najar *et al.*, 2018a). Similarly, for isolation of mesophilic and psychrophilic bacteria, the media were incubated at 37 °C over-night (Kyndt *et al.*, 2022) and 15 °C for 48-72 hrs. (Sherpa *et al.*, 2018, 2021) respectively as displayed in **Fig. 3**. After enrichment the isolation of pure colonies were done by spread plate and streak plate methods (Najar *et al.*, 2018b). The serial dilution method used 10^{-3} to 10^{-9} dilutions of physiological saline (0.7 % NaCl) for bacterial enumeration and the colonies were counted by growing them on Plate Count Agar. The isolated pure colonies

were stored in triplicates in 50 % glycerol and preserved at -80 °C. Isolated pure colonies were characterized based on their colony morphology, Gram staining, and other biochemical tests using Hi-Media Kit (HiMedia Carbo kit KB009) (Najar *et al.*, 2018a; Najar *et al.*, 2020b; Najar *et al.*, 2022b).

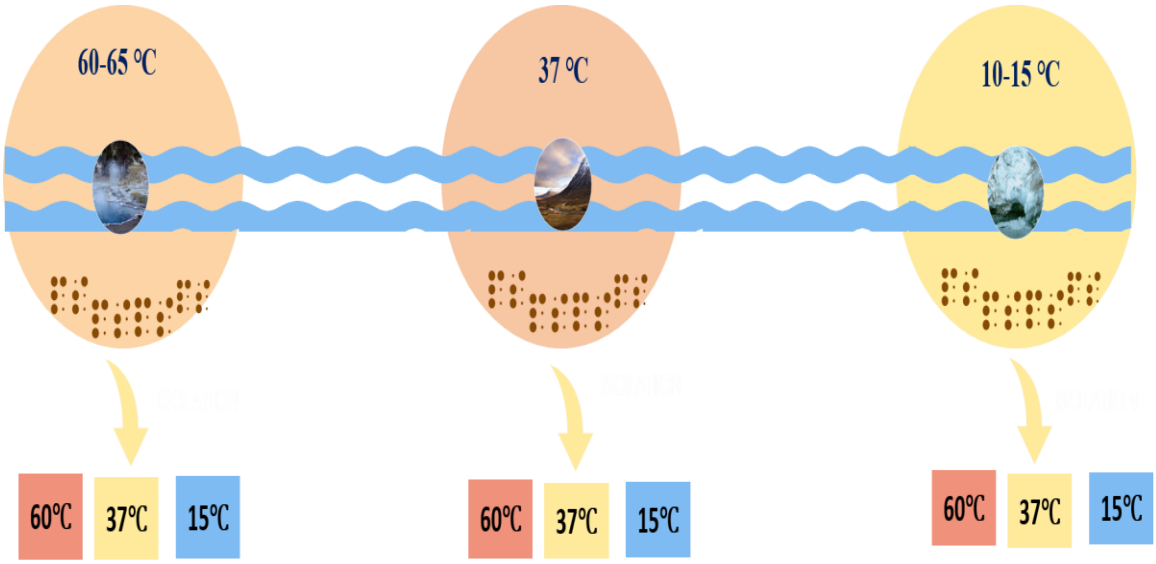


Fig. 3. Showing the isolation temperature of collected samples from distinct natural thermal zones

4.2.2. Bacterial Enumeration

To determine the number of live cells in each sample, bacterial enumeration was done. The serial dilution was performed using fresh culture. First, a test tube with 0.7 % NaCl concentration was used to dilute the sample from 10^{-3} to 10^{-9} the dilution factor. After that, 0.5 mL of each dilution was spread onto a Plate Count Agar (PCA) in triplicates and incubated at various temperatures (60 °C for hot region samples, 37 °C for warm region samples, and 15 °C for cold region samples) for 48–72 hours. Plates with more than 300 colonies were rejected as **Too Numerous to Count (TNTC)**, while plates with less than 20 colonies were also discarded as **Too Few to Count (TFTC**). The plates with ≥20 and ≤300 colonies were taken into account.

The bacterial enumeration procedure adhered to the protocol outlined by (Brugger *et al.*, 2012). Then the number of colonies obtained was calculated by using the formula:

Total viable count of the sample = Number of colonies (average of three replicates)

Volume Plated X Dilution factor

4.2.3. Biochemical characterization of the Isolates

Microorganism detection relies heavily on biochemical assays, which have several benefits including faster identification times, lower costs, and more reliable results. These assays are at the forefront of the fast advancement in the field of microbial detection.

4.2.3.1. Indole Test

The indole test was conducted to assess the ability of specific bacteria to break down the amino acid tryptophan into indole. This breakdown is facilitated by the enzyme tryptophanase, produced by certain indole-positive bacteria (MacWilliams, 2009b).

Procedure

- Inoculate the bacterial culture in peptone water containing peptone and NaCl.
- Incubate the culture overnight at 37 °C.
- Add 0.5 ml of Kovac's indole reagent (Hi-media, Mumbai, India) to the culture.

Result Interpretation

- ✓ Positive response: Occurrence of a pink/red color ring at the interface of the reagent and the broth.
- ✓ Negative response: No color change observed.

Positive/Negative control

- ❖ Indole positive: *Escherichia coli*
- ❖ Indole negative: *Klebsiella pneumoniae*

4.2.3.2. Methyl Red Test

The Methyl Red (MR) test was used to assess the ability of microbes to generate and sustain stable strong acids that arise from the fermentation of glucose. MR is part of the IMViC (Indole, Methyl Red, Voges-Proskauer, and Citrate) test. Methyl red is a pH indicator that turns a red color at pH levels below 4.4, yellow at pH levels overhead 6.2, and orange in between (McDevitt, 2009).

Procedure

- Inoculate the bacterial culture in MR-VP medium (Hi-media, Mumbai, India).
- Incubate the culture overnight at 37 °C.
- Add a few drops of methyl red indicator (Hi-media, Mumbai, India) to the medium.

Result Interpretation

- ✓ Positive response: Bright red color development
- ✓ Negative response: Yellow color development

Positive/Negative control

- ❖ Positive response: *Escherichia coli*
- ❖ Negative response: *Klebsiella pneumoniae*

4.2.3.3. Voges-Proskauer (VP) Test

The VP test was used to detect the presence of acetoin, a metabolic byproduct produced during glucose fermentation. It is commonly performed as part of the IMViC (Indole, Methyl Red, Voges-Proskauer, and Citrate) test. The VP test utilizes a particular reagent well known as the Voges-Proskauer reagent, which comprises alpha-naphthol and potassium hydroxide (KOH). The test is performed in two stages, involving the addition of different reagents (Lehman, 2014).

Procedure

- The MR-VP medium was prepared in-house and inoculated with the bacterial culture.
- The cultures were incubated overnight at 37 °C.
- Barritt's reagent (Hi-media) was added, which is a mixture of 5 % α-naphthol and 40 % potassium hydroxide solution, to the medium and mix.

Result Interpretation

- ✓ Positive response: Pink-red color development at the surface of the medium.
- ✓ Negative response: Yellow color development at the surface of the medium.

Positive/Negative control

- ❖ Positive reaction: *Klebsiella pneumoniae*
- ❖ Negative reaction: *Escherichia coli*

4.2.3.4. Citrate Utilization Test

The test is used to evaluate the ability of a microorganism to utilize citrate as its only source of carbon for growth. The test is conducted using a medium called Simmons' citrate agar, which includes ammonium dihydrogen phosphate as the sole source of nitrogen and sodium citrate as the only carbon source. The medium also includes bromothymol blue, a pH indicator that adapts its color to the pH of its surroundings (MacWilliams, 2009a).

Procedure

- Agar slants of Simmons citrate agar were prepared (Hi-media, Mumbai, India) in test tubes.
- Bacterial stab culture was placed into the butt of the slant and streaked on the slant using a loop.
- The test tubes were incubated at 37 °C overnight.

Result Interpretation

- ✓ Positive response: Growth with a deep blue color in the slant
- ✓ Negative response: Absence of growth and no color change in the medium

Positive/Negative control

- ❖ Citrate positive: *Klebsiella pneumoniae*
- ❖ Citrate negative: *Escherichia coli*

4.2.3.5. Carbohydrate Fermentation Test

The test for fermentation of carbohydrates evaluates the ability of a microorganism to break down a particular carbohydrate. It utilizes a pH indicator in a sugar fermentation medium. If the microorganism can metabolize the carbohydrate, fermentation takes place, producing acid and changing the pH indicator's color. Durham tubes are used for detecting gas produced by some species. In the tubes, the production of gas generates clear bubbles (Reiner, 2012).

Procedure

- Prepared sugar fermentation broth with distinct sugars, inserted the Durham's tubes with an indicator (phenol red)
- All the test tubes were incubated at 30 °C for 24-48 hrs.
- Observed the result

Result Interpretation

- ✓ Positive response: The presence of a yellowish color change and gas formation in the Durham tubes indicated a positive response.
- ✓ Negative response: No any color variations were observed.

Positive/Negative control

- ❖ A control tube with only sugar fermentation broth was comprised for assessment.

4.2.3.6. Catalase Test

The catalase test was conducted to identify the breakdown of hydrogen peroxide (H_2O_2) into oxygen (O_2) and water. Organisms capable of producing catalase generate bubbles (oxygen) when exposed to hydrogen peroxide (Reiner, 2010).

Procedure

- A loopful of fresh culture was transferred onto a clean glass slide, and a few drops of H_2O_2 solution were added.
- The formation of bubbles was promptly observed.

Result Interpretations

- ✓ Positive response: Rapid formation of bubbles.
- ✓ Negative response: No bubble formation.

Positive/Negative control

- ❖ A few drops of H_2O_2 solution were added onto a clean glass slide.

4.2.3.7. Nitrate Test

The nitrate test is used to determine an organism's ability to convert nitrate to nitrite (Ryan *et al.*, 2001).

Procedure:

- To perform the test, nitrate broth was prepared and sterilized by autoclaving before inoculating it with a bacterial culture.
- Then the bacterial culture was inoculated and incubated under specific temperatures overnight.

- After proper incubation, nitrite reagent A and nitrite reagent B are added to the tubes, typically 6-8 drops each.
- The formation of bubbles was promptly observed.

Result from Interpretations

- ✓ Positive response: If the nitrate broth becomes red following the addition of nitrate I and nitrate II within a minute.
- ✓ Negative response: When adding zinc powder, if a red color is seen for at least three minutes after adding zinc

Positive/Negative control

The test tubes were sterile nitrate broth without a bacterial culture in them.

4.2.3.8. Urease Test

The purpose of the urease test was to determine whether the bacterial isolates had any urease activity. Amino acids are converted to urea by the process of decarboxylation (Lehman, 2014). When urea is hydrolyzed, ammonia (NH_3) and carbon dioxide (CO_2) are the byproducts (Habchi *et al.*, 2018). The amount of alkalinity in the medium rises when ammonia is present. The pH indicator phenol red's color changes can be used to identify this pH shift. At a pH of 6.8, phenol red turns a light orange color; at a pH of 8.1, it turns magenta or pink.

Procedure

- A pure colony was streaked onto Christensen's urea agar slant.
- Then the tubes were incubated at 37 °C overnight
- Observed the result

Result /Interpretations

- ✓ Positive response: The slant color turned pink.
- ✓ Negative response: The medium color was not changed and was still yellow.

Positive/Negative control

- ❖ Positive response: *Proteus mirabilis*
- ❖ Negative response: *Escherichia coli*

4.2.3.9. Oxidase Test

The oxidase test is a valuable method used to determine the presence of the cytochrome c oxidase enzyme in microorganisms (Ludwig *et al.*, 2001). This particular enzyme is essential for the electron transport chain in aerobic respiration, acting as the terminal electron acceptor. It facilitates the transfer of electrons to molecular oxygen (O_2), ultimately leading to the production of water (H2O). The oxidase test relies on the oxidation reaction of a colorless reagent called N, N, N′, N′-Tetramethyl-p-phenylenediamine dihydrochloride (TMPD) (Chauhan *et al.*, 2015). This reagent serves as an artificial electron donor and is specifically oxidized by the cytochrome c oxidase enzyme when oxygen is present.

Procedure

- Hi-Media's Oxidase disc (Catalogue No. DD018-1VL) was utilized, and it was put on sterile filter paper.
- An isolated colony was selected, and the oxidase disc was rubbed with a sterile inoculation loop.
- The color change was observed

Result/Interpretations

- ✓ Positive response: When color quickly shifts to dark purple in an instant of 5 to 10s
- ✓ Negative response: No color changes are seen beyond 60s

Positive/Negative control

- ❖ Oxidase positive: *Pseudomonas aeruginosa*
- ❖ Oxidase negative: *Escherichia coli*

4.2.3.10. Amylase Activity

The amylase test was used to assess the presence and activity of the enzyme amylase with the tested bacterial isolates (Yassin *et al.*, 2021). Amylase is an important enzyme that facilitates the hydrolysis of starch, a complex polysaccharide, into smaller, soluble simple sugar molecules such as maltose and glucose. The amylase test operates on the principle that amylase can break down starch (Fulton *et al.*, 2008). This enzymatic breakdown can be detected by observing changes in the appearance of the medium containing starch.

Procedure

- Starch agar plates were prepared by subjecting them to autoclaving, ensuring their sterility.
- Then the media was poured into a sterile petri plate, once the plates solidified, a bacterial culture was inoculated at a sterile inoculation loop on the agar surface.
- The plates were then incubated at a temperature of 55 °C overnight, allowing the bacterial cells to grow and interact with the starch present in the medium.
- Following the incubation period, the plates were flooded with Gram's iodine solution. The iodine solution was allowed to remain in contact with the plates for a brief duration, typically a few seconds, and the result was noted.

Result from Interpretations

- ✓ Positive response: When the medium's color changed and the zone appeared noticeable
- ✓ Negative response: No any color changes and zones were observed

4.2.4. 16S rRNA Sequencing and Phylogeny for Bacterial Identification

A total of 261 bacterial isolates were obtained from 12 soil samples taken at two distinct sampling locations from three different thermal zones. Based on morphological characterization, 63 bacterial isolates were selected for partial 16S rRNA gene-based identification. The genomic DNA was extracted using the Qiagen QIAamp DNA Mini Kit (Qiagen, USA) as per the manufacturer's instructions. The 16S rRNA gene amplifications were done by using two universal primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′). The polymerase chain reaction (PCR) was performed in 25µL volume using 12.5µL Hi-Chrome Master mix, 1µL each of 20 pM forward and reverse primers, 8.5µL sterile DNase-free nucleated water, and 2µL of the DNA template. The PCR cycle was designed as, 5 min at 94 °C; 35 cycles of 1 min at 94 °C, 1 min at 55 °C, and 1.5 min at 72 °C; and a final extension step of 10 min at 72 °C. Gel electrophoresis was used to analyze the amplified PCR products. The PCR product was purified using a QIAquick PCR purification Kit (Qiagen, USA) for sequencing. The purified 16S rDNA was then sequenced using a BigDye™Terminator v3.1 cycle sequencing kit (Applied Biosystems, USA) as manufacturer's instructions using an Automated DNA Sequencer (ABS/Genetic 3500 Analyzer). The sequence obtained was assembled manually using Finch TV 1.4. Assembled sequences were compared with the nr/nt database of NCBI using BLASTn sequence homology search for taxonomic identification. Then the identified sequences and their related genome sequences were aligned by Clustal Omega (Sievers and Higgins, 2014). The phylogenetic tree was constructed using the Neighbor-joining method (Tamura *et al.*, 2004) with Tamura–Nei evolutionary distance measurement (Tamura *et al.*, 2004) in MEGA 11 software, and the phylogenetic tree was modified using iTOL interactive tree of life software (Letunic and Bork, 2021).

4.2.5. Antibiotic Susceptibility Testing (AST) of the bacterial Isolates

The experiment utilized the Kirby Bauer Disc Diffusion method to assess the susceptibility of bacterial isolates to various antibiotics. To establish a standardized level of turbidity known as McFarland 0.5, a single colony of the pure isolates was transferred into a sterile solution of 5 ml NaCl (0.9 %). The resulting suspension was thoroughly mixed and uniformly spread across the surface of Mueller-Hilton Agar (MHA) using a sterile cotton swab.

Once the agar surface was evenly coated, a sterile antibiotic disc was aseptically placed onto the swabbed plate. This antibiotic disc contained a specific antibiotic that was being tested for its effectiveness against the bacterial isolate. To serve as a negative control, the plate was also swabbed with sterile 0.4 µm membrane filter paper discs that had been soaked in sterile autoclaved water. We conducted antibiotic sensitivity testing on a single bacterial isolate using 28 different antibiotics from fifteen classes. The antibiotics used were as follows: Gentamicin (10 mcg), Streptomycin (10 mcg), Kanamycin (30 mcg), Ampicillin (10 mcg), Methicillin (10 mcg), Cephalothin (30 mcg), Amoxycillin (30 mcg), Cefotaxime (30 mcg), Vancomycin (5 mcg), Erythromycin (15 mcg), Azithromycin (15 mcg), Linezolid (30 mcg), Ciprofloxacin (5 mcg), Ofloxacin (5 mcg), Norfloxacin (10 mcg), Tetracycline (30 mcg), Chloramphenicol (30 mcg), Sulfafurazole (300 mcg), Imipenem (10 mcg), Ceftazidime (30 mcg), Cefixime (5 mcg), Cefuroxime (30 mcg), Ceftriaxone (30 mcg), Aztreonam (30 mcg), Rifampin (5 mcg), Novobiocin (30 mcg), Clindamycin (2 mcg), and Trimethoprim (10 mcg). After incubating the MHA plates for 48 hours at three different temperatures: 60 °C, 37 °C, and 15 °C, we measured the diameter of the zone of inhibition. The susceptibility tests were performed following CLSI guidelines, as described (Nguyen *et al.*, 2016; Sherpa *et al.*, 2020; Najar *et al.*, 2022a).

4.2.6. Minimum Inhibitory Concentration (MIC) assay

The MIC is the lowest concentration of an antimicrobial agent that prevents the visible growth of microorganisms after a specified incubation period. The determination of MIC is crucial in evaluating the susceptibility of specific isolates to different antimicrobial agents. In this study, twelve antibiotics from distinct classes were utilized in powder form to determine the MIC value of the isolates. To determine the MIC values of the selected bacterial isolates, a standardized microdilution method, following the protocol established by Elshikh *et al.*, (2016), was employed. This method involved the use of resazurin dye as an indicator. Resazurin dye, a non-fluorescent blue compound, is commonly used in microdilution assays (Elshikh *et al.*, 2016). It acts as a metabolic indicator, changing color from blue to pink when reduced in the presence of metabolically active cells. The reduction of resazurin color indicates cell viability and growth, facilitating the visual detection of microbial growth inhibition. For the preparation and storage of testing reagents, 0.015 g of resazurin was dissolved and vortexed to obtain a concentration of 0.015 %. The solution was then filtered with a sterilized (0.22 µm filter) and stored at 4 °C for future use (Elshikh *et al.*, 2016).

To determine the MIC, a series of twofold dilutions of the antimicrobial agent were prepared in a microtiter plate. Each well was subsequently loaded with 50 ml of MHB growth medium and standardized inoculums of the selected isolates. The inoculums were prepared following the standard guidelines set by the Clinical and Laboratory Standards Institute (CLSI, 2018), with the inoculums adjusted to an equivalent of 10^8 colony-forming units per ml (CFU/ml) and set at 600 nm. After inoculation, the plate was covered and labeled. Then the microtiter plate was incubated under appropriate conditions, allowing the microorganisms to grow in the presence of different concentrations of the antimicrobial agent. Following the incubation period, resazurin dye was added to each well and allowed to incubate for 3-4 hrs.

A pink color change in the wells indicated metabolic activity and microbial growth, whereas a blue color indicated growth inhibition. The MIC was determined as the lowest concentration of the antimicrobial agent at which no pink color change was observed, indicating complete inhibition of microbial growth. The experimental setup included a positive control well-containing growth medium without the antimicrobial agent and a negative control well-containing growth medium without the inoculums.

4.2.7. Multiple Antibiotic Resistance (MAR) index

For monitoring bacterial infections and assessing antibiotic resistance, the Multiple Antibiotic Resistance (MAR) index is a very helpful and efficient tool. It gives valuable insights into the patterns of antimicrobial resistance and the capacity to locate possible high-risk sources of contaminants. As a result, decision-making by governments and healthcare professionals is made possible, leading to the promotion of more efficient infection control practices and monitoring of antibiotics. We may make great progress in reducing the growing threat of drug resistance and ensuring public health by adding the MAR index to regular surveillance and monitoring procedures.

MAR index is determined by dividing the overall number of antibiotics that have been exposed to an isolate by the number of antibiotics that were found resistant. Bacterial isolates with a MAR index of more than 0.2 are likely to have been exposed to several antibiotics and may also indicate a higher chance of MDR, which makes treatment more challenging. Its ability to identify high-risk sources of contamination and provide valuable insights underscores its significance for informed decision-making and public health planning. By incorporating the MAR index into routine surveillance protocols, we can proactively address antibiotic resistance, safeguard the effectiveness of antibiotics, and ultimately protect public health.

4.3. Culture-independent study

4.3.1. Elemental analysis

All the collected soil samples collected were subjected to a drying process in a hot air oven at 50 °C overnight to eliminate moisture. Once dried, the samples were finely ground into a powder using a mortar and pestle, resulting in fine soil dust. The sample digestion process was then carried out on 1 g of the powdered samples, with triplicate measurements. This process involved the use of two concentrated acids: 69 % EMPARTA ACS nitric acid (HNO_3) from Merck and 48 % EMPARTA ACS hydrochloric acid (HCl), also from Merck. The digestion was performed using a microwave-assisted acid digestion method as described by Enamorado-Báez *et al.*, (2013) (Enamorado-Báez *et al.*, 2013). During digestion, 2 mL of HNO_3 and 5 mL of HCl acid were mixed with 1 g of powder samples and placed in microwave vessels made of Polytetrafluoroethylene (PTFE) (Enamorado-Báez *et al.*, 2013). These PTFE vessels were then appropriately positioned in an Anton Paar Microwave Reaction System Multiwave PRO, and the procedure was set to run for 30 mins at 200 °C. After completion, the PTFE vessels were allowed to cool, and the samples were filtered using a 0.2 μm syringe filter from Sigma-Aldrich. Finally, the samples were diluted 50 times in MilliQ water, following the method described by Enamorado-Báez *et al.*, (2013).

Once the digestion was completed, Inductively Coupled Plasma Mass Spectrometry (ICPMS) analysis was carried out using an Agilent 7900 instrument. A total of 19 elements, namely Li, Mg, K, Ca, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Mo, Ag, Cd, Pb, Na, P, and Cr, were measured. To assess and determine the characteristics of the soil samples, a Piper analysis was conducted, following the method described by Teng *et al.*, (2016).

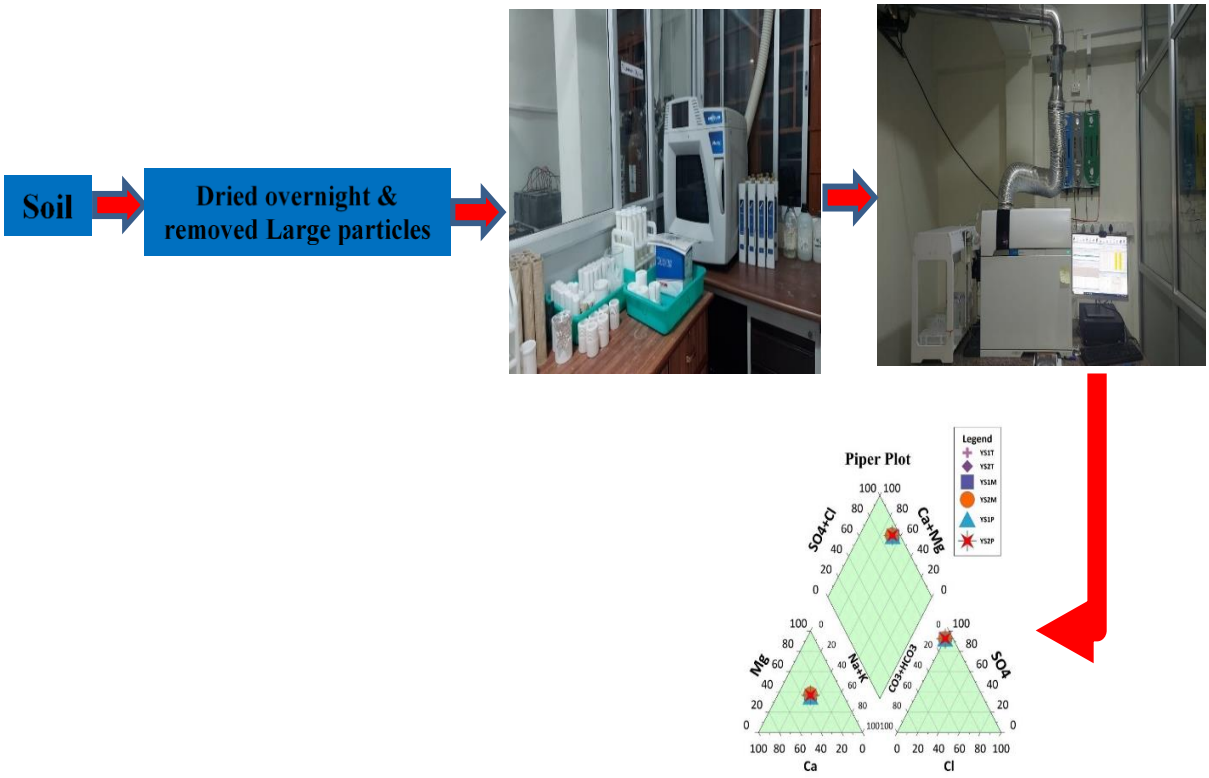


Fig. 4. Illustrating the process and methods of elemental analysis

4.3.2. Metagenomic DNA extraction

The eDNA (environmental soil DNA) was extracted following the manufacturer's instructions using the XpressDNA Soil Kit (50) (Cat: MG20So-50) from MagGenome Technologies Pvt Ltd, based in Chennai, India. To assess the quality of the extracted DNA, quantification was performed using a Qubit Fluorometer from Thermofisher Scientific, USA, which has a detection limit of 10–100 ng/µL. Furthermore, the extracted DNA was examined through 0.8 % agarose gel electrophoresis.

4.3.3. Amplicon Sequencing

The 16S rRNA genes for the V3 and V4 regions were amplified using two primers: 16S rRNA-F (5'-GCCTACGGGNGGCWGCAG-3') and 16S rRNA-R (5'-ACTACHVGGGTATCTAA-TCC-3') (Klindworth *et al.*, 2013). The amplicon libraries were created using the Nextera XT Index Kit (Illumina Inc.), following the 16S rRNA metagenomic sequence library preparation

technique described by Klindworth *et al.*, (2013). To purify the amplicon library, AMPure XP beads were employed. The concentration of the amplified library was determined using a Qubit fluorometer, and the library (10 pM, 500 uL) was transferred to the MiSeq cartridge for cluster creation and sequencing, guided by the Qubit fluorometer and bioanalyzer data. Paired-end sequencing was performed on the Illumina HiSeq 4000 platform.

After sequencing, the high-quality 16S rRNA gene amplicon sequence reads were trimmed to remove barcode and adaptor sequences, as described by Faircloth *et al.*, (2014). Subsequently, pre-processing steps were carried out on the adapter-trimmed sequences, including de-replication, removal of singletons, OTU clustering, and chimera filtering using SolexaQA. Sequences with a Phred score below 20, ambiguous bases with mismatched primers, and short read lengths of 100 bp were eliminated. The operational taxonomic units (OTUs) were annotated and standardized at a 97 % similarity level using UPARSE OTU clustering and QIIME (Kopylova *et al.*, 2016). Standardization was performed using both a built-in script and METAGEN aid. The generated representative OTUs were taxonomically classified and aligned using the Green Genes database (https://greengenes.lbl.gov). The workflow resulted in the classification of the reads at various taxonomic levels, including kingdom, phylum, class, order, family, genus, and species. Sequences that lacked a homologous pair were labeled as unidentified.

4.3.4. Shotgun Metagenomic Sequencing

The DNA from soil samples was extracted using the XpressDNA Soil Kit- MG20So-50 (MagGenome Pvt Ltd.) to obtain total environmental DNA (eDNA). To evaluate the purity of the extracted DNA, a NanoDrop 1000 UV-VIS Spectrophotometer (Thermo Scientific) was utilized (Cheng *et al.*, 2021). Subsequently, the eDNA was purified using the Genomic DNA Purification Kit- Catalogue No. K0512 (Thermo Scientific). The eDNA samples were then used to construct conventional paired-end DNASeq libraries on an Illumina 4000 platform,

following the manufacturer's instructions. The sequencing process employed 101bp paired-end modules. To ensure quality control, the NGSQC tool set (Patel and Jain, 2012) was employed to analyze the reads from each library, resulting in the identification of high-quality (HQ) reads in a fastq format. A stringent criterion of 80:30 was applied to filter out low-quality reads, retaining only HQ reads with more than 80 % high-quality bases and individual Phred scores greater than 30. The HQ-filtered paired-end library reads were then used for independent genome assemblies of each sample.

4.3.5. Metagenomic data analysis

The DNA sequences obtained were utilized for bacterial taxonomic identification. The identification process involved analyzing the annotations, functions, and composition of bacterial communities using the MG-RAST (https://mg-rast.org) platform (Keegan *et al.*, 2016). To determine the taxonomic classification of the representative Operational Taxonomic Units (OTUs), the SILVA database (Pruesse *et al.*, 2007) and the RefSeq database (Pruitt *et al.*, 2007) were employed. Through this analysis, the reads were classified into various taxonomic levels, including kingdom, phylum, class, order, family, genus, and species. Sequences that did not exhibit any similarity to known bacterial counterparts were categorized as unknown. The comprehensive workflow produced a classification system for the reads, enabling the identification of bacterial taxa.

4.3.6. Antibiotic-Resistant Gene detection from metagenomic sequences

The detection of antibiotic-resistant genes in metagenomic assembled sequences was performed using the Comprehensive Antibiotic Resistance Database (CARD) (https://card.mcmaster.ca/home), as described by Alcock *et al.*, (2020). To conduct the CARD analysis, the default parameters of the Metagenomic Read Mapping Service from the Bacterial and Viral Bioinformatics Resource Center, previously known as PATRIC (Wattam *et al.*,

2017), were utilized. Additionally, alternative pipelines, namely Galaxy ARGA and Galaxy ABRicate, were employed for identifying antibiotic resistance genes (ARGs) within the metagenomic assembled sequences (Afgan *et al.*, 2018). These pipelines were implemented using the Galaxy platform. To validate the predicted resistance genes, a similarity search was conducted in the NCBI non-redundant database.

4.3.7. Detection of Heavy Metal Resistance, Gyrase, and Virulence Genes in Metagenomic Sequences

In this study, we looked at the existence of virulence, gyrase, and metal resistance genes (MRGs) in metagenomic sequences. We used the KBase database and BacMetScan V.1.0 as the two main tools for performing this. As described by Najar *et al.*, in their experiments carried out in 2022a and 2020b, we used the Annotate Metagenome Assembly with Prokka - v1.14.5 tool as support. A specified bioinformatics program called BacMetScan V.1.0 was created exclusively for locating and analyzing metal resistance genes in genomic sequences. It makes use of a sizable database made up of known metal resistance genes. To find and characterize the presence of metal resistance genes in the examined samples, BacMetScan compares the metagenomic sequences with this huge database using cutting-edge algorithms.

The KBase database, on the other hand, is a freely available resource that offers a variety of biological data and computer programs for study and analysis. The KBase database was used in this work to gather further details and insights on the revealed virulence, gyrase, and metal resistance genes. The database made it possible to analyze these genes in great detail and study their probable roles in the metagenomic samples. We used the program Annotate Metagenome Assembly with Prokka - v1.14.5 to aid with the investigation. This program was designed primarily to annotate and analyze metagenomic sequences.

4.3.8. Statistical Analysis

We conducted elemental analysis, specifically utilizing the Piper plot construction method, with the aid of Grapher v20 software. For Principal Component Analysis and Bubble plot generation, we employed R software (Team, 2013). Graph Pad Prism was utilized for regression and correlation analysis, t-test, and ANOVA. To calculate the Shannon diversity indices and chao1, we employed EstimateS and PAST software, respectively (Chao *et al.*, 2006). R software, specifically the iNEXT package, was used to plot the rarefaction curve at the species level of diversity. For comparative bacterial diversity analysis at the genus level, we utilized a heat map generated by R software, employing the Bray Curtis Dissimilarity matrix and the ggplot package's heatmap.2 function.

Results

Chapter 5

5. Results

5.1. Sampling site geographic locations and assessment of physical parameters

The geographical coordinates of the two sites are 27° 91'77.6"N 88° 69'42.1"E with an elevation of 4395 mamsl. The soil samples of different zones from two sites were coded as YS1T and YS2T (for the hot region); YS1M and YS2M (for the warm region) and YS1P and YS2P (for the cold region). The temperature and pH of the three thermal zones of each site were recorded. The temperature was 61±2 °C (hot region), 37±2 °C (warm region), and 4±2 °C (cold region) for site 1. Similarly, for site 2 the temperature was 55±2 °C (hot region), 37±2 °C (warm region), and 8±2 °C (cold region). The pH was found to be 8.28±2 (hot region), 8.9±2 (warm region), and 9±2 (cold region) for site 1. Similarly, for site 2 the pH was 8.6±2 (hot region), 9.16±2 (warm region), and 9.4±2 (cold region) as shown in **Table 1.** Thus, from the recorded pH it can be said that these areas were alkaline. The alkalinity can be contributed by the geological attributes.

Based on the physical parameters, the pH levels of samples from different thermal gradient zones were consistently alkaline and mostly similar. The only variation observed was in temperature, ranging from hot to cold regions. This suggests that the pH does not significantly impact the distinct thermal gradient sampling zones, but rather, it is the changing temperature that may influence the microbial diversity in these sampling regions.

Table 1 The physical parameters (temperature and pH) of the distinct natural thermal zones.

Sample Sites		**Temperature**	**pH**
Site-I	Hot region	61 °C	8.28
	Warm region	37 °C	8.90
	Cold region	4 °C	9.00
Site-II	Hot region	55 °C	8.6
	Warm region	37 °C	9.16
	Cold region	8 °C	9.4

5.2. Elemental Analysis

ICP-MS analysis was employed to assess the concentration of 19 elements. The results revealed that the two sampling sites consisted of three distinct zones categorized as hot, warm, and cold regions. Notably, these zones exhibited a noteworthy abundance of elements, particularly Mg, K, Ca, Mn, Fe, and Li. Additionally, the concentrations of each element were found to be relatively consistent across multiple samples obtained from different zones, as depicted in **Table 2**. To further analyze the data, a Piper analysis was conducted, and the resulting Piper plot indicated that all soil samples, including YS1T, YS2T, YS1M, YS2M, YS1P, and YS2P, were positioned at the same point on the plot. This observation suggests that the samples shared similar characteristics and were classified as calcium-sulfate type, as illustrated in **Fig. 5**. Consequently, the findings imply that there were no significant variations in the concentration levels of different elements among the various samples.

Table 2 Displays the concentrations of various elements in different samples, measured in parts per billion (ppb).

Elements	**YS1T**	**YS2T**	**YS1M**	**YS2M**	**YS1P**	**YS2P**
Li	2.749	2.895667	2.987333	2.889	1.326333	1.740333
Mg	397.1823	403.995	431.9793	356.1277	207.1903	303.046
K	501.8703	540.4657	455.2773	424.6263	224.267	350.4203
Ca	10.74833	11.03867	14.649	7.137667	11.827	13.653
Mn	29.34	36.73133	28.155	30.19	22.89967	32.56733
Fe	1644.289	1856.399	1723.003	1768.343	1029.268	1474.428
Co	0.511667	0.529667	0.514	0.431333	0.294333	0.400667
Ni	0.433667	0.532333	0.797	0.481667	0.119	0.287
Cu	0.600333	0.573333	0.572333	0.380333	0.436667	0.538

Zn	3.462667	3.688667	3.751667	3.46	1.612333	2.926667
As	0.424333	0.588667	0.342333	0.445	0.695667	0.96
Se	0.200333	0.283333	0.104333	0.109	0.196	0.313667
Mo	0.000333	0	0	0.058667	0.002	0.000333
Ag	0.001333	0.000667	0.004667	0	0	0.000667
Cd	0.004667	0.006333	0.003	0.005667	0.003667	0.002333
Pb	0.095667	0.104	0.144667	0.125	0.068667	0.1
Na	1085.622	1135.261	950.6573	990.0793	1007.26	1018.05
P	3691.354	3689.461	4666.264	3767.525	3352.231	3822.845
Cr	115.2717	111.5097	109.2917	110.8563	107.36	111.0167

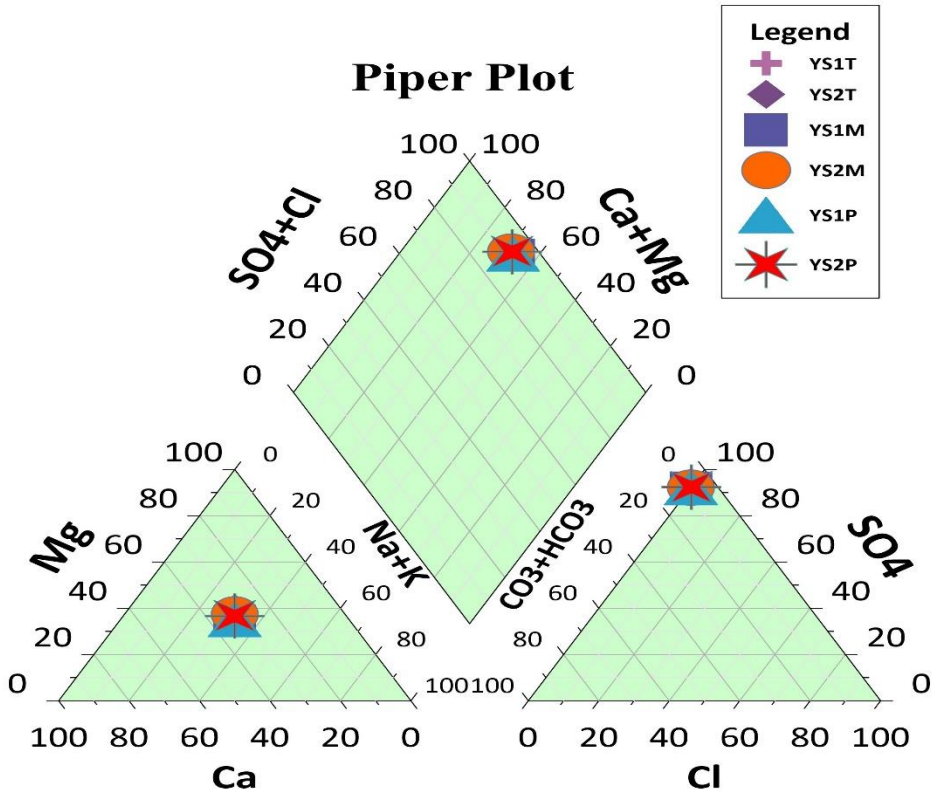


Fig. 5. Piper Diagram plotting various sampling points

5.3. Culture-dependent analysis of soil samples

5.3.1. Bacterial enumeration

Based on the microbial viable count obtained from the soil samples collected from distinct natural thermal gradient regions, namely the warm region, cold region, and hot region, interesting patterns emerged at various dilution factors. In the warm and cold regions, when the soil samples were diluted at a dilution factor of 10^{-3}, a significantly higher number of colonies were observed. This suggests that the microbial population in these regions is abundant and thriving. However, when the samples were further diluted to a dilution factor of 10^{-9}, the colonies were still countable, indicating that even at lower concentrations the microbial colonies were present and could be observed. It is worth noting that for the specific soil sample labeled as META12PSYH3, colonies were only countable in the range between dilution factors 10^{-3} and 10^{-7}. Contrastingly, the hot region exhibited the lowest number of colonies compared to the warm and cold regions. Even at the most diluted factor of 10^{-9}, colonies were easily countable, suggesting a relatively smaller microbial population in the hot region compared to the other two thermal regions. All the observed colonies from the distinct thermal zones' soil samples were documented in **Table 3**, highlighting the varying colony counts at different dilution factors and regions.

Table 3. Present the quantitative analysis of bacterial populations.

Sample Id	Dilution factor	Colony count
META01MESOH2	10^{-3}	TNTC
	10^{-5}	187
	10^{-7}	74
	10^{-9}	34
META02MESOH2	10^{-3}	TNTC
	10^{-5}	213
	10^{-7}	88
	10^{-9}	41
META03THERMH2	10^{-3}	289
	10^{-5}	129

	10^{-7}	56
	10^{-9}	TFTC
META04THERMH2	10^{-3}	263
	10^{-5}	135
	10^{-7}	61
	10^{-9}	TFTC
META05PSYH2	10^{-3}	TNTC
	10^{-5}	295
	10^{-7}	171
	10^{-9}	74
META06PSYH2	10^{-3}	TNTC
	10^{-5}	184
	10^{-7}	79
	10^{-9}	31
META07THERMH3	10^{-3}	201
	10^{-5}	95
	10^{-7}	39
	10^{-9}	TFTC
META08THERMH3	10^{-3}	279
	10^{-5}	129
	10^{-7}	72
	10^{-9}	40
META09MESOH3	10^{-3}	TNTC
	10^{-5}	187
	10^{-7}	81
	10^{-9}	34
META10MESOH3	10^{-3}	TNTC
	10^{-5}	205
	10^{-7}	88
	10^{-9}	23
META11PSYH3	10^{-3}	TNTC
	10^{-5}	269
	10^{-7}	91
	10^{-9}	47
META12PSYH3	10^{-3}	TNTC
	10^{-5}	285
	10^{-7}	57
	10^{-9}	TFTC

5.3.2. Isolation and morphological characterization of bacterial isolates

A total of 261 bacterial isolates were obtained from various soil samples, each representing a different region's climate conditions. Specifically, there were 77 isolates from cold regions, 80 isolates from warm regions, and 104 isolates from hot regions. To obtain these bacterial isolates, each soil sample was subjected to culture at three distinct temperatures: 15 °C, which

aimed to isolate psychrophiles (organisms adapted to cold temperatures); 37 °C, targeting mesophiles (organisms adapted to moderate temperatures); and 60 °C, aimed at isolating thermophiles (organisms adapted to high temperatures).

The bacterial isolates were categorized into two groups using a differential staining technique, with reference to their Gram staining response. Based on the morphological characterization, the majority of the isolates were identified as Gram-positive bacteria that formed spores. These bacteria were predominantly rod-shaped, exhibiting colonies with a pale-yellowish or creamy-white appearance. However, due to the extensive number of isolates, only a subset of 63 representative bacterial isolates were selected for further investigation based on their morphological characteristics.

5.3.3. Bacterial tolerance to temperatures

The bacterial isolates were subjected to temperature tolerance tests spanning a range from 10 °C to 60 °C to assess their ability to grow under various temperature conditions. The results revealed distinct temperature preferences among the bacterial isolates. Thermophilic bacteria, known for their affinity towards high temperatures, demonstrated optimal growth at 50 °C. On the other hand, mesophilic bacteria, which thrive in moderate temperatures, exhibited their best growth at 37 °C. Psychrophilic bacteria, adapted to cold environments, displayed optimal growth at 20 °C as displayed in **Fig. 6**.

In the hot region, the prevalence and abundance of thermophilic bacteria were the highest as displayed in **Fig. 7 a**, accounting for 70.37 % of the isolates. Thermotolerant bacteria constituted 22.22 % of the isolates, while mesophilic bacteria accounted for 7.4 %. No psychrophilic or psychrotolerant bacteria were found in the hot region. Similarly, in the warm region, mesophiles were the most abundant as displayed in **Fig. 7 b**, comprising 68.42 % of the isolates. They were followed by psychrophiles at 15.78 %, thermophiles at 10.52 %, and

thermotolerant bacteria at 5.26 %. No psychrotolerant bacteria were detected in the warm region. In the cold region, all types of bacteria were present except for thermophiles. Psychrophiles were the dominant group as displayed in **Fig. 7 c**, constituting 70.58 % of the isolates, followed by mesophiles at 23.52 % and psychrotolerant bacteria at 5.88 %. No thermophiles were found in the cold region. These findings are visually represented in **Fig. 8** and indicate the distribution and abundance of different bacterial types in each region based on the temperature preferences of the isolates.

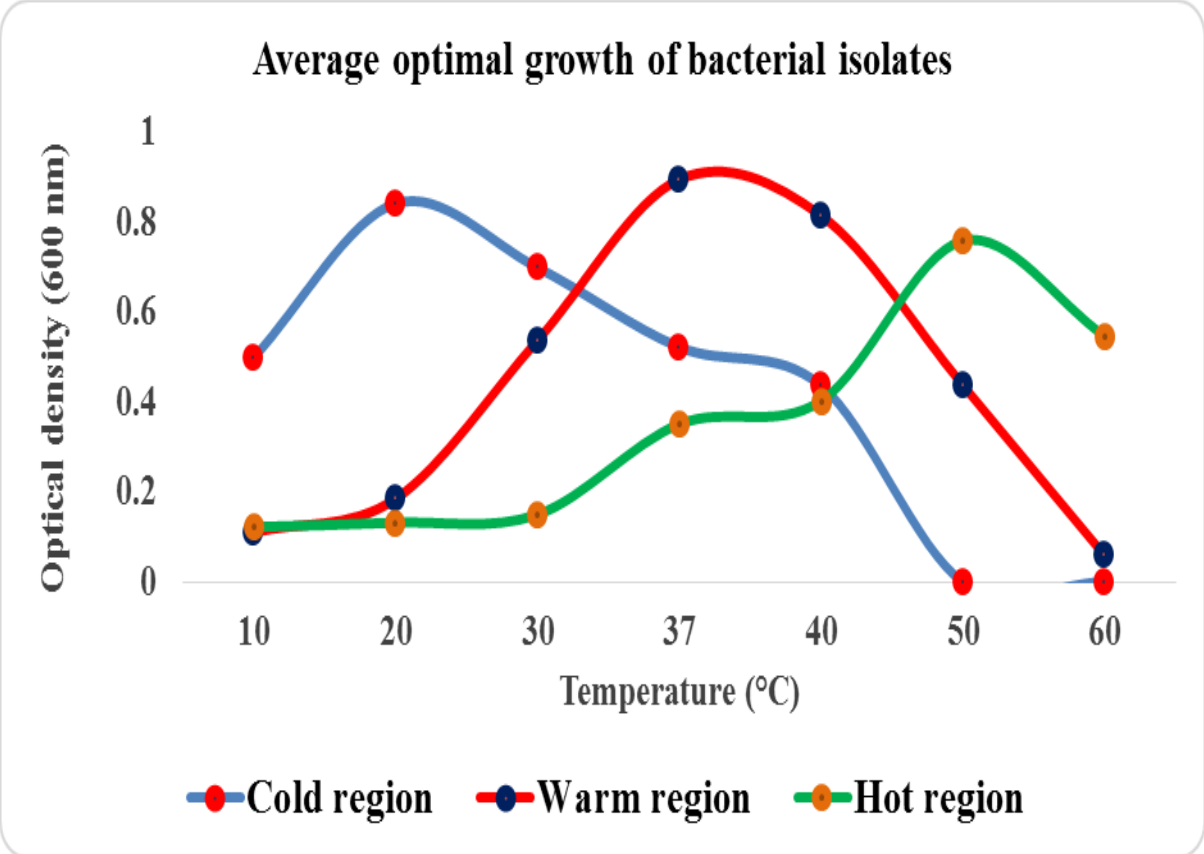


Fig. 6 Average optimal growth of the bacterial isolates at distinct temperatures

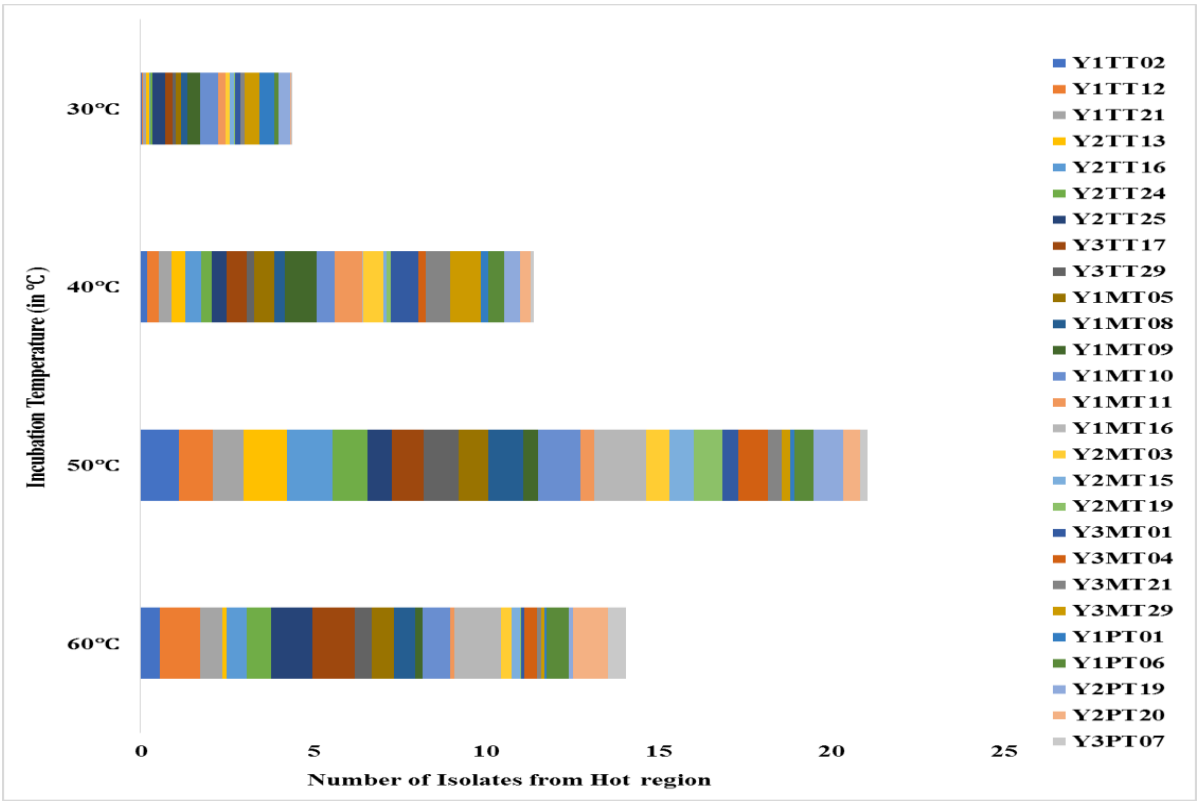


Fig. 7 a. Bacterial growth tolerance (hot region) to various temperatures

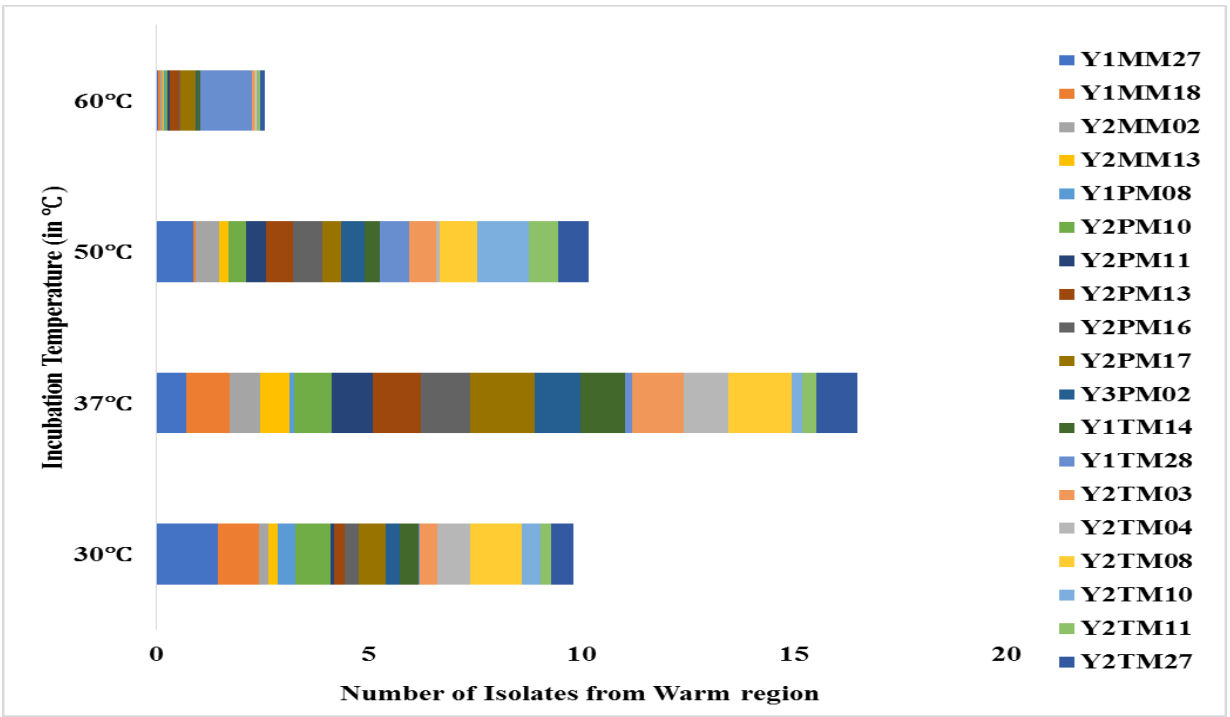


Fig. 7 b. Bacterial growth tolerance (warm region) to various temperatures

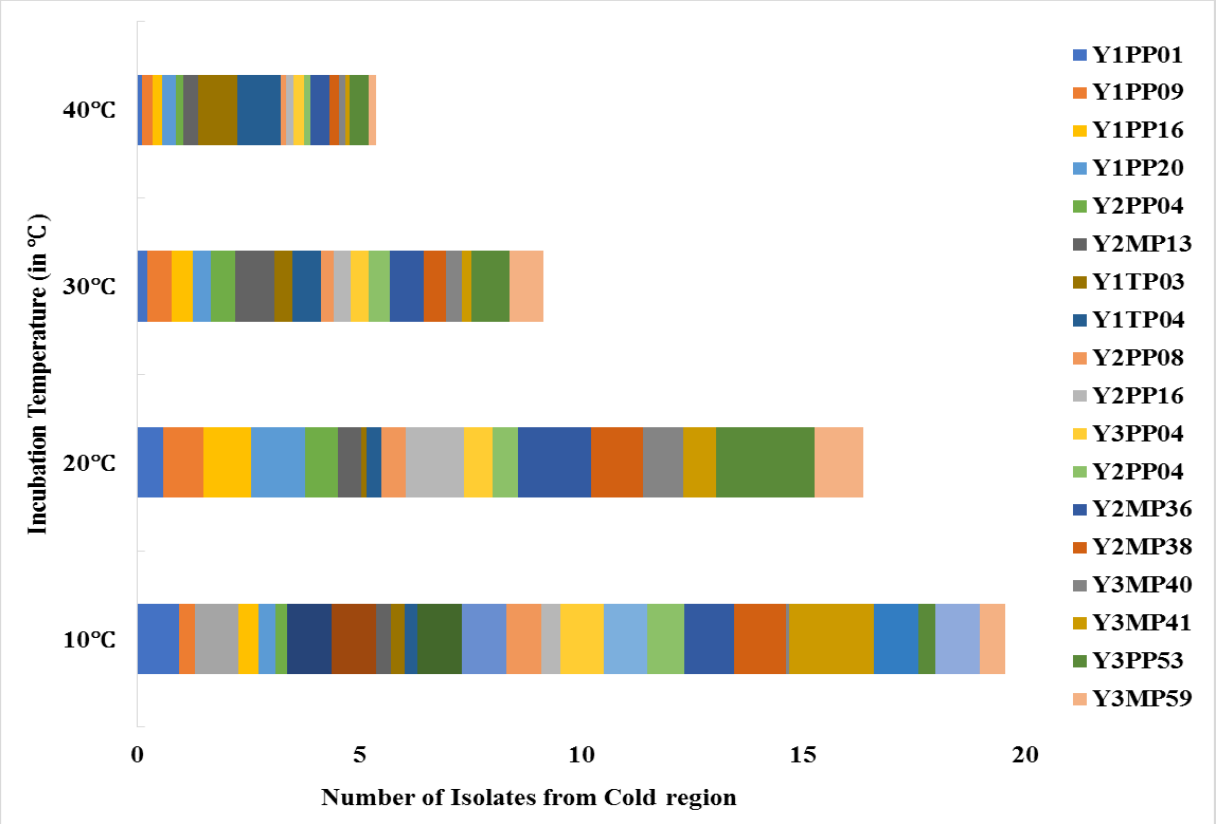


Fig. 7 c. Bacterial growth tolerance (cold region) to various temperatures

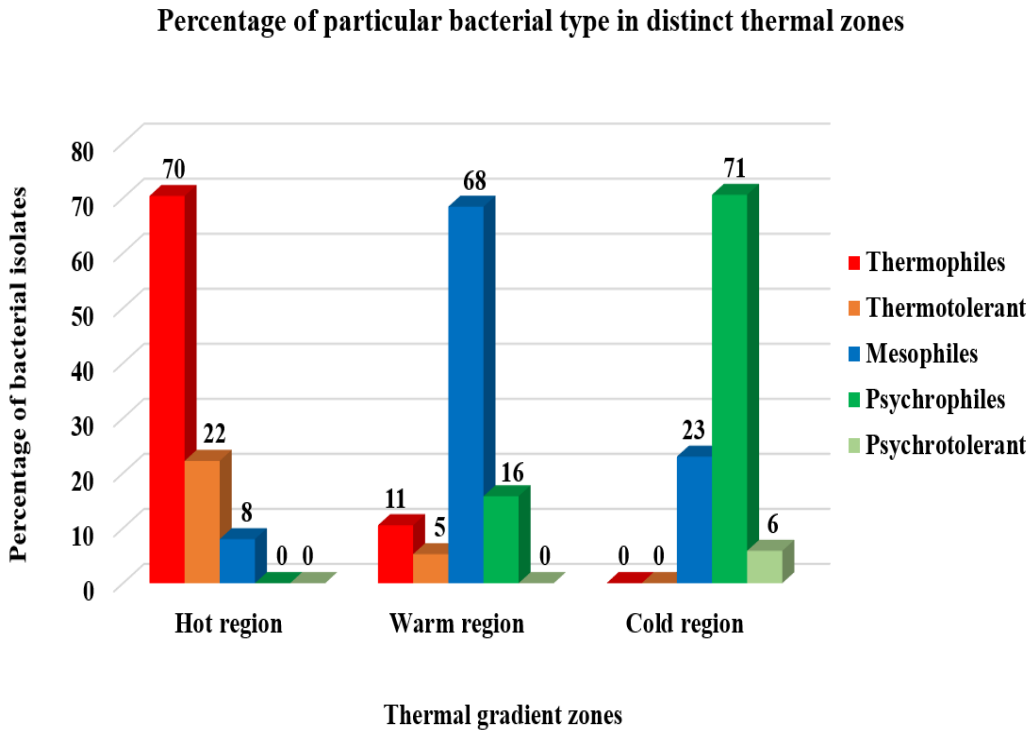


Fig. 8. Percentage of bacterial isolates in a particular thermal zone

5.3.4. Biochemical Tests

The results of the catalase test indicated that all the isolates tested were positive. Moreover, when examining the Carbohydrate fermentation, casein hydrolysis, gelatin hydrolysis, and lipid hydrolysis tests, it was found that over 90 % of the tested isolates displayed positive reactions. This suggests that the majority of the isolates could ferment carbohydrates and hydrolyze casein, gelatin, and lipids. Similarly, the citrate utilization and starch hydrolysis tests showed positive results in over 90 % of the isolates. However, upon closer inspection of the starch activity zones, only a small number of isolates exhibited larger zones, indicating higher starch hydrolysis activity.

The majority of the dominant bacterial isolates belonged to the Gram-positive group. H_2S testing revealed that only a few isolates tested positive, as indicated in the data presented in **Table 4** and visualized in **Fig. 9** illustrating the results of all the standard biochemical tests conducted. It is important to note that there were no substantial variations observed in the biochemical test results among the isolates from distinct thermal gradient zones. This suggests that the environmental conditions related to the thermal gradient zone did not have a significant impact on the biochemical characteristics of the isolates.

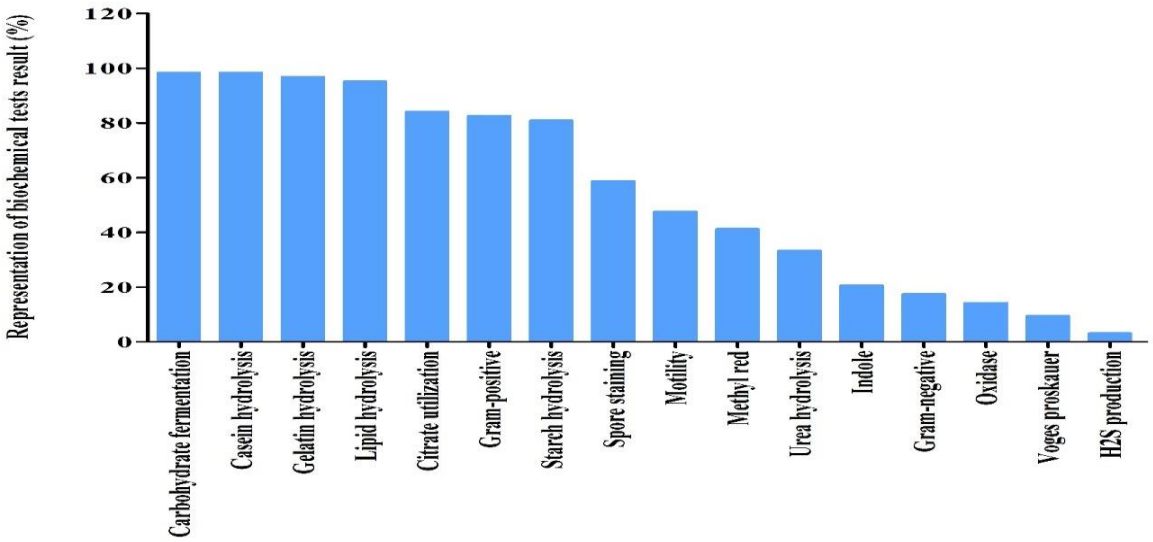


Fig. 9 Biochemical Tests

Table 4 Displays the result of the standard biochemical tests.

Isolates IDs	Gram staining	Motility	Spore staining	Indole	Methyl red	Voges proskauer	Citrate utilization	Catalase	Oxidase	H2S production	Carbohydrate	Urea hydrolysis	Casein hydrolysis	Starch hydrolysis	Lipid hydrolysis	Gelatin hydrolysis
Y1TT02	G +ve short rod	-	+	-	-	-	-	+	-	+	+	+	+	+	+	+
Y1TT12	G+ve rod	+	+	-	-	-	+	+	-	-	+	-	+	+	+	+
Y1TT21	Gram +	-	+	+	+	+	+	+	+	-	+	-	+	+	+	+
Y2TT13	Gram +	-	+	+	+	+	+	+	+	-	+	-	+	+	+	+
Y2TT16	G+ve rod	+	+	-	-	-	+	+	-	-	+	-	+	+	+	+
Y2TT24	Gram +	-	+	+	+	+	+	+	-	-	+	+	+	+	+	+
Y2TT25	Gram +	+	+	-	-	-	+	+	-	-	+	-	+	+	+	+
Y3TT17	G+ve rod	+	+	-	-	-	+	+	-	-	+	-	+	-	+	+
Y3TT29	G+ve rod	+	+	-	-	-	+	+	-	-	+	-	+	+	+	+
Y1MT05	G+ve rod	+	+	-	-	-	+	+	-	-	+	-	+	-	+	+
Y1MT08	Gram +	+	+	-	-	-	+	+	-	-	+	-	+	+	+	+
Y1MT09	Gram +	-	+	-	-	-	+	+	-	-	+	-	+	+	+	+
Y1MT10	G+ve rod	+	+	-	-	-	+	+	-	-	+	-	+	-	+	+
Y1MT11	Gram +	-	+	-	-	-	+	+	-	-	+	+	+	+	+	+
Y1MT16	G+ve rod	+	+	-	+	-	+	+	-	-	+	-	+	+	+	+
Y2MT03	Gram +	-	+	-	-	-	+	+	-	-	+	+	+	+	+	+
Y2MT15	Gram +	-	+	-	-	-	+	+	-	-	+	-	+	+	+	+
Y2MT19	Gram +	-	+	-	-	-	+	+	-	-	+	-	+	+	+	+
Y3MT01	Gram +	-	+	+	+	+	+	+	-	-	+	+	+	+	+	+
Y3MT04	Gram +	-	+	-	-	-	+	+	-	-	+	+	+	+	+	+
Y3MT21	Gram +	-	+	-	-	-	+	+	-	-	+	-	+	+	+	+
Y3MT29	Gram +	-	+	+	+	+	+	+	-	-	+	+	+	+	+	+
Y1PT01	Gram +	-	+	-	-	-	+	+	-	-	+	+	+	+	+	+
Y1PT06	Gram +	-	+	+	+	+	+	+	-	-	+	+	+	+	+	+
Y2PT19	Gram +	+	+	-	-	-	+	+	-	-	+	-	+	+	+	+
Y2PT20	Gram +	-	+	-	-	-	+	+	-	-	+	+	+	+	+	+
Y3PT07	G+ve rod	+	+	-	-	-	+	+	-	-	+	-	+	+	+	+
Y1MM27	Gram -	-	-	+	+	-	+	+	-	+	+	+	+	+	+	+
Y1MM18	Gram +	+	+	-	-	-	+	+	-	-	+	-	+	+	+	+
Y2MM02	Gram +	-	-	-	+	-	-	+	-	-	+	-	+	-	-	-

Y2MM13	Gram -	+	-	-	+	-	+	+	+	-	+	-	+	-	+	+
Y1PM08	Gram +, Rod	+	+	-	+	-	+	+	-	-	+	+	+	+	+	+
Y2PM10	Gram -	+	-	-	+	-	+	+	-	-	+	-	+	-	+	+
Y2PM11	Gram +	-	-	-	-	-	+	+	-	-	+	+	+	+	+	+
Y2PM13	Gram +	+	+	-	-	-	+	+	-	-	+	-	+	+	+	+
Y2PM16	Gram +	+	+	-	-	-	+	+	-	-	+	-	+	+	+	+
Y2PM17	G+ve rod	+	+	-	-	-	+	+	-	-	+	-	+	-	+	+
Y3PM02	Gram +	+	+	-	-	-	+	+	-	-	+	-	+	+	+	+
Y1TM14	Gram +	-	-	-	-	-	+	+	-	-	+	+	+	+	+	+
Y1TM28	Gram -	+	-	-	+	-	+	+	-	-	+	-	+	-	+	+
Y2TM03	Gram +	+	+	+	+	-	-	+	-	-	+	-	+	+	+	+
Y2TM04	Gram +	+	+	+	+	-	-	+	-	-	+	-	+	+	+	+
Y2TM08	Gram +	+	+	-	-	-	+	+	-	-	+	-	+	+	+	+
Y2TM10	Gram -	+	-	+	-	-	-	+	+	-	+	-	-	-	-	-
Y2TM11	Gram +	-	-	-	-	-	+	+	-	-	+	+	+	+	+	+
Y2TM27	G+ve rod	+	+	-	-	-	+	+	-	-	+	-	+	-	+	+
Y1PP01	Gram +	-	-	-	-	-	+	+	-	-	+	-	+	+	+	+
Y1PP09	Gram -	+	-	-	+	-	+	+	+	-	+	-	+	+	-	+
Y1PP10	Gram -	+	-	+	+	-	+	+	+	-	+	+	+	+	+	+
Y1PP16	Gram +	-	-	-	-	-	+	+	-	-	+	-	+	+	+	+
Y1PP20	Gram -	+	-	+	+	-	+	+	+	-	+	+	+	+	+	+
Y2PP04	Gram +	-	-	-	+	-	-	+	-	-	+	-	+	+	+	+
Y2PP14	Gram -	+	-	-	+	-	+	+	+	-	-	-	+	-	+	+
Y3PP04	Gram +	-	-	-	-	-	+	+	-	-	+	-	+	+	+	+
Y1MP03	Gram +	-	-	-	-	-	+	+	-	-	+	+	+	+	+	+
Y1MP04	Gram +	-	-	-	-	-	+	+	-	-	+	+	+	+	+	+
Y2MP01	Gram +	-	-	-	+	-	-	+	-	-	+	-	+	+	+	+
Y2MP13	Gram +	-	-	-	+	-	-	+	-	-	+	-	+	+	+	+
Y2MP16	Gram +	-	-	-	+	-	-	+	-	-	+	-	+	+	+	+
Y1TP03	Gram -	+	-	+	+	-	+	+	+	-	+	+	+	+	+	+
Y1TP04	Gram +	-	-	-	+	-	-	+	-	-	+	-	+	+	+	+
Y1TP01	Gram -	-	-	-	+	-	+	+	-	-	+	+	+	-	+	+
Y2TP11	Gram +	-	-	-	-	-	+	+	-	-	+	-	+	+	+	+

5.3.5. Identification and Phylogeny

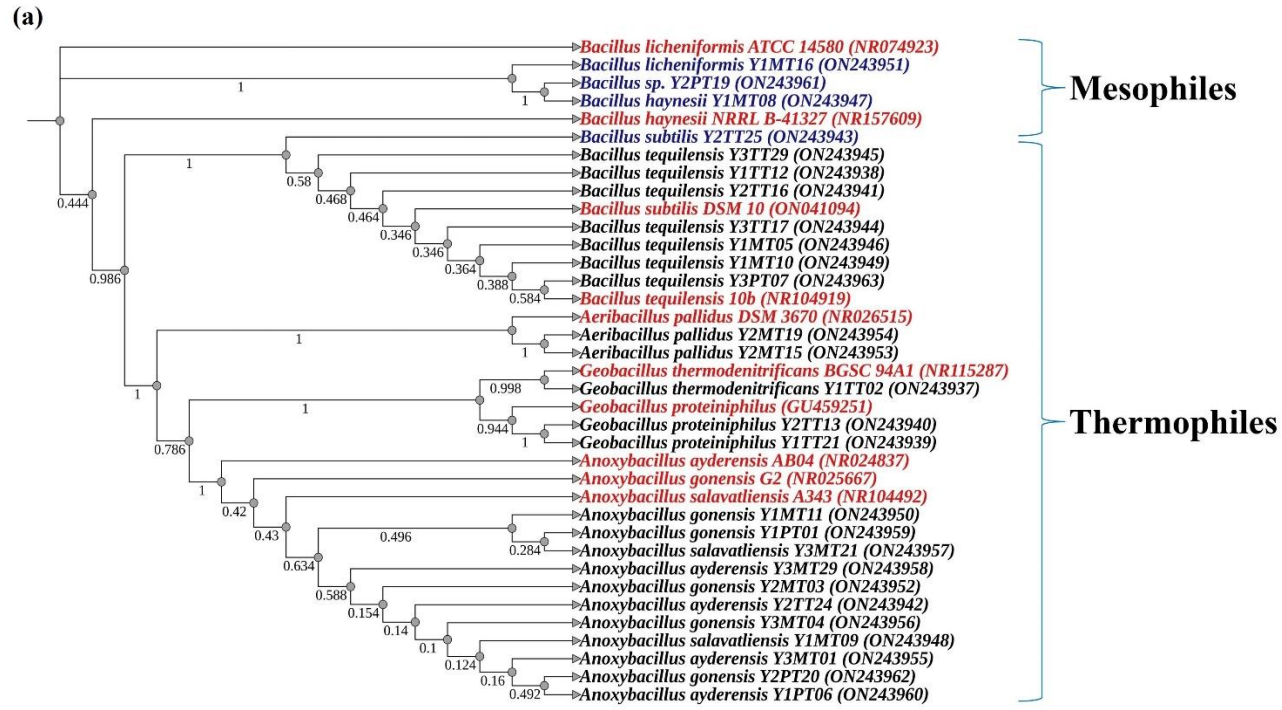
A total of 63 representative bacterial isolates obtained after biochemical groping were identified by partial 16s rRNA gene sequencing. Among the 63 isolates, 27 isolates were thermophiles belonging to the genera *Bacillus, Geobacillus*, *Anoxybacillus,* and *Aeribacillus* species. A total of 19 isolates were mesophiles belonging to *Bacillus*, *Cronobacter*, *Exiguobacterium, Lysinibacillus*, *Citrobacter*, *Brevundimonas*, and *Staphylococcus*. 17 isolates were psychrophiles from *Exiguobacterium, Janthinobacterium*, *Aeromonas, Arthrobacter,* and *Acinetobacter* genera **Table 5**. Hence, it can be observed that the isolates were mostly common soil bacteria. Phylogenetic tree analysis was done using the Tamura Nei model and the evolutionary history was inferred by the Neighbor-joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. Three phylogenetic trees were prepared and shown, each tree for a specific thermal zone. The identified bacteria from each zone containing thermophilic, mesophilic, and psychrophilic bacteria with their corresponding type strains were used in making a phylogenetic tree. It was shown that each bacterial isolate with their type strains formed distinct clades with their respective temperatures as shown in **Fig. 10a, b, c**. This suggests that these bacteria are truly thermophilic, mesophilic, and psychrophilic.

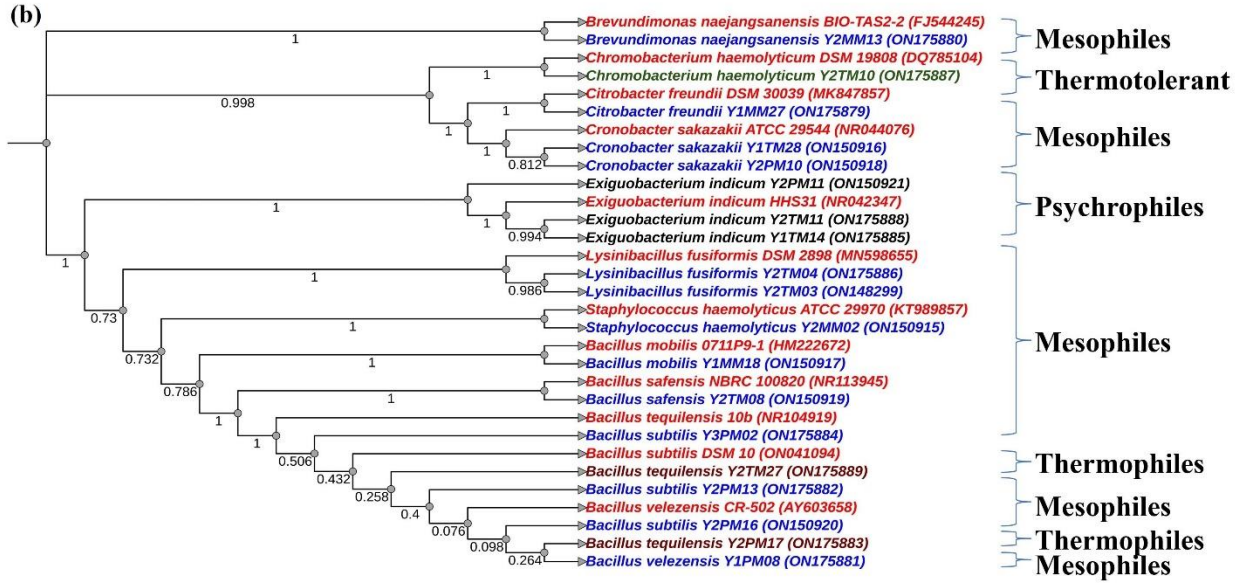
Table 5. Identified bacterial isolates by 16S rRNA sequencing

SL No.	Isolates ID	Name of the isolates	Base pair	Accession Number
1	Y1TT02	*Geobacillus thermodenitrificans*	1366	ON243937
2	Y1TT12	*Bacillus tequilensis*	1389	ON243938
3	Y1TT21	*Geobacillus proteiniphilus*	1409	ON243939
4	Y2TT13	*Geobacillus proteiniphilus*	1397	ON243940
5	Y2TT16	*Bacillus tequilensis*	1386	ON243941
6	Y2TT24	*Anoxybacillus ayderensis*	1386	ON243942
7	Y2TT25	*Bacillus subtilis*	1398	ON243943
8	Y3TT17	*Bacillus tequilensis*	1386	ON243944

9	Y3TT29	*Bacillus tequilensis*	1396	ON243945
10	Y1MT05	*Bacillus tequilensis*	1403	ON243946
11	Y1MT08	*Bacillus haynesii*	1386	ON243947
12	Y1MT09	*Anoxybacillus salavatliensis*	1405	ON243948
13	Y1MT10	*Bacillus tequilensis*	1374	ON243949
14	Y1MT11	*Anoxybacillus gonensis*	1410	ON243950
15	Y1MT16	*Bacillus licheniformis*	1396	ON243951
16	Y2MT03	*Anoxybacillus gonensis*	1410	ON243952
17	Y2MT15	*Aeribacillus pallidus*	1393	ON243953
18	Y2MT19	*Aeribacillus pallidus*	1402	ON243954
19	Y3MT01	*Anoxybacillus ayderensis*	1397	ON243955
20	Y3MT04	*Anoxybacillus gonensis*	1416	ON243956
21	Y3MT21	*Anoxybacillus salavatliensis*	1414	ON243957
22	Y3MT29	*Anoxybacillus ayderensis*	1413	ON243958
23	Y1PT01	*Anoxybacillus gonensis*	1420	ON243959
24	Y1PT06	*Anoxybacillus ayderensis*	1419	ON243960
25	Y2PT19	*Bacillus sp.*	1426	ON243961
26	Y2PT20	*Anoxybacillus gonensis*	1422	ON243962
27	Y3PT07	*Bacillus tequilensis*	1383	ON243963
28	Y1MM27	*Citrobacter freundii*	1378	ON175879
29	Y1MM18	*Bacillus mobilis*	1405	ON150917
30	Y2MM02	*Staphylococcus haemolyticus*	1320	ON150915
31	Y2MM13	*Brevundimonas naejangsanensis*	1321	ON175880
32	Y1PM08	*Bacillus velezensis*	1321	ON175881
33	Y2PM10	*Cronobacter sakazakii*	1339	ON150918
34	Y2PM11	*Exiguobacterium indicum*	1421	ON150921
35	Y2PM13	*Bacillus subtilis*	1382	ON175882
36	Y2PM16	*Bacillus subtilis*	1384	ON150920
37	Y2PM17	*Bacillus tequilensis*	1412	ON175883
38	Y3PM02	*Bacillus subtilis*	1367	ON175884
39	Y1TM14	*Exiguobacterium indicum*	1395	ON175885
40	Y1TM28	*Cronobacter sakazakii*	1230	ON150916
41	Y2TM03	*Lysinibacillus fusiformis*	1360	ON148299
42	Y2TM04	*Lysinibacillus fusiformis*	1369	ON175886
43	Y2TM08	*Bacillus safensis*	1343	ON150919
44	Y2TM10	*Chromobacterium haemolyticum*	1332	ON175887
45	Y2TM11	*Exiguobacterium indicum*	1416	ON175888
46	Y2TM27	*Bacillus tequilensis*	1404	ON175889
47	Y1PP01	*Arthrobacter agilis*	1331	ON237927
48	Y1PP09	*Janthinobacterium lividum*	1388	ON237928
49	Y1PP10	*Aeromonas sp.*	1371	ON237929
50	Y1PP16	*Exiguobacterium antarcticum*	1395	ON237930
51	Y1PP20	*Aeromonas salmonicida*	1342	ON237931
52	Y2PP04	*Exiguobacterium acetylicum*	1371	ON237932
53	Y2PP14	*Janthinobacterium tructae*	1357	ON237933
54	Y3PP04	*Exiguobacterium antarcticum*	1402	ON237934

55	Y1MP03	*Exiguobacterium indicum*	1354	ON237935
56	Y1MP04	*Exiguobacterium indicum*	1364	ON237936
57	Y2MP01	*Exiguobacterium acetylicum*	1364	ON237937
58	Y2MP13	*Exiguobacterium acetylicum*	1364	ON237938
59	Y2MP16	*Exiguobacterium acetylicum*	1407	ON237939
60	Y1TP03	*Aeromonas rivipollensis*	1327	ON237940
61	Y1TP04	*Exiguobacterium acetylicum*	1352	ON237941
62	Y1TP01	*Acinetobacter johnsonii*	1384	ON237942
63	Y2TP11	*Exiguobacterium antarcticum*	1387	ON237943





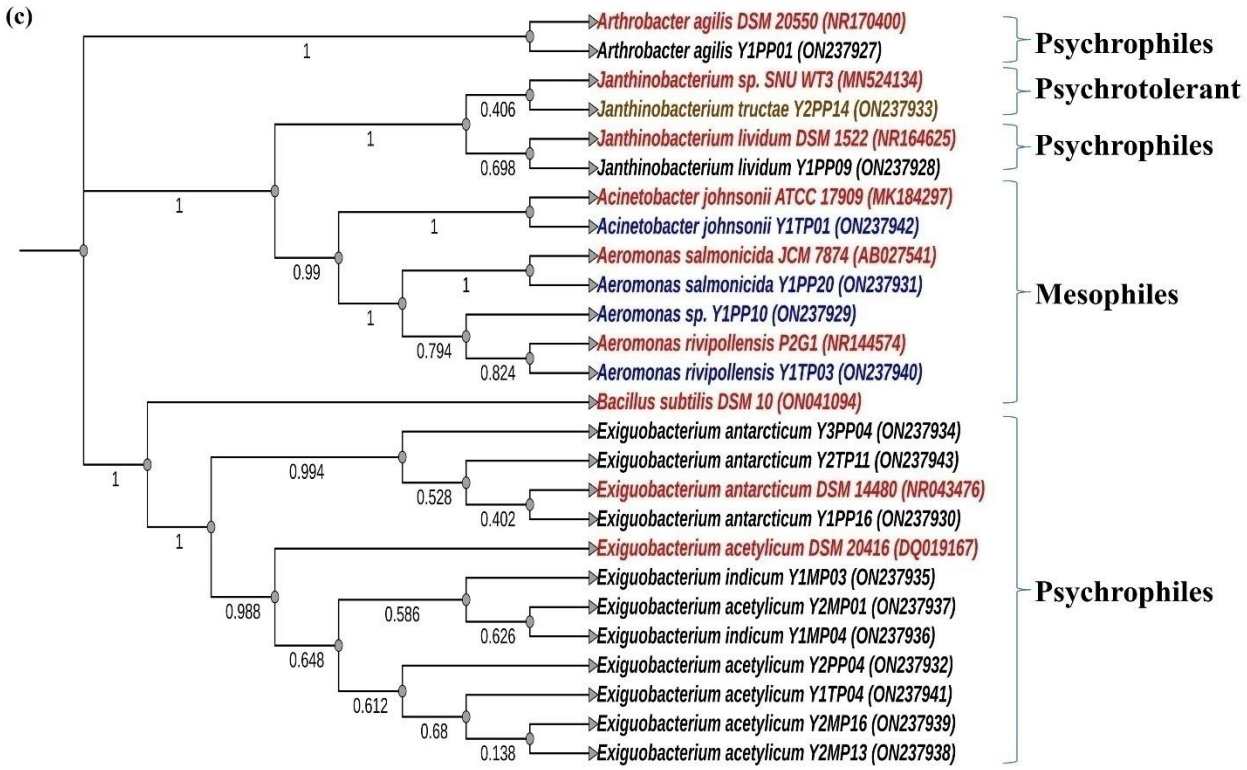


Fig. 10a, b, c. Phylogenetic tree analysis was conducted using the Neighbor-Joining method and the Tamura Nei model. The bootstrap test was performed with 500 replicates to determine the percentage of replicate trees where the associated taxa clustered together. The results are presented next to the branches. Three distinct phylogenetic trees were created and displayed, each representing bacterial isolates from a specific thermal zone. Tree (a) represents bacterial isolates from the hot region, tree (b) represents bacterial isolates from the warm region, and tree (c) represents bacterial isolates from the cold region.

The findings of culture-dependent phylum level diversity revealed the presence of three phyla: Bacillota, Pseudomonadota, and Actinomycetota across various thermal gradient regions including hot, warm, and cold. Among these phyla, Bacillota exhibited a higher abundance and were found in all temperate regions. On the other hand, Pseudomonadota were detected in both warm and cold regions. Interestingly, the cold region exhibited the highest diversity among all phyla.

Notably, the phylum abundances followed distinct patterns as temperatures changed. In the case of Bacillota, their dominance gradually decreased as the temperature decreased. This trend was consistent across all regions, indicating that Bacillota prefer warmer conditions and their numbers decline as the environment becomes colder. Conversely, Pseudomonadota displayed an opposite pattern, with their numbers increasing as temperatures decreased as displayed in **Fig. 11**. This suggests that Pseudomonadota thrive in colder environments and their populations thrive in such conditions. Actinomycetota, however, were exclusively found in the cold region, indicating their preference for colder temperatures and limited distribution to this specific environment. **Table 6** provides a representation of the phylum-level diversity data, highlighting the abundance and distribution of Bacillota, Pseudomonadota, and Actinomycetota across the different thermal gradient regions.

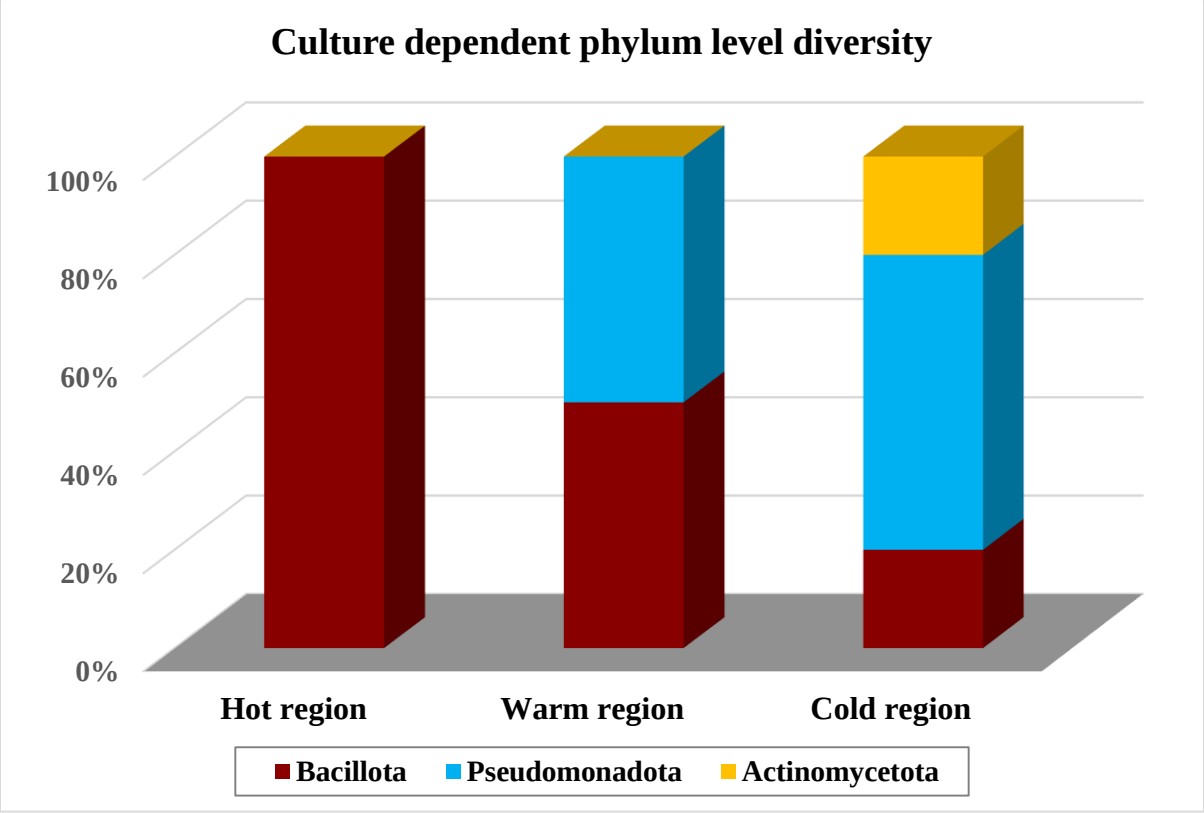


Fig. 11. Culture-dependent phylum level bacterial diversity at distinct thermal zones

Table 6. The distribution of Phylum level diversity across the distinct thermal gradient regions.

Phylum	Hot region	Warm region	Cold region
Bacillota	4	4	1
Pseudomonadota	0	4	3
Actinomycetota	0	0	1

The bacterial diversity at the genus level demonstrated a varied presence of bacteria across different temperature regions. The warm region exhibited a diverse group of bacteria, followed by the cold and hot regions. Among the hot regions, the dominant bacterial groups were *Anoxybacillus* and *Bacillus*. In the warm region, the most prevalent bacteria belonged to the *Bacillus* group, while in the cold region, *Exiguobacterium* was the most dominant. This pattern of genus-level bacterial diversity also revealed a correlation with temperature changes.

Exiguobacterium bacteria were abundant in the cold region, followed by the warm region. However, as the temperature increased, the number of *Exiguobacterium* bacteria decreased, indicating the influence of temperature on this group. Conversely, *Bacillus* exhibited an opposite trend, with an increase in numbers as the temperature rose as displayed in **Fig. 12**. Additionally, *Geobacillus* were exclusively observed in higher temperature regions only. The genus-level bacterial diversity is represented in **Table 7**.

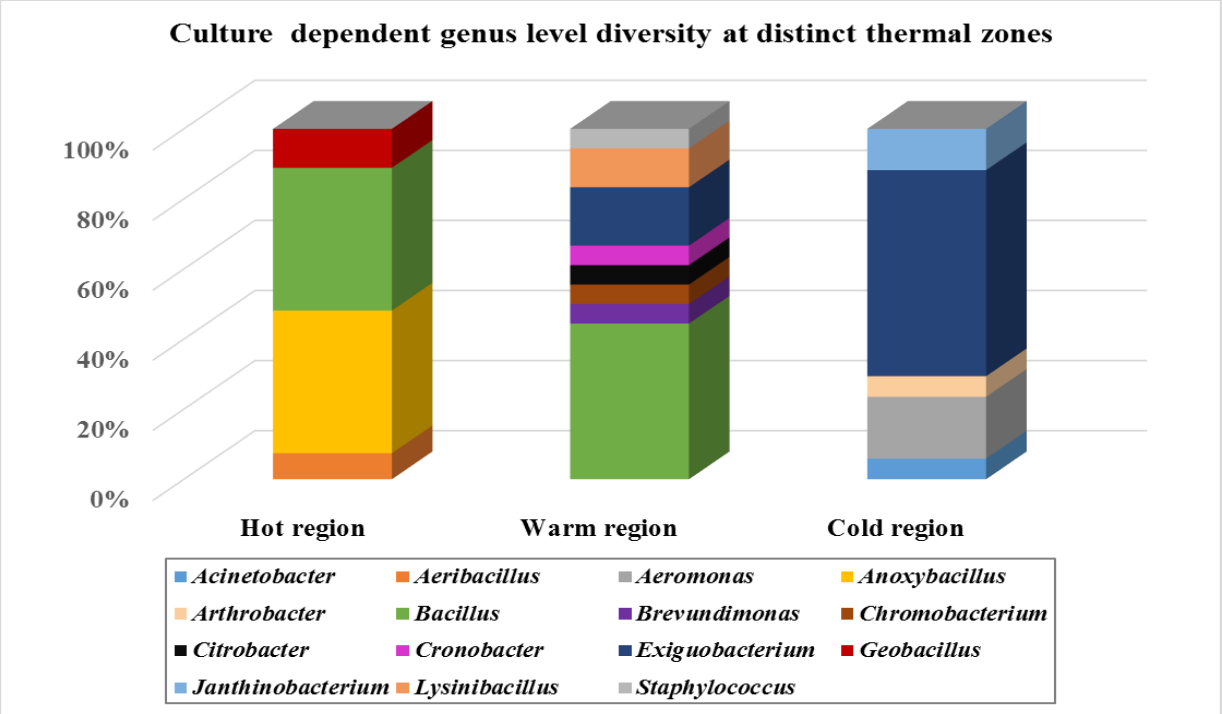


Fig. 12. Culture-dependent genus-level bacterial diversity at distinct thermal zones

Table 7 Presents the distribution of bacterial diversity at the genus level.

Genus	Hot region	Warm region	Cold region
Acinetobacter	0	0	1
Aeribacillus	2	0	0
Aeromonas	0	0	3
Anoxybacillus	11	0	0
Arthrobacter	0	0	1
Bacillus	11	8	0
Brevundimonas	0	1	0
Chromobacterium	0	1	0
Citrobacter	0	1	0
Cronobacter	0	1	0
Exiguobacterium	0	3	10
Geobacillus	3	0	0
Janthinobacterium	0	0	2
Lysinibacillus	0	2	0
Staphylococcus	0	1	0

5.3.6. Antibiotic Resistance Susceptibility pattern in bacterial isolates

A total of 28 antibiotics from 16 different classes were utilized in the present study **Table 8**. The results have shown that the total antibiotic resistance pattern in three thermal zones was warm region (36.84 %) > cold region (23.52 %) > hot region (7.4 %) as shown in **Fig. 13a**. However, the antibiotic resistance pattern contributed by a particular bacterial type was mesophiles (92.3 %) > psychrophiles (7.7 %) > thermophiles (0). Mesophilic bacteria contribute the highest antibiotic resistance in all three thermal zones respectively as shown in **Fig. 13 b**. Thermophilic bacteria were devoid of any antibiotic resistance and, thus do not contribute antibiotic resistance to any thermal zone. Moreover, psychrophilic bacteria contribute very less antibiotic resistance that too only in a cold region. The total cumulative abundance of antibiotic classes was found among three thermal zones. It was found that Monobactams (19.12 %), Cephalosporin (17.65 %), β-Lactams (13.24 %), Rifamycin (8.82 %), Macrolide (7.35 %) were found to be most abundant antibiotics in these thermal zones as shown in **Fig. 14**. Other low abundant antibiotics resistance classes were Oxazolidnodes (5.88 %), Aminocoumarin (5.88 %), Diaminopyrimidines (4.41 %), Glycopeptide (4.41 %), Chloramphenicol (2.94 %), Lincosamide (2.94 %), Aminoglycoside (2.94 %), Fluoroquinolone (1.47 %), Sulfonamide (1.47 %), Tetracycline (1.47 %) and Carbapenem (0) as shown in **Table 9**. However, 80 % of this antibiotic resistance is contributed by mesophiles. The zone of inhibition of the tested isolates from the hot region is shown in **Table 10** and the warm and cold regions are shown in **Table 11 & Table 12**.

Table 8. Various antibiotics from different classes are utilized for Antibiotic Susceptibility Testing (AST)

SL No.	Antibiotics	Classes of antibiotics	Concentration of antibiotics
1.	Gentamicin (Gen)	Aminoglycoside	10 mcg

2.	Streptomycin (S)	Aminoglycoside	10 mcg
3.	Kanamycin (K)	Aminoglycoside	30 mcg
4.	Ampicillin (AMP)	Beta-lactam	10 mcg
5.	Methicillin (MET)	Beta-lactam	10 mcg
6.	Amoxycillin and Clavulanic acid (AMC)	Beta-lactam	30 mcg
7.	Vancomycin (VA)	Glycopeptide	5 mcg
8.	Erythromycin (E)	Macrolide	15mcg
9.	Azithromycin (AZM)	Macrolide	15mcg
10.	Linezolid (LZ)	Oxazolidnodes	30 mcg
11.	Ciprofloxacin (CIP)	Fluoroquinolone	5 mcg
12.	Ofloxacin (OF)	Fluoroquinolone	5mcg
13.	Norfloxacin (NX)	Fluoroquinolone	10 mcg
14.	Tetracycline (TE)	Tetracycline	30 mcg
15.	Chloramphenicol (C)	Chloramphenicol	30 mcg
16.	Sulfafurazole (SF)	Sulfafurazole	300 mcg
17.	Imipenem (IPM)	Carbapenem	10mcg
18.	Ceftazidime (CAZ)	Cephalosporin	30 mcg
19.	Cefixime (CFM)	Cephalosporin	5 mcg
20.	Cefuroxime (CXM)	Cephalosporin	30 mcg
21.	Ceftriaxone (CTR)	Cephalosporin	30 mcg
22.	Cephalothin (CEP)	Cephalosporin	30 mcg
23.	Cefotaxime (CTX)	Cephalosporin	30 mcg
24.	Aztreonam (AT)	Monobactam	30 mcg
25.	Rifampin (RIF)	Rifamycin (Antitubercular Agent)	5 mcg
26.	Novobiocin (NV)	Aminocoumarin	30 mcg
27.	Clindamycin (CD)	Lincosamide	2 mcg
28.	Trimethoprim (TR)	Diaminopyrimidines	10 mcg

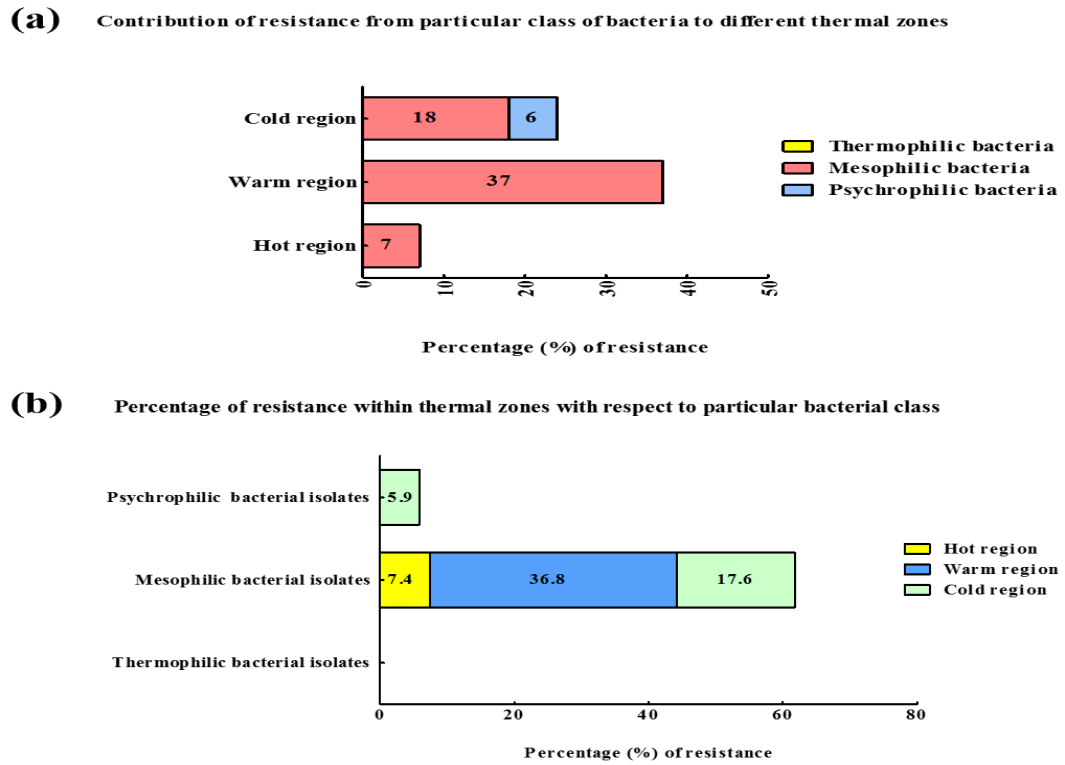


Fig. 13 a-Percentage of antibiotic resistance within thermal zones concerning particular bacterial class; **b-** Contribution of antibiotic resistance from a particular class of bacteria to thermal zones.

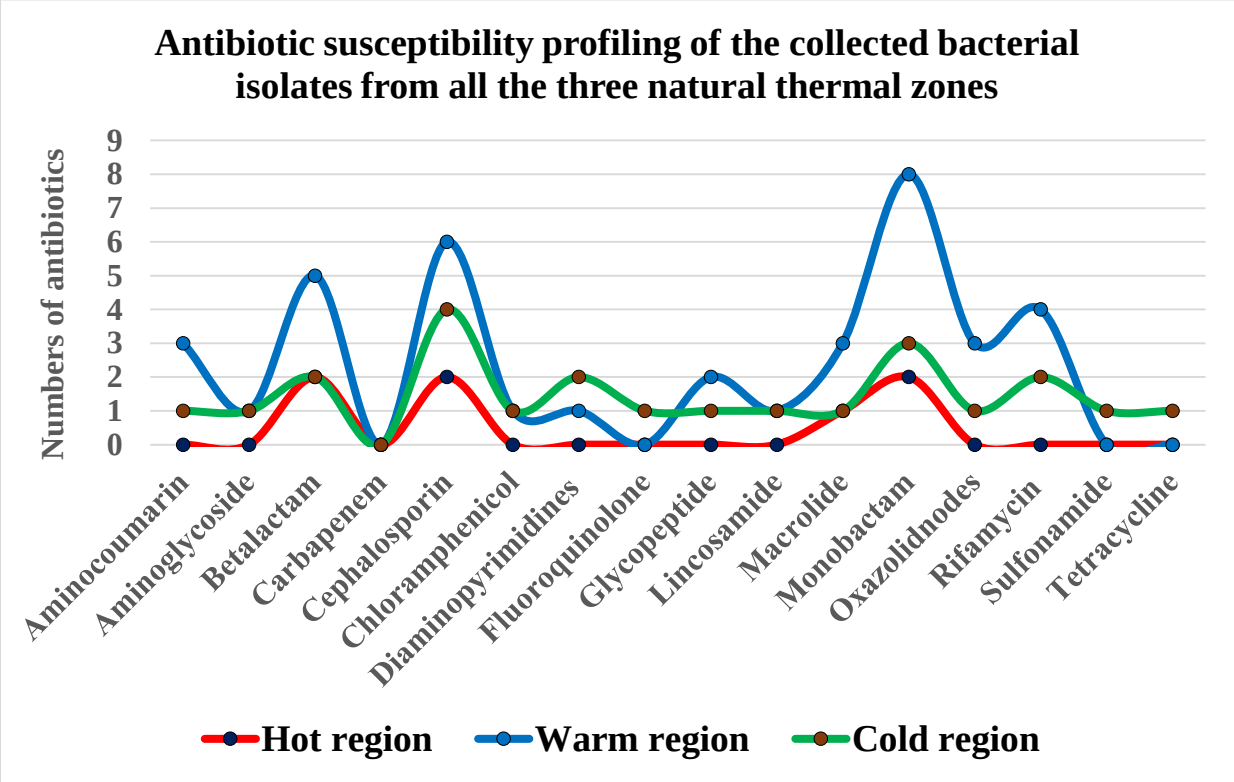


Fig. 14. Percentage of Antibiotic resistance to distinct classes in respective thermal zones.

Table 9 Displays the percentage of antibiotic resistance (determined using AST) observed in isolated bacteria, categorized by distinct thermal regions.

Classes of antibiotics	Hot region	Warm region	Cold region	Total Percentage
Monobactam	2.94	11.76	4.41	19.12
Cephalosporin	2.94	8.82	5.88	17.65
Betalactam	2.94	7.35	2.94	13.24
Rifamycin	0.00	5.88	2.94	8.82
Macrolide	1.47	4.41	1.47	7.35
Oxazolidnodes	0.00	4.41	1.47	5.88
Aminocoumarin	0.00	4.41	1.47	5.88
Diaminopyrimidines	0.00	1.47	2.94	4.41
Glycopeptide	0.00	2.94	1.47	4.41
Chloramphenicol	0.00	1.47	1.47	2.94
Lincosamide	0.00	1.47	1.47	2.94
Aminoglycoside	0.00	1.47	1.47	2.94
Fluoroquinolone	0.00	0.00	1.47	1.47
Sulfonamide	0.00	0.00	1.47	1.47
Tetracycline	0.00	0.00	1.47	1.47
Carbapenem	0.00	0.00	0.00	0.00

Table 10. Antibiotic resistance profile of isolates from hot regions based on specific antibiotics and their classes.

ISOLATES ID		Y1TT02	Y1TT12	Y1TT21	Y2TT13	Y2TT16	Y2TT24	Y2TT25	Y3TT17	Y3TT29	Y1MT05	Y1MT08	Y1MT09	Y1MT10	Y1MT11	Y1MT16	Y2MT03	Y2MT15	Y2MT19	Y3MT01	Y3MT04	Y3MT21	Y3MT29	Y1PT01	Y1PT06	Y2PT19	Y2MT20	Y3PT07
Aminoglycoside	**GEN**	32	30	24	27	25	28	26	28	23	24	23	29	22	28	25	28	30	29	26	28	30	33	30	24	32	30	30
	S	30	30	22	25	26	28	20	24	22	25	24	23	28	30	27	30	40	23	22	40	34	32	28	20	30	22	24
	K	22	28	21	26	26	25	26	25	23	24	20	27	29	33	29	27	26	29	24	24	31	32	30	32	26	30	22
Betalactam	**AMP**	34	30	23	20	24	40	14	36	22	21	25	28	34	22	26	29	40	12	40	40	32	33	33	26	24	21	40
	MET	37	33	26	24	21	28	13	30	22	19	22	24	29	19	23	33	31	R	34	36	30	30	32	22	33	21	32
	AMC	34	31	35	36	34	36	29	38	34	32	32	31	38	34	33	35	34	16	40	36	38	37	32	34	36	34	40
Glycopeptide	**VA**	30	27	26	21	24	24	24	28	19	20	26	25	23	20	22	22	32	19	24	32	26	23	28	30	26	22	30
Macrolide	**E**	32	30	24	21	32	28	22	28	17	26	30	31	29	30	30	25	38	10	32	32	23	30	30	24	30	28	32
	AZM	30	22	26	19	18	24	18	24	20	28	26	24	28	28	28	30	32	14	24	30	29	25	30	28	26	26	22
Oxazolidnodes	**LZ**	34	30	34	30	31	30	34	31	34	36	34	30	39	40	37	34	40	34	34	40	36	33	39	31	32	33	36
Fluoroquinolone	**CIP**	31	35	34	30	32	36	22	30	33	31	30	31	30	34	30	30	40	36	32	32	31	30	32	40	33	38	36
	OF	35	30	34	32	28	32	34	28	33	30	30	31	34	33	35	30	40	35	28	30	34	35	32	32	31	34	30
	NX	33	32	30	34	27	30	32	32	32	28	26	30	28	35	30	29	36	36	30	30	30	33	33	36	31	35	34
Tetracycline	**TE**	21	22	25	28	35	36	28	28	22	30	34	30	24	27	23	20	38	34	34	38	27	25	34	30	28	24	32
Chloramphenicol	**C**	32	34	20	26	32	32	21	30	20	25	31	19	26	28	30	33	38	31	30	30	30	27	31	32	26	30	30
Sulfonamide	**SF**	36	36	30	30	36	40	29	34	34	34	39	35	39	33	30	27	40	36	36	30	35	30	31	36	31	38	40
Carbapenem	**IPM**	34	39	37	36	40	40	32	38	34	38	42	38	40	34	38	39	40	40	40	40	38	35	31	32	36	30	40
Cephalosporin	**CAZ**	25	22	18	20	26	34	14	27	16	20	26	21	21	23	20	22	28	12	28	34	23	27	26	27	27	30	32
	CFM	28	22	30	30	34	32	14	30	13	34	30	30	32	28	26	20	22	11	28	32	28	26	29	18	27	18	32
	CXM	23	20	22	20	32	20	19	38	18	21	32	23	26	22	20	22	30	R	40	30	32	30	33	34	32	36	36
	CTR	25	24	26	23	25	22	21	30	20	28	19	20	24	22	27	23	29	R	22	25	20	26	32	35	36	36	32
	CEP	32	34	32	32	23	40	11	36	22	24	26	27	35	32	30	36	32	15	40	39	33	30	39	36	39	34	40
	CTX	30	32	28	30	30	33	17	31	22	28	25	29	34	28	26	29	30	R	39	34	30	34	32	36	38	35	34

Monobactam	**AT**	18	24	26	22	38	24	12	19	13	24	19	16	18	18	22	25	22	10	28	40	24	30	23	16	26	20	30
Rifamycin (Antitubercular Agent)	**RIF**	30	30	25	20	28	32	27	30	19	29	32	22	30	20	22	22	26	24	32	34	27	22	29	36	35	34	34
Aminocoumarin	**NV**	20	22	19	22	17	20	18	24	17	22	30	25	28	22	20	32	30	20	26	30	28	25	24	28	24	28	26
Lincosamide	**CD**	30	28	20	22	28	26	30	28	20	28	32	30	36	30	32	33	40	18	34	40	38	34	30	35	37	33	29
Diaminopyrimidines	**TR**	26	20	22	22	18	20	28	21	27	24	33	28	39	32	30	28	34	21	33	30	30	20	35	21	36	20	24

Table 11. Antibiotic resistance profile of isolates from warm regions based on specific antibiotics and their classes.

Isolates ID		Y1MM27	Y1MM18	Y2MM02	Y2MM13	Y1PM08	Y2PM10	Y2PM11	Y2PM13	Y2PM16	Y2PM17	Y3PM02	Y1TM14	Y1TM28	Y2TM03	Y2TM04	Y2TM08	Y2TM10	Y2TM11	Y2TM27
Aminoglycoside	**GEN**	22	24	24	22	21	22	20	20	28	24	26	23	18	30	28	24	27	25	16
	S	17	21	18	16	19	21	18	21	19	24	16	20	16	20	R	22	15	24	30
	K	18	22	19	24	24	23	18	21	25	21	24	24	15	20	14	22	18	25	28
Betalactam	**AMP**	12	R	20	36	22	28	21	32	32	30	22	R	19	27	23	10	27	24	38
	MET	R	14	23	28	28	18	27	28	29	30	24	20	R	28	15	20	19	20	22
	AMC	20	16	29	37	34	28	34	35	36	34	37	18	14	34	32	22	32	22	30
Glycopeptide	**VA**	R	21	23	23	18	20	21	22	21	20	27	21	14	29	22	20	22	21	19
Macrolide	**E**	R	20	24	22	24	24	27	28	23	18	26	22	17	29	24	22	29	21	18
	AZM	11	18	20	21	20	20	19	20	20	24	22	16	11	28	20	19	20	21	10
Oxazolidnodes	**LZ**	R	26	34	33	32	32	34	34	34	34	37	34	R	40	14	34	33	26	40
Fluoroquinolone	**CIP**	30	22	24	31	24	30	23	28	28	30	34	34	28	38	25	32	35	28	25
	OF	26	19	24	26	30	30	32	30	30	26	33	30	25	36	21	20	29	28	30
	NX	26	21	24	24	28	20	16	24	28	30	24	30	20	34	23	29	28	21	30
Tetracycline	**TE**	21	23	23	25	24	23	20	28	27	30	17	19	14	27	25	30	29	28	32
Chloramphenicol	**C**	24	22	27	23	30	14	25	32	24	28	31	27	22	27	23	29	26	22	26
Sulfonamide	**SF**	30	23	24	32	32	33	23	32	26	30	34	30	23	33	24	21	36	34	20

Carbapenem	**IPM**	24	27	30	38	34	28	32	38	40	32	40	33	23	38	28	38	38	38	42
Cephalosporin	**CAZ**	27	R	18	20	18	R	26	21	26	22	24	26	22	26	R	R	20	20	24
	CFM	22	24	R	17	23	R	28	20	22	18	28	22	24	R	14	R	16	22	22
	CXM	20	14	22	18	22	10	30	30	24	30	36	24	19	21	18	R	18	20	26
	CTR	25	14	14	29	30	14	30	32	27	22	33	35	24	24	24	29	35	16	35
	CEP	24	20	24	35	30	24	29	28	39	40	39	39	10	30	26	10	37	35	38
	CTX	25	14	16	29	30	18	24	35	27	34	31	34	22	21	22	30	33	19	33
Monobactam	**AT**	24	R	R	18	17	R	R	17	22	20	16	18	29	12	R	R	14	18	19
Rifamycin (Antitubercular Agent)	**RIF**	R	20	R	24	24	15	21	32	26	26	19	20	10	23	23	12	22	20	28
Aminocoumarin	**NV**	R	18	18	18	22	R	17	22	19	32	18	21	R	21	22	15	24	19	32
Lincosamide	**CD**	30	22	29	28	30	17	22	24	30	34	28	26	R	38	20	27	26	19	26
Diaminopyrimidines	**TR**	27	R	28	30	26	19	34	31	31	27	34	28	22	35	30	30	29	22	30

Table 12. Antibiotic resistance profile of isolates from cold regions based on specific antibiotics and their classes.

ISOLATES ID		**Y1PP01**	**Y1PP09**	**Y1PP10**	**Y1PP16**	**Y1PP20**	**Y2PP04**	**Y2PP14**	**Y3PP04**	**Y1MP03**	**Y1MP04**	**Y2MP01**	**Y2MP13**	**Y2MP16**	**Y1TP03**	**Y1TP04**	**Y1TP01**	**Y2 TP11**
Aminoglycoside	**GEN**	28	30	31	32	21	30	30	30	28	34	19	35	22	28	30	12	32
	S	21	23	27	24	19	36	19	20	25	21	29	29	30	26	40	15	30
	K	19	21	27	28	23	22	24	20	25	29	28	23	30	24	26	11	28
Betalactam	**AMP**	20	24	26	32	22	22	32	27	24	27	31	R	33	21	40	R	25
	MET	17	19	22	30	28	16	29	28	21	17	30	R	21	19	31	R	34
	AMC	34	36	27	34	30	30	40	33	22	34	32	14	29	26	34	R	34
Glycopeptide	**VA**	28	24	22	32	18	30	20	29	21	25	22	30	25	20	32	R	28
Macrolide	**E**	24	27	18	28	24	28	26	29	20	26	27	35	26	26	38	13	28
	AZM	23	26	20	24	20	24	21	28	20	25	26	23	22	28	32	12	24
Oxazolidnodes	**LZ**	40	34	28	40	32	33	39	40	26	35	36	26	34	36	40	10	30

Fluoroquinolone	**CIP**	38	35	34	40	26	39	22	38	28	28	29	36	33	31	40	14	34
	OF	36	33	31	40	30	36	28	36	28	32	36	32	29	30	40	10	31
	NX	34	36	34	30	28	34	28	34	24	34	30	25	22	28	36	R	33
Tetracycline	**TE**	32	30	30	32	24	32	30	27	28	34	22	26	24	30	38	17	28
Chloramphenicol	**C**	30	28	30	38	30	30	29	27	22	27	24	38	28	25	38	10	26
Sulfonamide	**SF**	31	33	34	39	32	28	32	33	34	36	36	24	30	34	40	12	31
Carbapenem	**IPM**	37	34	37	38	34	32	40	38	38	33	38	38	32	37	40	26	36
Cephalosporin	**CAZ**	19	20	21	24	18	21	17	26	20	19	24	R	21	25	28	R	30
	CFM	21	23	20	R	23	R	17	20	22	15	30	R	31	30	22	R	32
	CXM	24	22	22	17	22	19	32	21	22	20	24	R	28	29	30	19	32
	CTR	22	24	20	24	30	24	35	24	24	27	24	11	28	28	29	R	34
	CEP	32	34	24	29	30	26	34	30	25	29	33	11	32	22	32	R	39
	CTX	30	31	23	28	30	23	30	21	26	20	36	18	38	28	30	R	32
Monobactam	**AT**	17	20	19	22	17	14	17	18	20	15	20	R	19	24	22	R	26
Rifamycin (Antitubercular Agent)	**RIF**	29	31	30	34	24	R	23	23	22	30	32	29	30	29	26	10	35
Aminocoumarin	**NV**	27	20	24	30	22	28	22	21	19	20	26	22	26	23	30	R	23
Lincosamide	**CD**	32	30	28	40	30	40	26	38	24	36	34	34	34	28	40	R	37
Diaminopyrimidines	**TR**	30	30	21	32	26	21	35	35	22	26	32	R	32	24	34	R	36

5.3.7. Multiple Antibiotic Resistance (MAR) Index

We examined the MAR index of selected isolates from three different natural thermal conditions. A total of 27 isolates were selected from the hot region, 19 from the warm region, and 17 from the cold region. These isolates were tested against 28 distinct antibiotics representing various antibiotic classes. In the hot region, all isolates exhibited a MAR index value of less than 0.2, indicating a very low level of antibiotic resistance as shown in **Fig. 15**. Conversely, in the warm region, two isolates (Y1MM27 and Y1TM28) out of the nineteen showed a MAR index above the standard limit, suggesting the presence of contamination in this area as shown in **Fig. 16**. Similarly, in the cold region, two isolates (Y2MP13 and Y1TP01) also displayed a MAR index value above the standard limit **Fig. 17**. Comparing the different thermal zones, the cold region had the highest level of contamination, followed by the warm region and then the hot region.

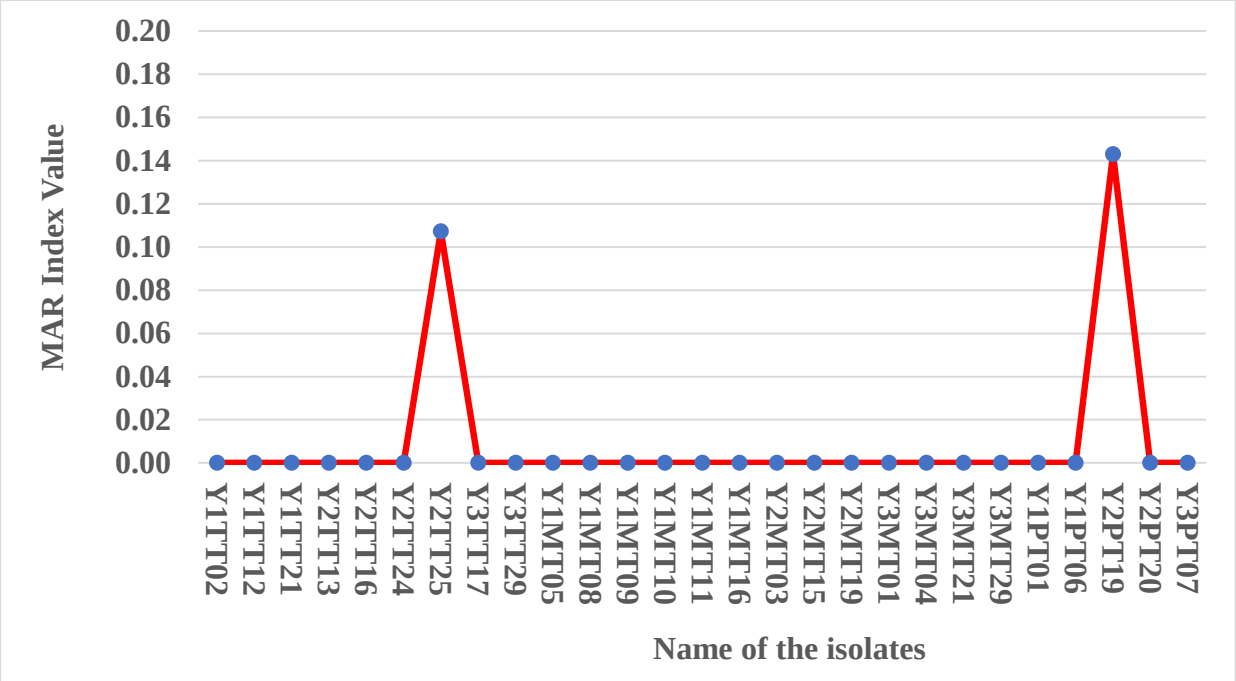


Fig. 15 Displays the MAR Index value of the isolates gathered from the hot region.

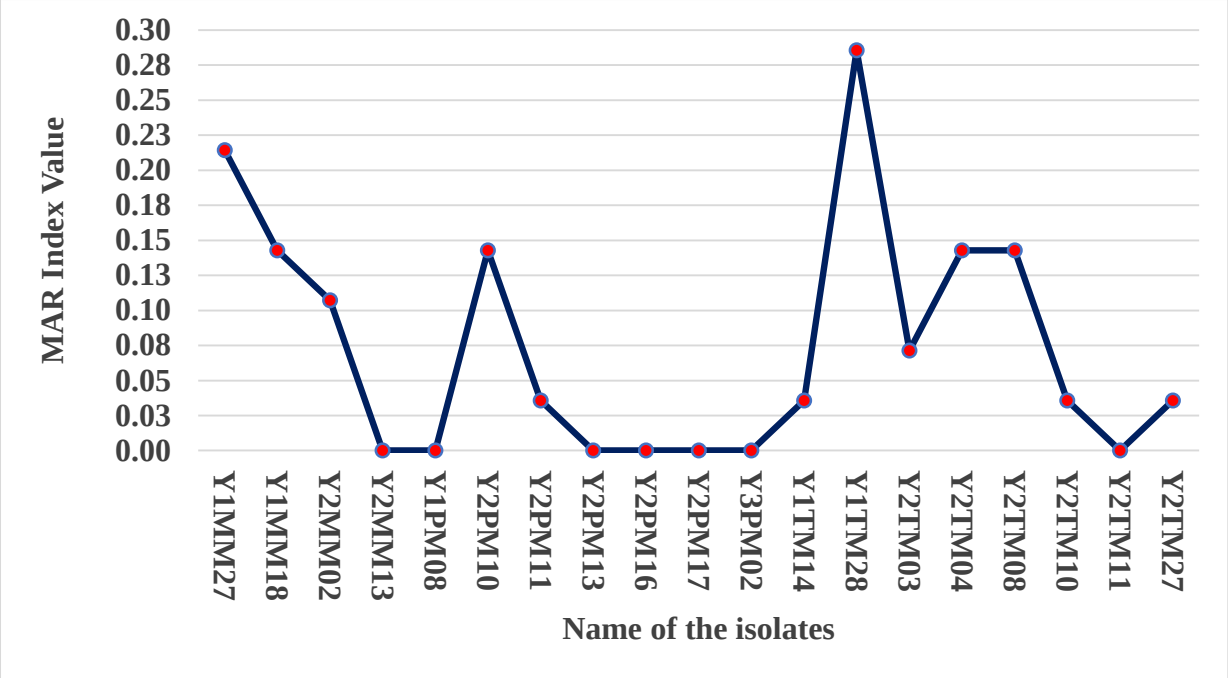


Fig. 16 Displays the MAR Index value of the isolates gathered from the warm region.

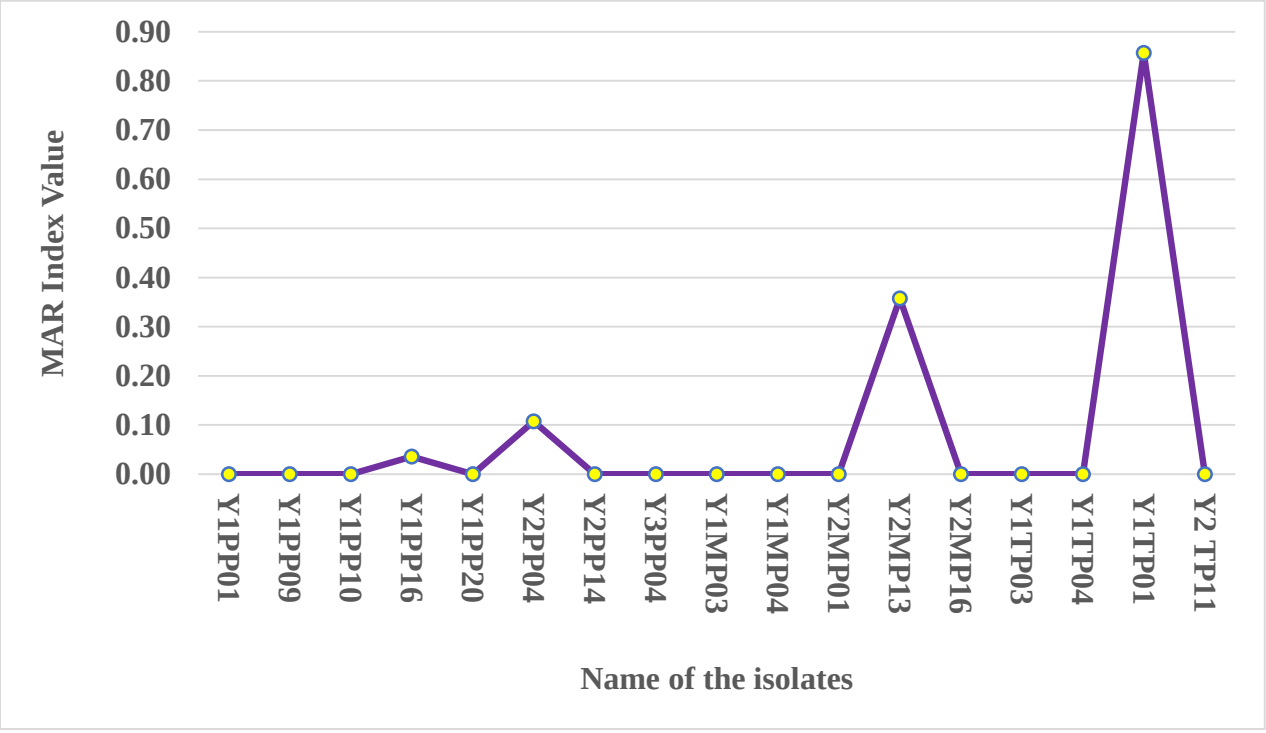


Fig. 17 Displays the MAR Index value of the isolates gathered from the cold region.

5.3.8. Minimum Inhibitory Concentration (MIC) assay

The findings derived from the Minimum Inhibitory Concentration (MIC) assay revealed that the mesophilic bacterial isolates displayed considerably greater resilience towards the antibiotics tested visualized in **Fig. 18**. To effectively impede the growth of these mesophilic bacteria, higher doses of antibiotics were necessary. Notably, Ceftazidime, Erythromycin, and Streptomycin were identified as particularly ineffective against the mesophilic isolates as stated

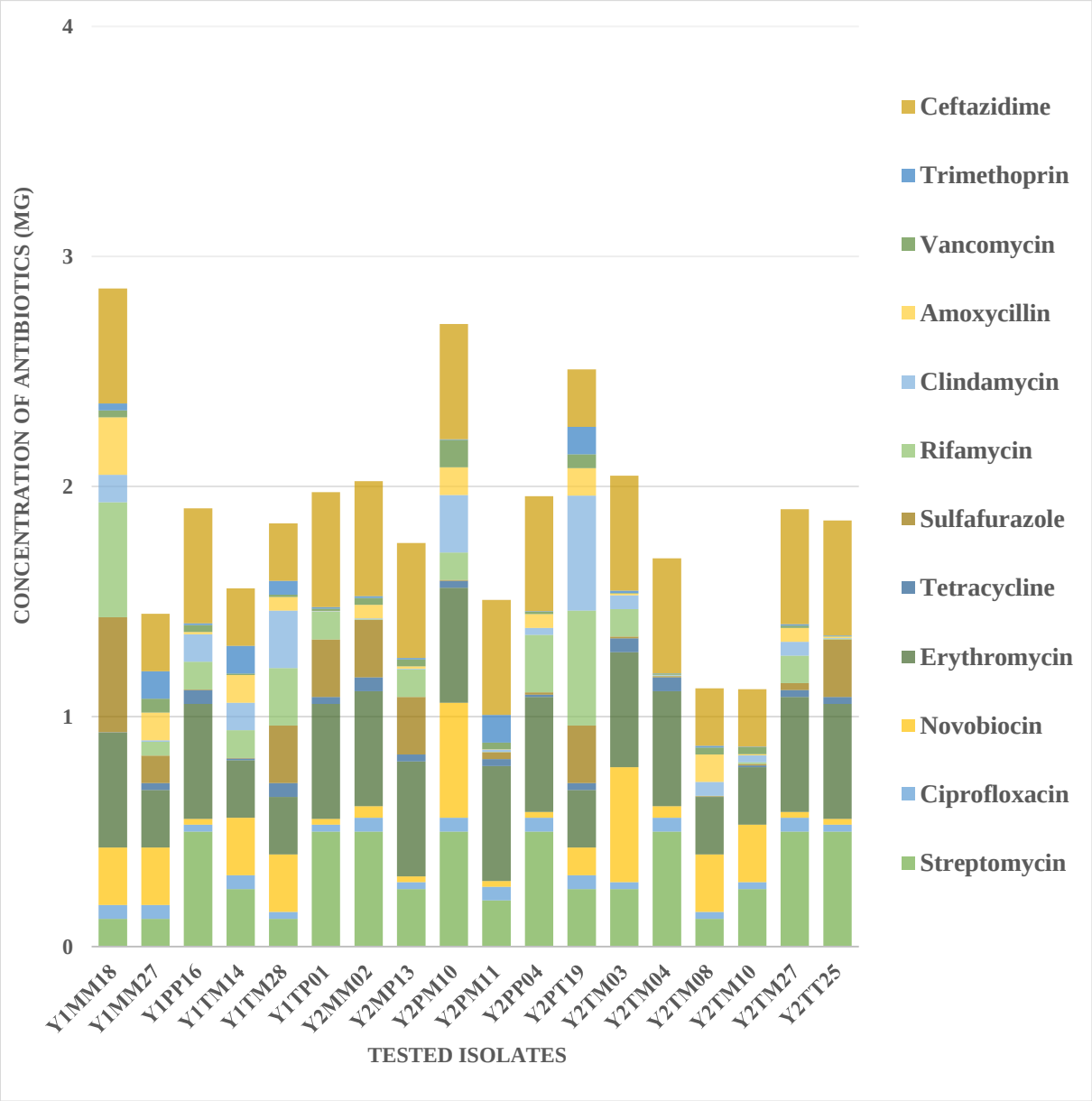


Fig. 18 Minimum Inhibitory concentration assay of the selected bacterial isolates.

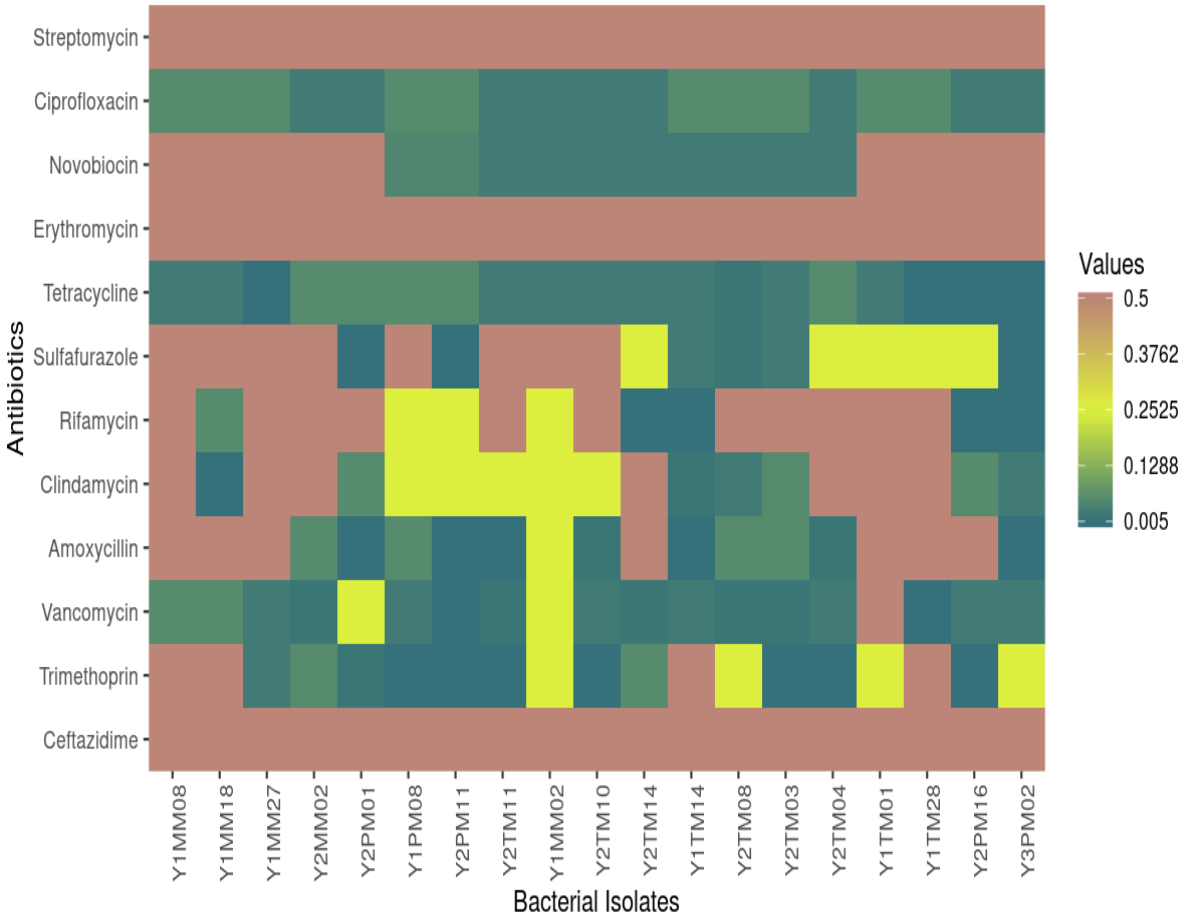


Fig. 19 The Minimum Bactericidal Concentration (MBC) of the tested bacterial isolates.

Table 13 a. The Minimum Inhibitory Concentration (MIC) assay results of the selected bacterial isolates against the antibiotics are presented.

Antibiotics	Y1MM18	Y1MM27	Y1PP16	Y1TM14	Y1TM28	Y1TP01	Y2MM02	Y2MP13	Y2PM10	Y2PM11	Y2PP04	Y2PT19	Y2TM03	Y2TM04	Y2TM08	Y2TM10	Y2TM27	Y2TT25
Streptomycin	0.12	0.12	0.5	0.25	0.12	0.5	0.5	0.25	0.5	0.2	0.5	0.25	0.25	0.5	0.12	0.25	0.5	0.5
Ciprofloxacin	0.06	0.06	0.03	0.06	0.03	0.03	0.06	0.03	0.06	0.06	0.06	0.06	0.03	0.06	0.03	0.03	0.06	0.03
Novobiocin	0.25	0.25	0.025	0.25	0.25	0.025	0.05	0.025	0.5	0.025	0.025	0.12	0.5	0.05	0.25	0.25	0.025	0.025
Erythromycin	0.5	0.25	0.5	0.25	0.25	0.5	0.5	0.5	0.5	0.5	0.5	0.25	0.5	0.5	0.25	0.25	0.5	0.5
Tetracycline	0.001	0.03	0.06	0.007	0.06	0.03	0.06	0.03	0.03	0.03	0.01	0.03	0.06	0.06	0.001	0.007	0.03	0.03
Sulfafurazole	0.5	0.12	0.003	0.003	0.25	0.25	0.25	0.25	0.003	0.03	0.01	0.25	0.007	0.005	0.003	0.007	0.03	0.25
Rifamycin	0.5	0.06	0.12	0.12	0.25	0.12	0.003	0.12	0.12	0.001	0.25	0.5	0.12	0.003	0.001	0.007	0.12	0.003
Clindamycin	0.12	0.007	0.12	0.12	0.25	0.003	0.003	0.003	0.25	0.01	0.03	0.5	0.06	0.003	0.06	0.03	0.06	0.003
Amoxycillin	0.25	0.12	0.01	0.12	0.06	0.001	0.06	0.01	0.12	0.001	0.06	0.12	0.007	0.001	0.12	0.005	0.06	0.006
Vancomycin	0.03	0.06	0.03	0.007	0.01	0.01	0.03	0.03	0.12	0.03	0.01	0.06	0.003	0.005	0.03	0.03	0.01	0.003
Trimethoprin	0.03	0.12	0.007	0.12	0.06	0.007	0.007	0.007	0.003	0.12	0.003	0.12	0.01	0.001	0.007	0.003	0.007	0.003
Ceftazidime	0.5	0.25	0.5	0.25	0.25	0.5	0.5	0.5	0.5	0.5	0.5	0.25	0.5	0.5	0.25	0.25	0.5	0.5

Table 13 b. The Minimum Bactericidal Concentration (MBC) of the tested bacterial isolates

Antibiotics	Y1MM08	Y1MM18	Y1MM27	Y2MM02	Y2PM01	Y1PM08	Y2PM11	Y2TM11	Y1MM02	Y2TM10	Y2TM14	Y1TM14	Y2TM08	Y2TM03	Y2TM04	Y1TM01	Y1TM28	Y2PM16	Y3PM02
Streptomycin	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Ciprofloxacin	0.06	0.06	0.06	0.03	0.03	0.06	0.06	0.03	0.03	0.03	0.03	0.06	0.06	0.06	0.03	0.06	0.06	0.03	0.03
Novobiocin	0.5	0.5	0.5	0.5	0.5	0.05	0.05	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.5	0.5	0.5	0.5
Erythromycin	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Tetracycline	0.03	0.03	0.007	0.06	0.06	0.06	0.06	0.03	0.03	0.03	0.03	0.03	0.02	0.03	0.06	0.03	0.007	0.007	0.007
Sulfafurazole	0.5	0.5	0.5	0.5	0.007	0.5	0.005	0.5	0.5	0.5	0.25	0.03	0.02	0.03	0.25	0.25	0.25	0.25	0.007
Rifamycin	0.5	0.06	0.5	0.5	0.5	0.25	0.25	0.5	0.25	0.5	0.007	0.007	0.5	0.5	0.5	0.5	0.5	0.007	0.007
Clindamycin	0.5	0.007	0.5	0.5	0.06	0.25	0.25	0.25	0.25	0.25	0.5	0.02	0.03	0.06	0.5	0.5	0.5	0.06	0.03
Amoxycillin	0.5	0.5	0.5	0.06	0.007	0.06	0.007	0.007	0.25	0.02	0.5	0.007	0.06	0.06	0.02	0.5	0.5	0.5	0.005
Vancomycin	0.06	0.06	0.03	0.02	0.25	0.03	0.005	0.02	0.25	0.03	0.02	0.03	0.02	0.02	0.03	0.5	0.007	0.03	0.03
Trimethoprin	0.5	0.5	0.03	0.06	0.02	0.007	0.007	0.007	0.25	0.007	0.06	0.5	0.25	0.007	0.007	0.25	0.5	0.007	0.25
Ceftazidime	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5

5.4. Culture-independent study

5.4.1. Bacterial diversity analysis through shotgun metagenomic sequences

A total of 12 soil samples, two from each zone (i.e. hot, warm, and cold region) of the two sites were chosen for metagenomic analysis. Thus among 12 samples, 4 samples correspond to each thermal zone. However, for data analysis, the mean of two samples (results) from each zone of both the sites (site-I and site-II) were represented in the results. Thus, a total of six metagenomic samples such as METAYHS1T/METAYHS2T (hot region), METAYHS1M/METAYHS2M (warm region), and METAYHS1P/METAYHS2P (cold region) were analyzed. The total read length was >20Mbp for most of the samples. The average raw and trimmed data of the metagenomes were >3GB as depicted in **Table 14**.

Table 14. Metagenomic samples with Read length and Sequence Data in GBs.

Sample ID	Total Reads	Total Bases	Mean Read Length R1	Mean Read Length R2	Raw Data In GB
META01MESOH2	16061250	2553238250	159	159	2.55323825
META02MESOH2	19525564	3104564626	159	159	3.10456468
META03THERMH2	63420032	10091235088	159	159	10.0912351
META04THERMH2	5932294	943314246	159	159	0.94331425
META05PSYH2	13425280	2142569520	159	159	2.14256952
META06PSYH2	12816698	2832854982	159	159	2.83285498
META07THERMH3	12900128	2846128302	159	159	2.8461283
META08THERMH3	13325392	2126682328	159	159	2.12668233
META09MESOH3	14542830	2313104920	159	159	2.31310492
META10MESOH3	15388582	2446284538	159	159	2.44628454
META11PSYH3	12335456	2256332504	159	159	2.2563325
META12PSYH3	14566562	2316083358	159	159	2.31608336

5.4.1.1. Distribution of Microbial Diversity

Microbial populations thrive in all three natural thermal gradient regions, including hot, warm, and cold environments. Bacteria dominated the sampled areas, comprising over 97 % of the population in sampling site-I and more than 96 % in sampling site-II. Archaea were the second most prevalent group, accounting for more than 1 % in sampling site-I and more than 2 % in sampling site-II. We observed distinct patterns of microbial population distribution based on each thermal region. The temperature strongly influenced the composition of microorganisms. In the transition from hot to cold, bacterial and eukaryotic microbial diversity increased. Conversely, the abundance of Archaea increased as temperatures rose from cold to hot. Likewise, the abundance of viruses was higher in the hot region compared to the warm and cold regions as visualized in **Fig. 20 and 21**.

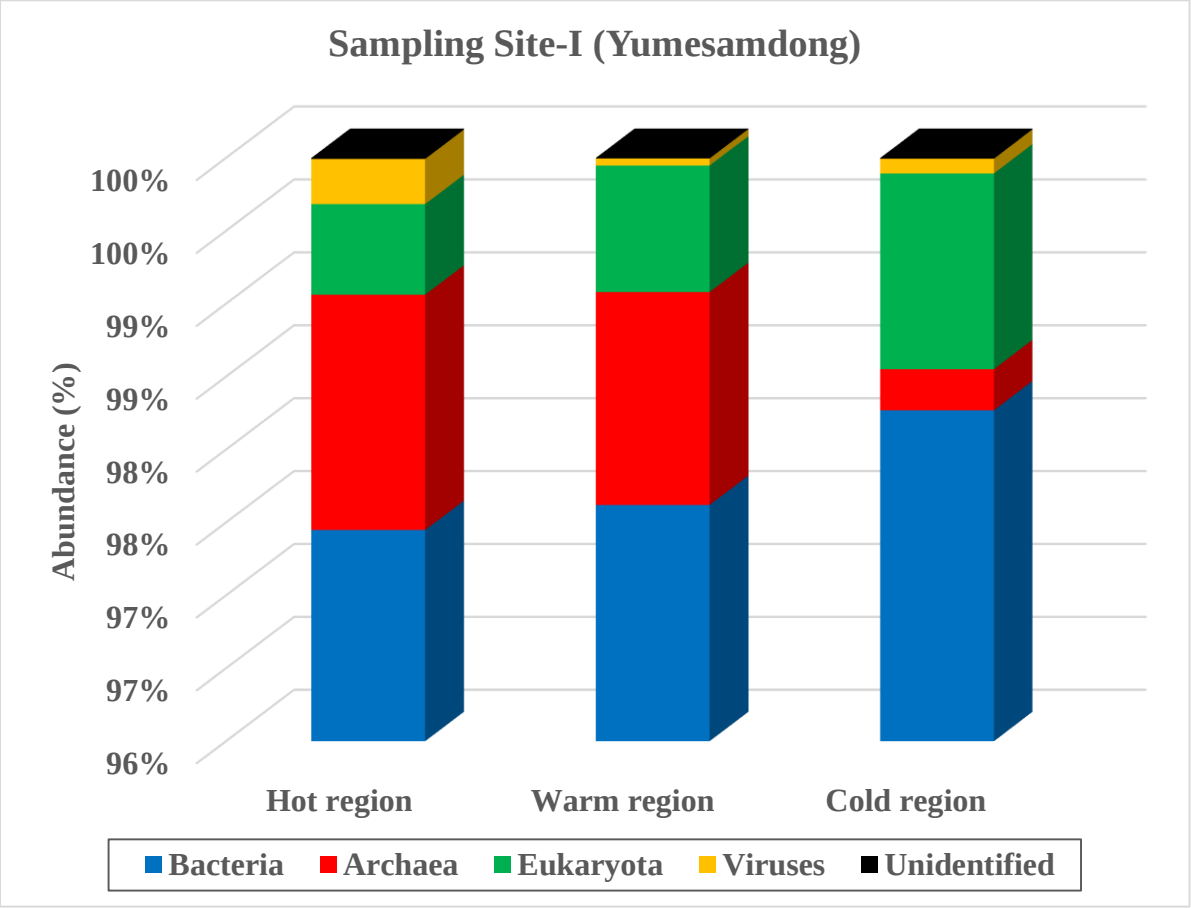


Fig. 20 Distribution of microbial diversity to distinct thermal zones of sampling site-I

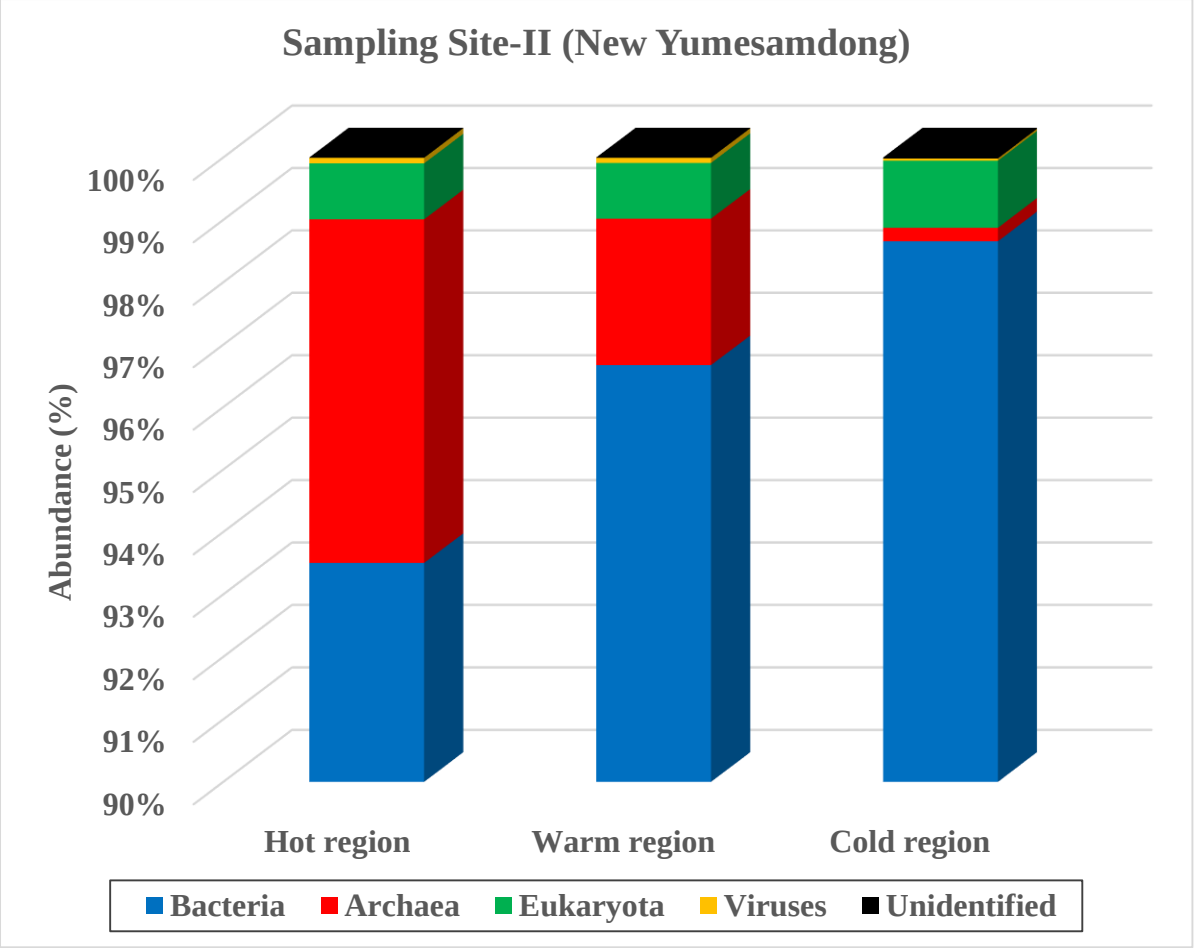


Fig. 21 Distribution of microbial diversity to distinct thermal zones of sampling site-II

5.4.1.2. Diversity Indices and Rarefaction Curve

The analysis revealed interesting findings at the diversity indices of both sampling sites. By calculating the mean of diversity indices, it was observed that the warm region exhibited the highest alpha diversity (29.6) compared to the hot (17.96) and cold (17.99) regions. Moreover, the Shannon and Simpson indices were also found to be higher in the warm region as indicated in **Table 15** and illustrated in **Fig. 22**.

To analyze the uniqueness of the sampling sites more comprehensively, a rarefaction curve was generated. The results indicate a distinct difference between the two sampling sites. The first sampling area, referred to as the warm region (METAYHS1M), exhibited the highest number of individuals, followed by the cold region (METAYHS1P) and the hot region

(METAYHS1T), as illustrated in **Fig. 23a**. Conversely, in the second sampling zone, the cold region (METAYHS2P) demonstrated a higher projection with a greater number of individuals, followed by the warm region (METAYHS2M) and the hot region (METAYHS2T), as depicted in **Fig. 23b**. It is noteworthy that, in both sampling areas, the hot region displayed significantly lower projections compared to the other zones.

Based on the findings from diversity indices and rarefaction curve analysis, it can be inferred that the warm region, which encompasses temperatures ranging from 10 to 40℃, offers optimal conditions for bacterial growth and determines the composition of bacterial communities. Furthermore, it is plausible to hypothesize that comparable microenvironments could exert differential impacts on microbial diversity, thereby underscoring the significance of temperature in shaping bacterial communities.

Table 15 Diversity indices of different thermal zones.

Diversity Indices	Hot region	Warm region	Cold region
Fisher alpha	17.965	29.655	17.99
Shannon index	3.165	3.73	3.28
Simpson index	0.93	0.965	0.945
Chao-1	59.69	58.135	46.915

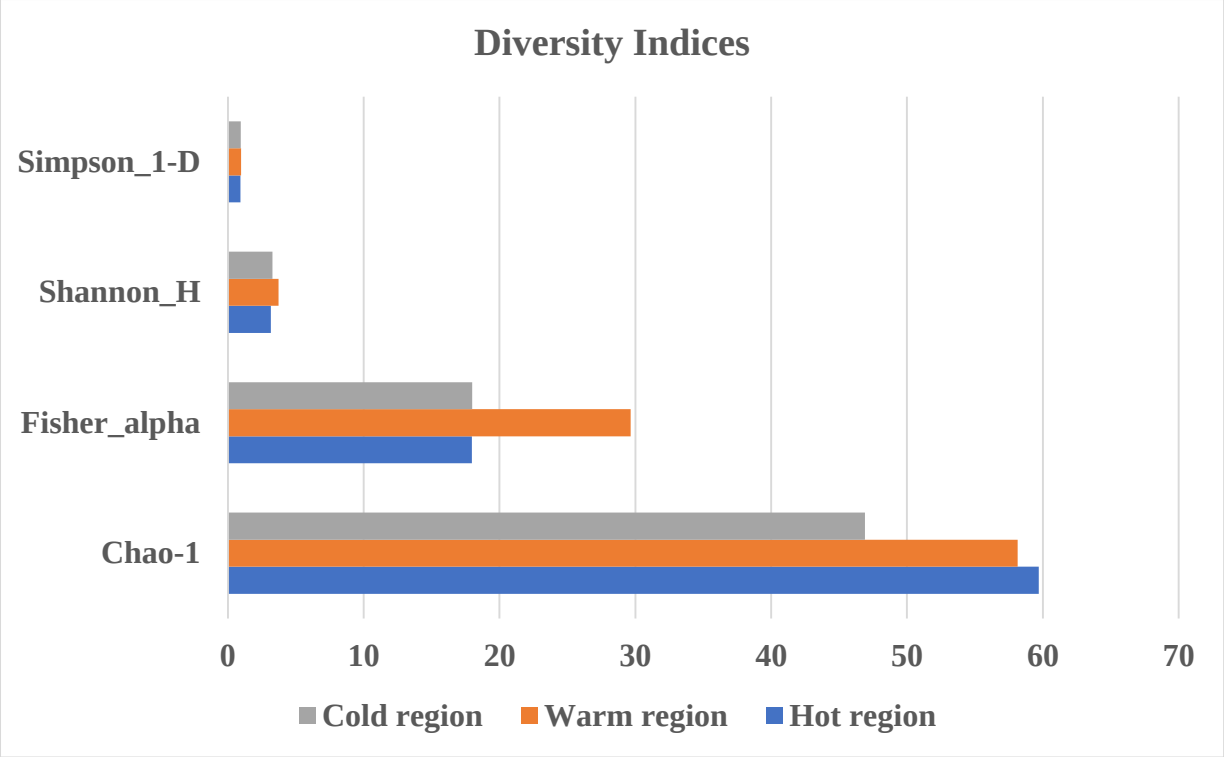


Fig. 22 Diversity indices of distinct thermal zones.

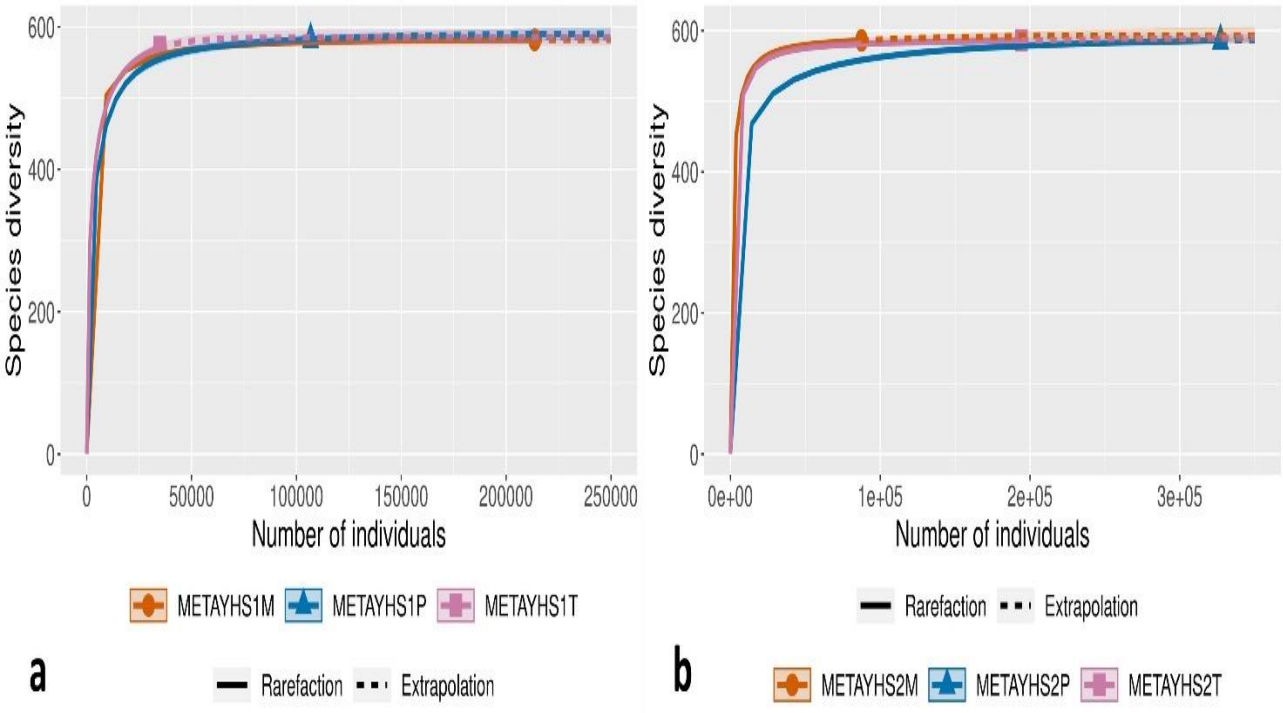


Fig. 23a, b. Rarefaction curve.

5.4.1.3. Principal Component Analysis

A detailed analysis using Principal Component Analysis (PCA) revealed several noteworthy findings regarding the correlation between temperature and bacterial groups. **Fig. 24** illustrates these correlations, which provide valuable insights into the dynamics of bacterial diversity in extreme thermal conditions. The PCA results indicate a positive correlation between temperature and two bacterial groups, namely Bacillota and Chloroflexota. This suggests that as the temperature increases, the abundance or prevalence of these bacterial groups tends to rise. On the other hand, a negative correlation is observed between temperature and two other bacterial groups, Pseudomonadota and Bacteroidota (Bacteroidetes). This indicates that as the temperature increases, the abundance or prevalence of these bacterial groups tends to decrease. **Fig. 24** further demonstrates that thermophilic bacteria, which thrive in high-temperature environments, tend to cluster around the hot region. This clustering phenomenon signifies that the thermophilic bacteria have a strong affinity for and are adapted to the extreme heat conditions found in that region. Conversely, mesophilic bacteria, which prefer moderate temperatures, tend to cluster around the warm region. Notably, the mesophilic bacteria exhibit a relatively higher diversity compared to the cold region, indicating a wider range of bacterial species and genetic variation in the warm region.

Based on these findings, it can be inferred that temperature plays a pivotal role in shaping bacterial diversity in extreme thermal conditions. The results strongly suggest that temperature acts as the primary factor governing the distribution and composition of bacterial communities in these harsh environments. The positive and negative correlations with specific bacterial groups emphasize the influence of temperature on their abundance and highlight its role as a selective force shaping the bacterial community structure.

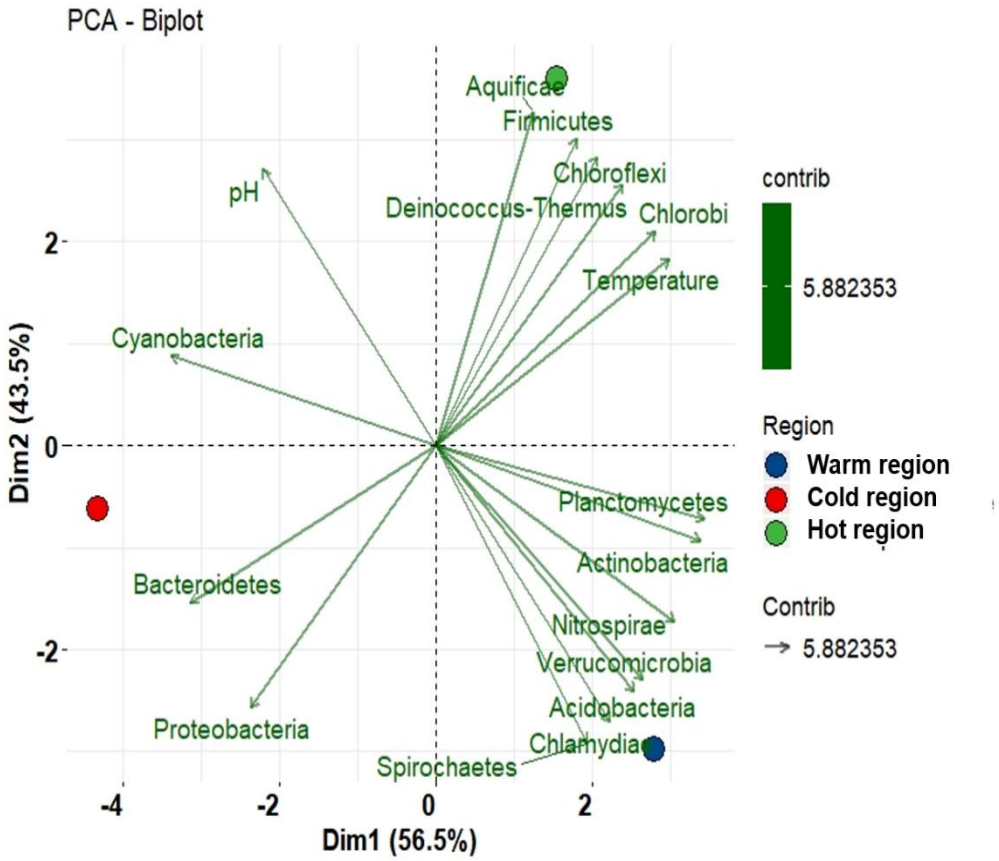


Fig. 24 Principal Component Analysis showing the correlation of various bacterial phyla in a specific thermal zone with temperature.

5.4.1.4. Phylum-level bacterial diversity

Phylum level diversity shows the abundance of Pseudomonadota followed by Bacillota, Chloroflexota, Bacteroidota, and Deinococcota (Deinococcus-Thermus) in the samples of the hot region. In the case of warm regions, the prevalence of Pseudomonadota was followed by Bacteroidota, and Bacillota were found but with a relatively higher percentage of Pseudomonadota as compared to hot regions. Also, various other phyla showed considerable reduction in abundance such as Bacillota, Chloroflexota, and Deinococcota. However, there were other diverse phyla observed such as Acidobacteriota (Acidobacteria), Actinomycetota, and Verrucomicrobiota (Verrucomicrobia) indicating higher biodiversity in the warm region.

Similarly, in the case of the cold region, the higher prevalence of Pseudomonadota followed by Bacteroidota was found with the increase in Cyanobacteriota (Cyanobacteria) population and reduction in many other phyla as shown in **Fig. 25**. PCA confirmed this trending pattern. The thermophilic bacteria formed a cluster around the hot region. The mesophilic bacteria clustered around warm regions and were relatively rich in diversity as compared to cold regions as shown in Fig. 24.

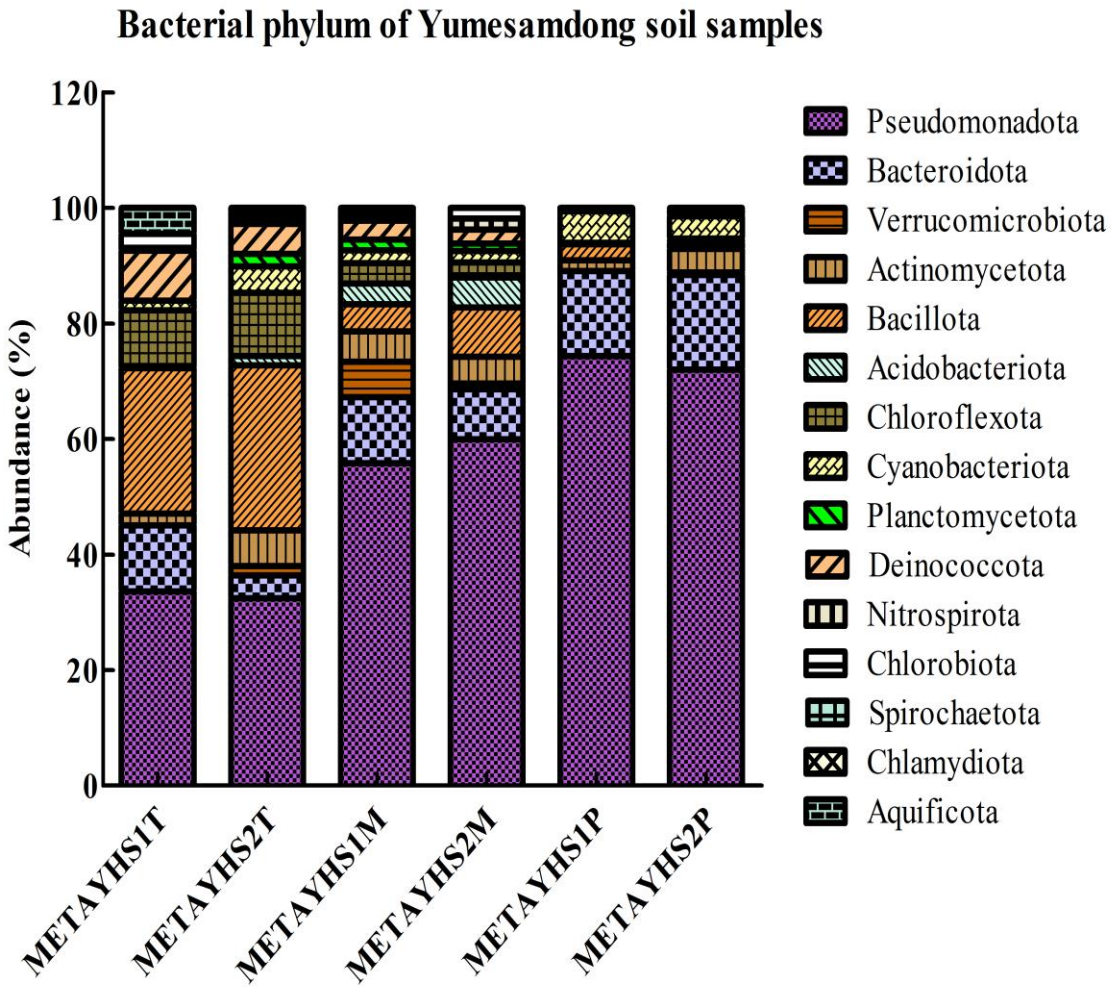


Fig. 25 Phylum level diversity of various soil samples of three thermal zones.

5.4.1.5. Genus-level bacterial diversity

Genus level diversity shows the richness of thermophilic bacteria such as *Meiothermus, Deinococcus, Geobacillus*, *Thermus*, *Bacillus*, *Anoxybacillus*, and *Acinetobacter*, etc. in hot regions. In the case of the warm region, the mesophilic bacteria observed were *Geobacter*,

Thiobacillus, *Nitrobacter*, Candidatus *Solibacter*, *Acidobacterium*, *Nitrococcus*, *Rhodobacter*, *Roseobacter*, *Nitrospira* and *Marivirga*, etc. Similarly, in the case of the cold region, the psychrophilic bacteria abundance was from Bacteroides, *Gramella*, *Flavobacterium*, *Nitrosomonas*, *Burkholderia*, *Acidovorax*, *Comamonas*, *Pseudomonas*, *Yersinia*, *Sphingomonas*, etc as shown in **Fig. 26.**

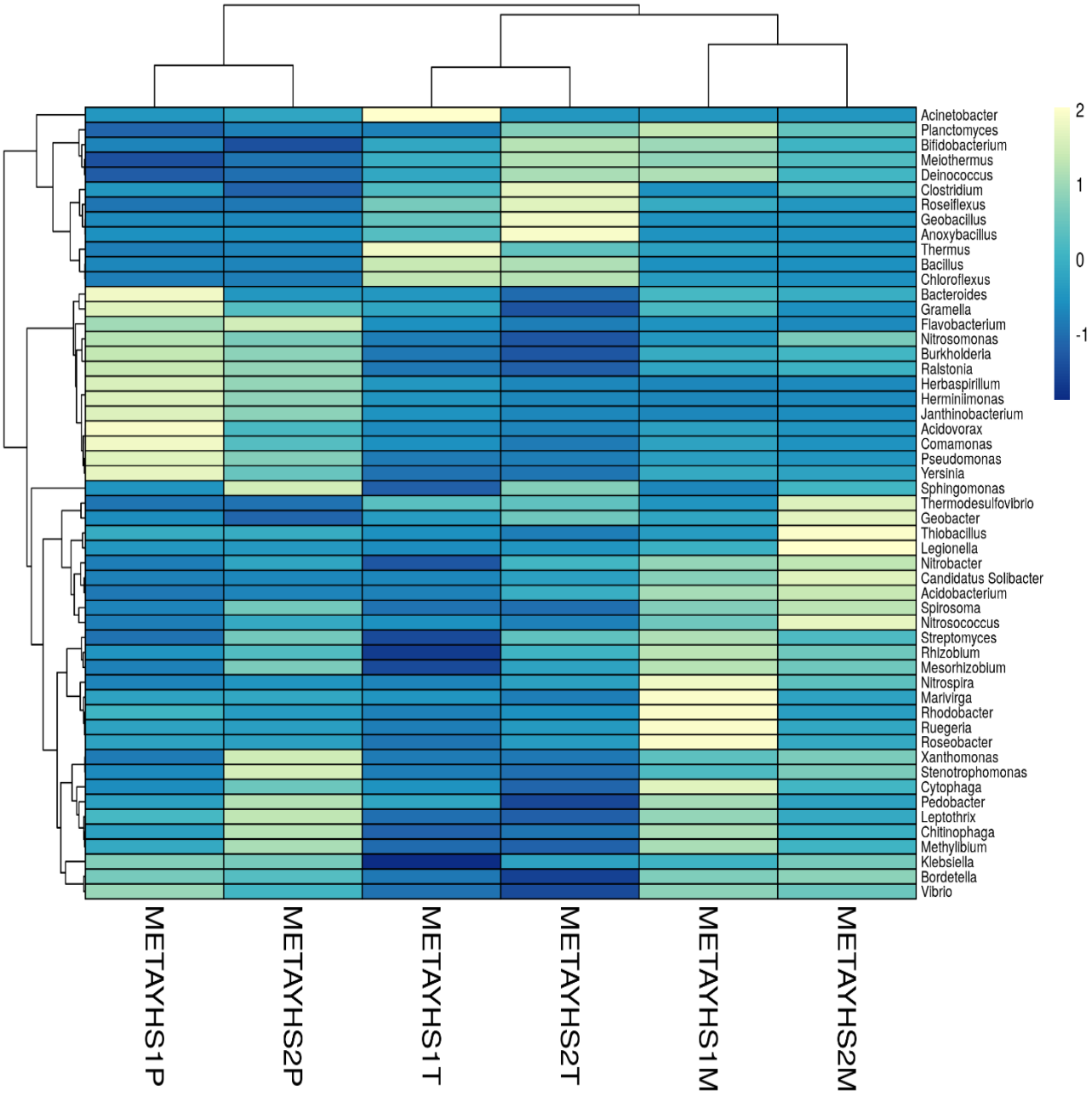


Fig. 26 Heat Map showing the genus level bacterial diversity among different thermal zones.

5.4.1.6. Correlation between abundant bacterial phyla and various physicochemical parameters

Regression analysis was done to check the impact of various elements on bacterial diversity found in three different temperature zones. The abundant phyla such as Pseudomonadota, Bacillota, Bacteroidota, and Chloroflexota were taken into consideration for this analysis. Similarly, the elements with good concentrations and which are believed to play some role in shaping microbial diversity such as Mg, K, Ca, Na, P, and Cl were chosen for this analysis. However, the results have shown that none of the elements except Cl where, showing any correlation with the bacterial diversity with the value of ($r^2 \leq 0.5$, $p<0.05$). Chlorine gave a positive correlation with Pseudomonadota ($r^2 \leq 0.8$, $p=0.01$) and Bacteroidota ($r^2 \leq 0.2$, $p=0.2$) and a negative correlation with Bacillota ($r^2 \leq 0.7$, $p=0.03$) and Chloroflexota (Chloroflexi) ($r^2 \leq 0.7$, $p=0.01$) as shown in **Fig. 27a, b, c, d**. Thus, this may be broadly concluded that there is a negligible impact of various elements on the bacterial diversity patterns of these studied thermal zones.

Similarly, the role of temperature and pH in shaping bacterial diversity was also studied by using regression analysis. The results have shown that there is no correlation between pH and dominant phyla such as Pseudomonadota, Bacillota, Bacteroidota, and Chloroflexota with mean $r^2 \leq 0.6$, $p<0.05$. However, temperature showed a significant negative correlation with Pseudomonadota and Bacteroidota ($r^2 \geq 0.8$, $p<0.05$) and a significant positive correlation with Bacillota and Chloroflexota ($r^2 \geq 0.8$, $p<0.05$) **Fig. 27e, f.** PCA also shows a positive correlation of temperature with Bacillota and Chloroflexota and a negative correlation with Pseudomonadota and Bacteroidota as shown in **Fig. 24**. These results suggest that the temperature is the major factor governing bacterial diversity in such extreme thermal conditions.

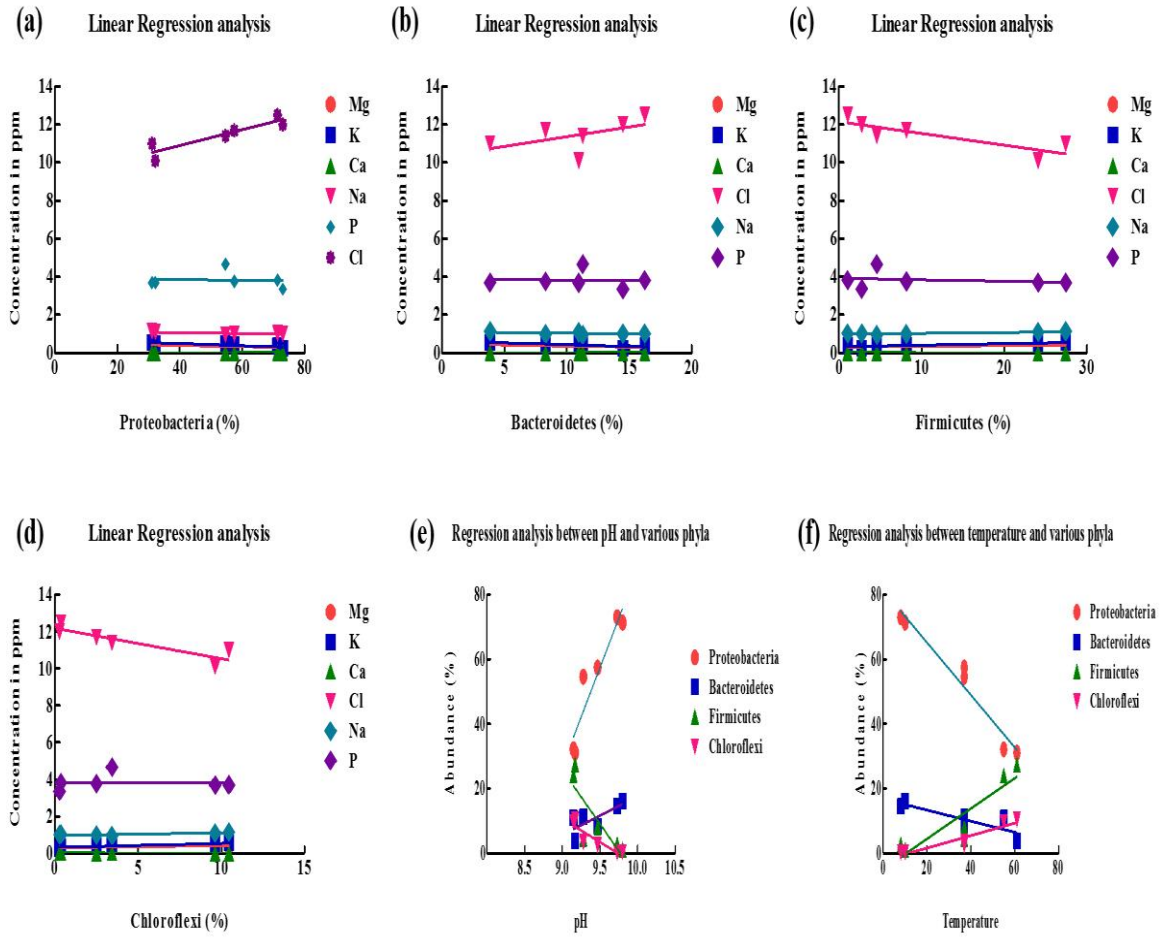


Fig. 27a, b, c, d: Linear Regression analysis showing the correlation with the elements and the dominant bacterial phyla. Whereas **Fig. 27e, f: s**hows the correlation between temperature and pH with various bacterial phyla.

5.4.2. Fungal diversity analysis through metagenomic sequences

5.4.2.1. Diversity Indices

The PAST software packages were used to estimate various diversity indices, namely Simpson_1-D, Shannon_H, Fisher_alpha, and Chao-1. The results indicated distinct patterns across different regions. The Simpson_1-D index, a measure of biodiversity, exhibited higher values in both warm and cold regions, while the hot regions displayed the lowest values. In terms of the Shannon_H index, which quantifies diversity, the warm region displayed the highest value at 3.52, followed closely by the cold region at 3.49. The hot region had the lowest

value at 3.28. Regarding the Fisher_alpha index, which assesses species richness, the hot region had the highest value, indicating a greater diversity of species. The cold region had the second highest value, suggesting a moderate level of species richness. In contrast, the warm region had the lowest Fisher_alpha index value, indicating a relatively lower level of species richness. The Chao-1 index, which estimates species richness based on abundance, revealed that the hot region had the highest value, indicating a larger number of species. On the other hand, the warm region exhibited the lowest Chao-1 index value, suggesting a lower species richness compared to the other temperate regions. **Table 16** displayed the data for the diversity indices, while **Fig. 28** depicted the corresponding graph.

Table 16 Displays the fungal diversity Indices.

	Simpson_1-D	**Shannon_H**	**Fisher_alpha**	**Chao-1**
Hot region	0.9513	3.281	26.875	49.34
Warm region	0.96215	3.521	17.995	46.06
Cold region	0.9621	3.4935	20.765	48.69

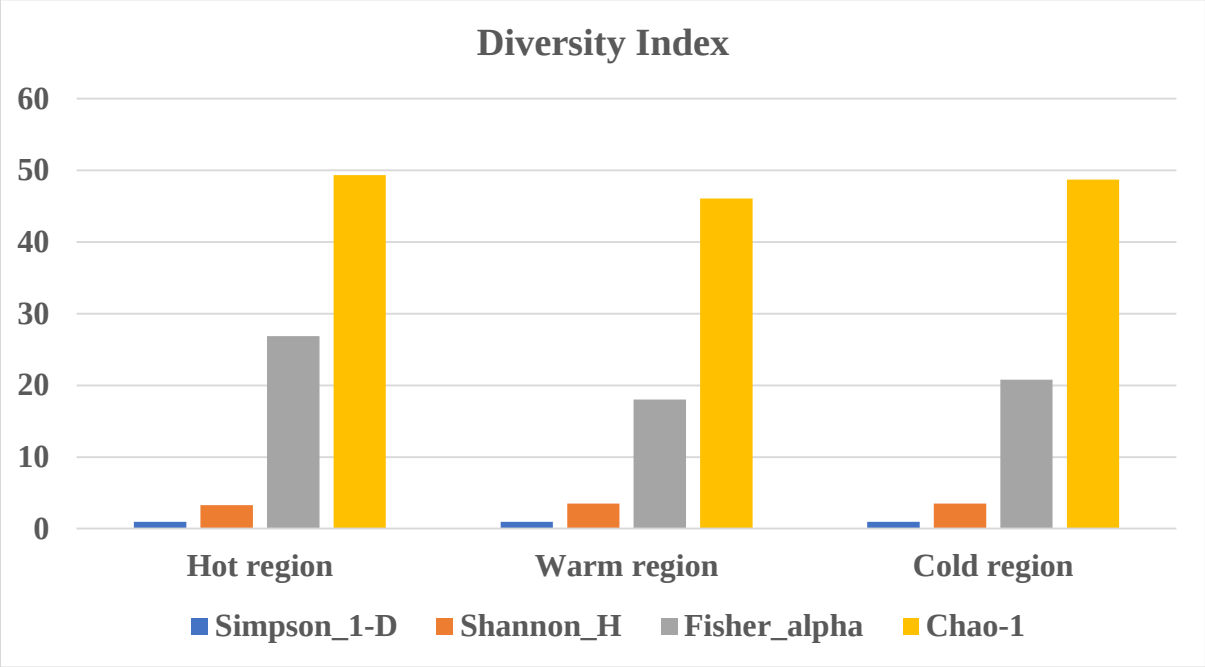


Fig. 28 Diversity Indices of Fungi at distinct thermal zones

5.4.2.2. Rarefaction curve and Principle Component Analysis (PCA)

A rarefaction curve is a graphical tool used to analyze species richness across distinct thermal gradient regions. The hot region, exhibited the highest projection when considering the number of individuals present at the species-level diversity. The high number of individuals observed in the hot region suggests a diverse and rich community of species. On the other hand, the cold region showed the lowest projection in terms of the number of individuals present. This indicates that the cold region had a lower species richness compared to the other regions. The warm region, positioned in second place, had more individuals than the cold region but still had a lower projection than the hot region. This suggests that the warm region had a moderate level of species richness, falling between the hot and cold regions as depicted in **Fig. 29**.

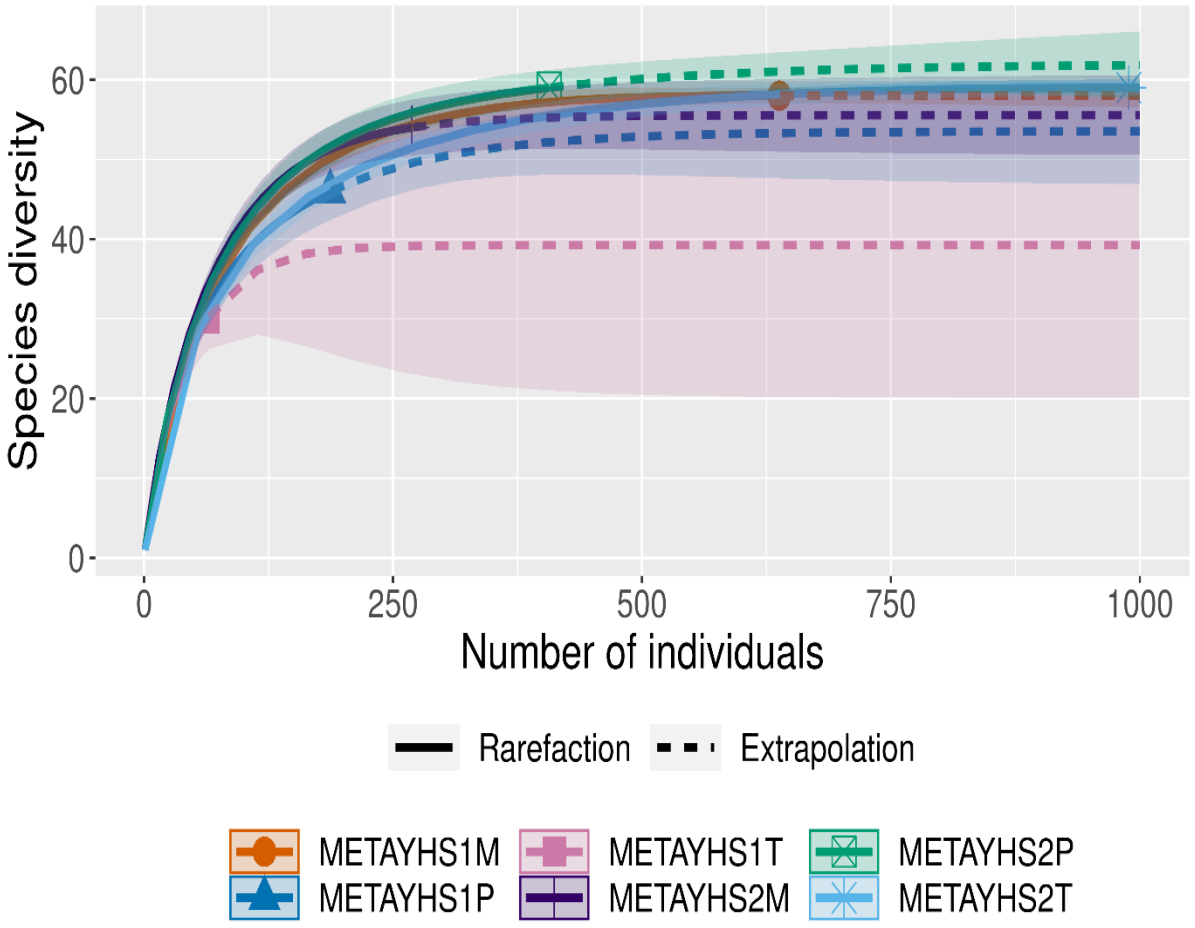


Fig. 29 Rarefaction curve of fungal diversity richness at distinct samples (thermal region)

Principal Component Analysis (PCA) was utilized to assess the connection between fungal diversity in distinct thermal gradient regions. The analysis demonstrated that the primary principal component (PC1) explained a significant portion of the overall variation, accounting for 89.39 % of the total diversity observed across the studied regions. These findings revealed a notable correlation between fungal diversity in all three temperate zones, with a p-value below 0.05, indicating a statistically significant relationship. Consequently, it can be inferred that there is a meaningful association among fungal communities across hot, warm, and cold regions. Moreover, the results indicated a positive correlation between the warm and cold regions, suggesting similarities in fungal community composition, while the hot region displayed distinct fungal dynamics compared to the other regions. The correlation between the different thermal gradient regions is visually represented in **Fig. 30**.

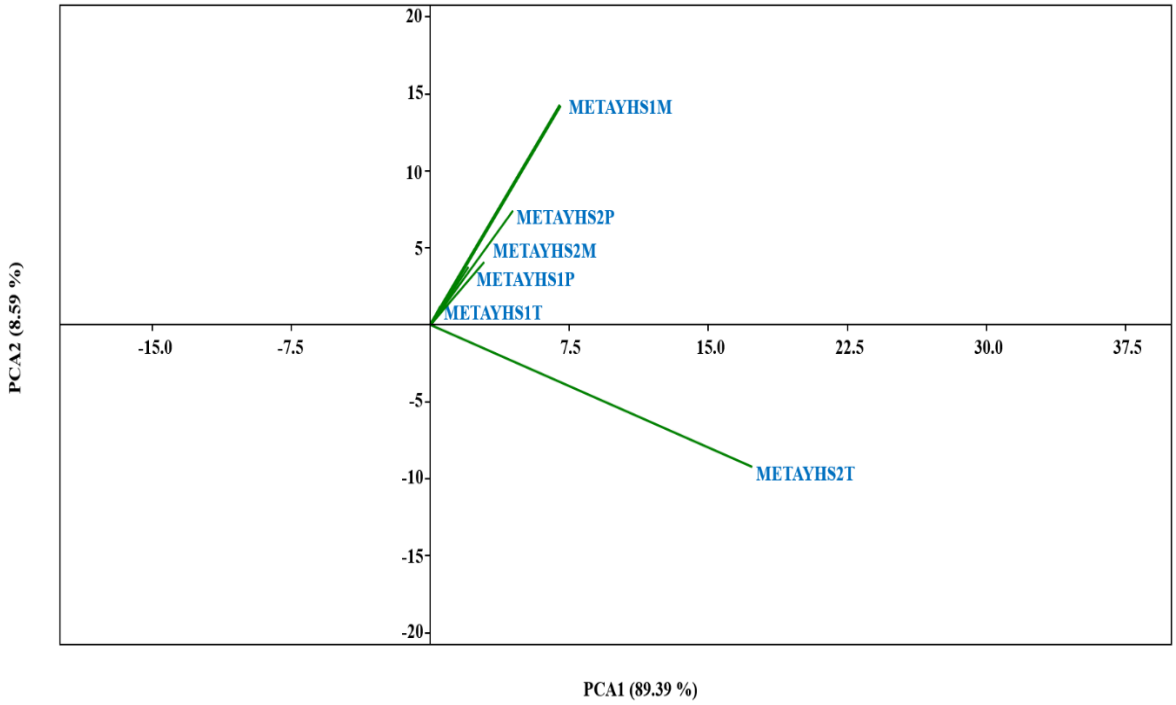


Fig. 30 Principal component analysis of distinct samples

5.4.2.3. Phylum level diversity

All the soil samples collected exhibited the presence of fungal phyla, namely Ascomycota and Basidiomycota. The highest proportion of fungi was found in the hot region, with Ascomycota

accounting for 36.83 % and Basidiomycota for 8.52 %. In the warm region, the microbial community contained 31.86 % Ascomycota and 7.46 % Basidiomycota. Conversely, the cold region had significantly lower percentages of Ascomycota (11.77 %) and Basidiomycota (1.84 %), as shown in **Fig. 31**. Notably, we observed a decline in fungal abundance as the temperature decreased from the hot to the cold region, passing through the warm region. This temperature-dependent trend highlighted the crucial role of temperature in determining fungal diversity.

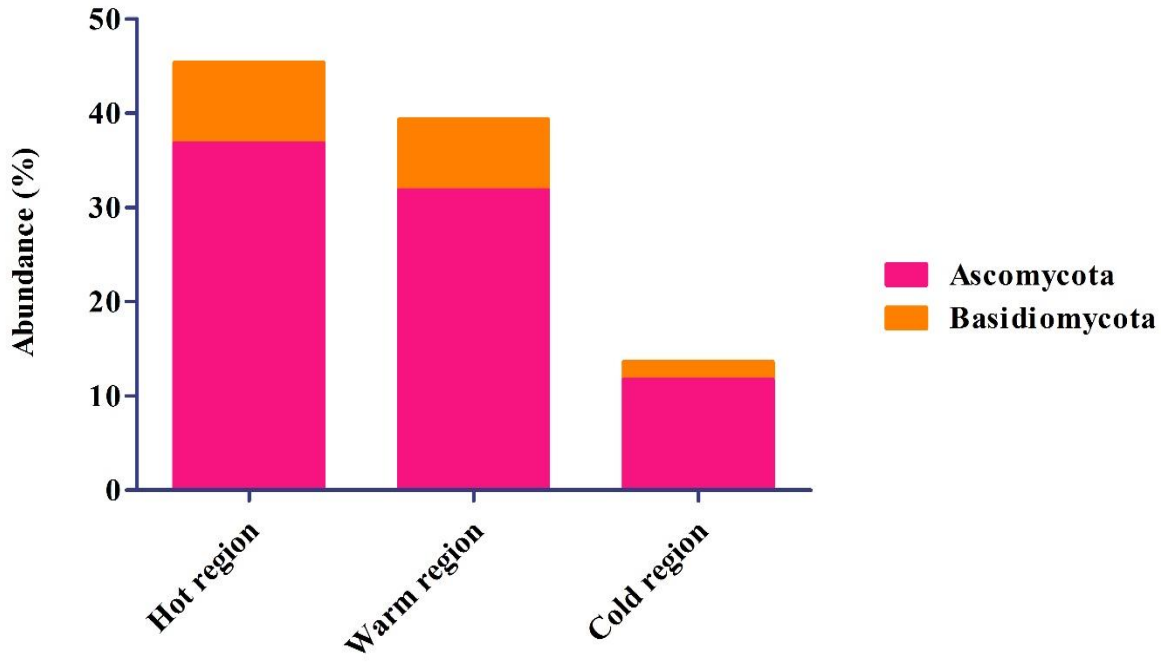


Fig. 31 Phylum level fungal diversity at distinct thermal regions.

5.4.2.4. Genus level fungal diversity

The results regarding the diversity at the phylum level indicate that fungi are the most abundant in the hot region, followed by the mesophilic and cold regions. These findings are consistent with the observations made at the genus level. The prevalent genera in all three thermal zones (hot, warm, and cold) were *Ricinus*, *Hydra*, *Homo*, and *Neosartorya*, as illustrated in **Fig. 32**. The genus-level fungal diversity of the sample is illustrated in **Fig. 33**.

In the hot region, the most common fungal genera were *Neosartorya* (13.1 %), *Aspergillus* (10.82 %), *Malassezia* (5.7 %), *Gibberella* (5.5 %), and *Penicillium* (5.12 %). Within the warm region, the genera with the highest diversity were Gibberella (8.14 %), *Neosartorya* (7.48 %), *Schizosaccharomyces* (6.71 %), *Aspergillus* (6.6 %), and *Ustilago* (5.39 %). As for the cold region, the dominant genera were Aspergillus (8.08 %), *Gibberella* (7.24 %), *Magnaporthe* (7.07 %), *Neosartorya* (6.4 %), and *Yarrowia* (5.39 %).

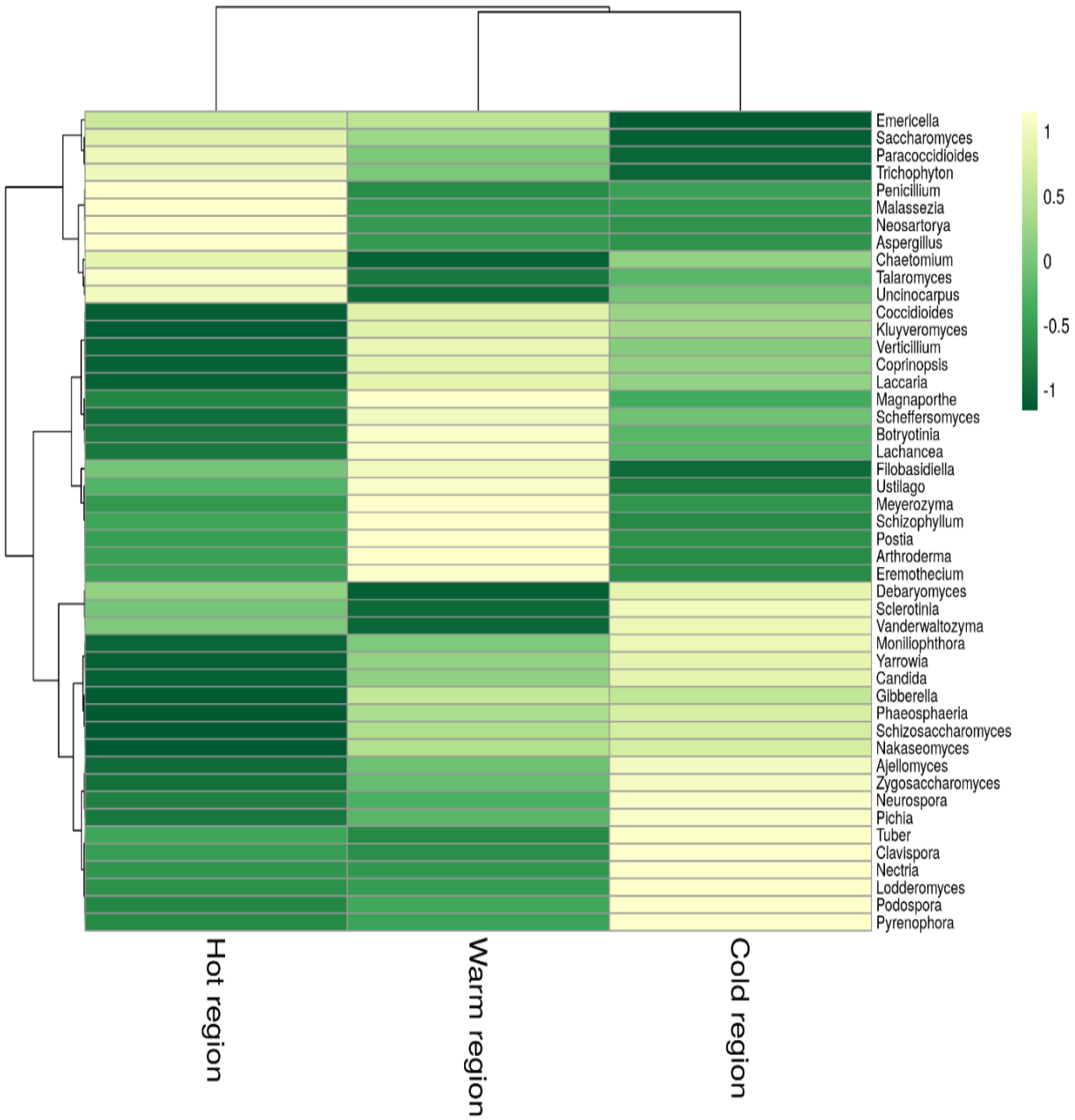


Fig. 32 Heat Map showing the fungal diversity at the genus level.

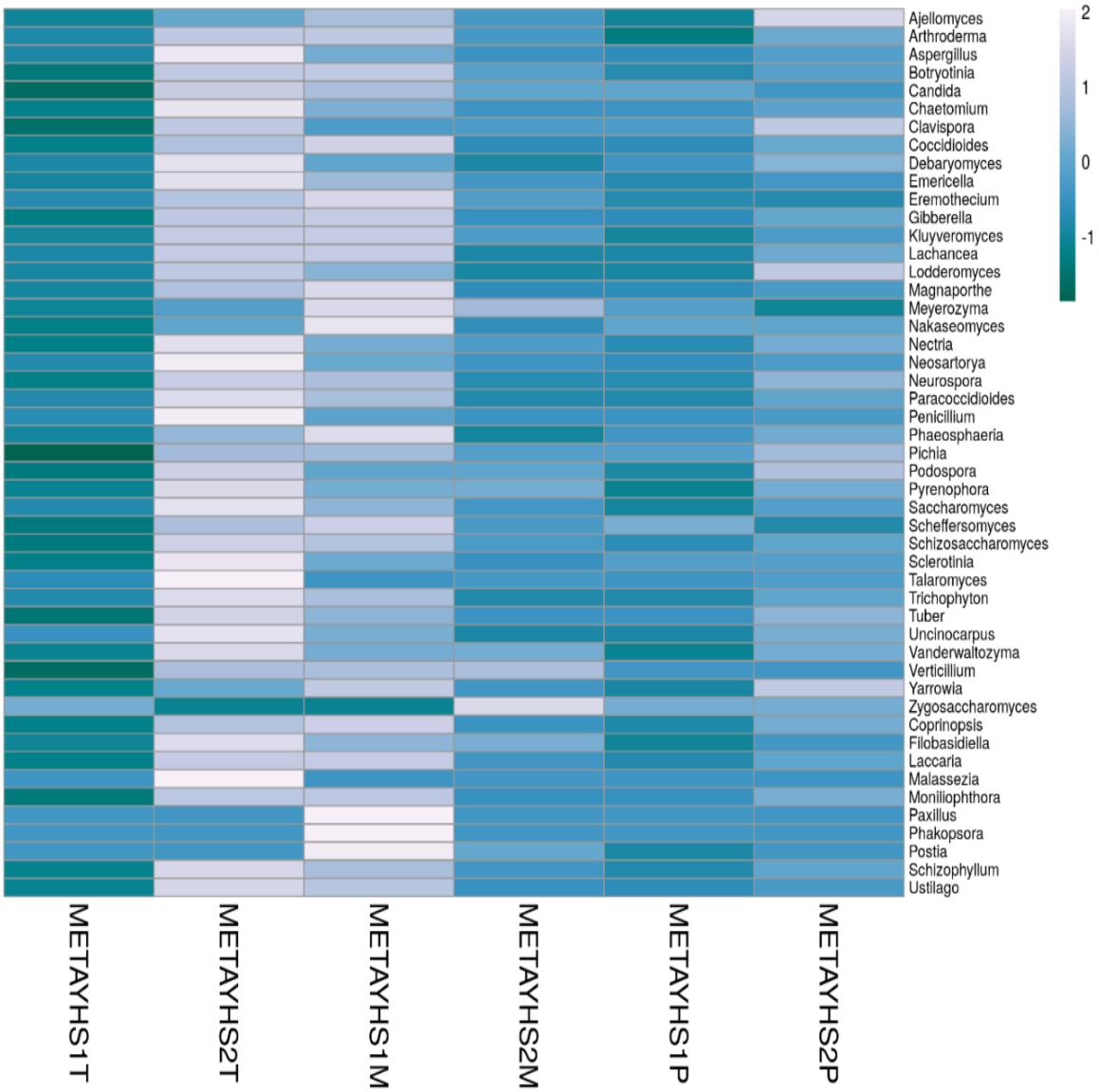


Fig. 33 Heat Map showing the genus level fungal diversity in distinct samples.

5.4.2.5. Species Level Fungal diversity

The dominant species varied across different temperature zones. In the hot region, the most prevalent species were *Neosartorya fumigata* (10.62 %), *Malassezia globosa* (5.7 %), *Gibberella zeae* (5.5 %), *Talaromyces stipitatus* (4.74 %), and *Ustilago maydis* (4.55 %). Moving to the warm region, the dominant species shifted, with *Gibberella zeae* (8.14 %) being the most prevalent, followed by *Neosartorya fumigata* and *Schizosaccharomyces pombe* (5.72 %), *Ustilago maydis* (5.39 %), and *Magnaporthe oryzae* (4.51 %). Conversely, in the cold region, the dominant species were *Gibberella zeae* (8.08 %), *Neosartorya fumigata* (6.39 %),

Schizosaccharomyces pombe (6.4 %), *Neurospora crassa* (5.39 %), *Yarrowia lipolytica* (4.54 %), and *Ustilago maydis* (4.21 %), as depicted in **Fig. 34**.

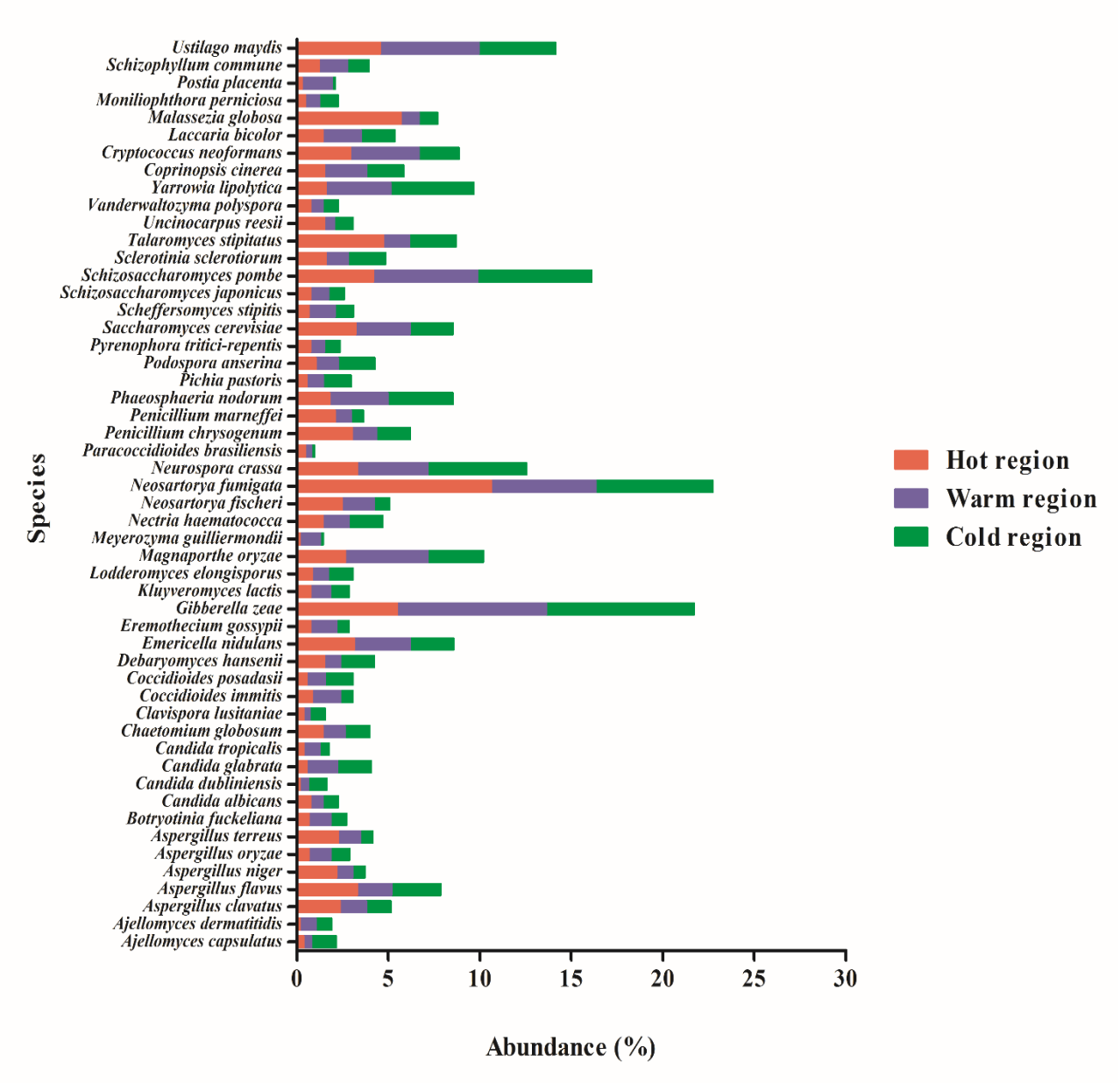


Fig. 34 Species-level fungal diversity at distinct thermal regions.

5.4.3. Detection of ARGs through metagenomic

The soil metagenomic sequences were analyzed for antibiotic resistance genes (ARGs) using different databases. From all the 12 metagenomic sequences (two metagenomic sequences from each three zones), a total of 151 resistant genes were predicted. All the resistant genes showed similar identities with the mesophilic bacteria. Thus, only the mesophiles contributed

antibiotic resistance to these thermal zones. The percentage contribution of antibiotic resistance by mesophiles to hot, warm, and cold regions were 23.1 %, 35.7 %, and 41 % respectively as shown in **Fig. 35a, b**. The percentage contribution of some major antibiotic resistance gene classes is shown in **Table 17.** On the other hand, no antibiotic-resistant genes were found matching thermophiles and psychrophiles thus suggesting that thermophiles and psychrophiles are mainly devoid of antibiotic resistance in such high-altitude pristine areas. It was found that the resistance against various antibiotic classes was prevalent among aminoglycosides (81 %), beta-lactams (13 %), lincosamides (12 %) peptides (10 %), etc. as shown in **Table 17**.

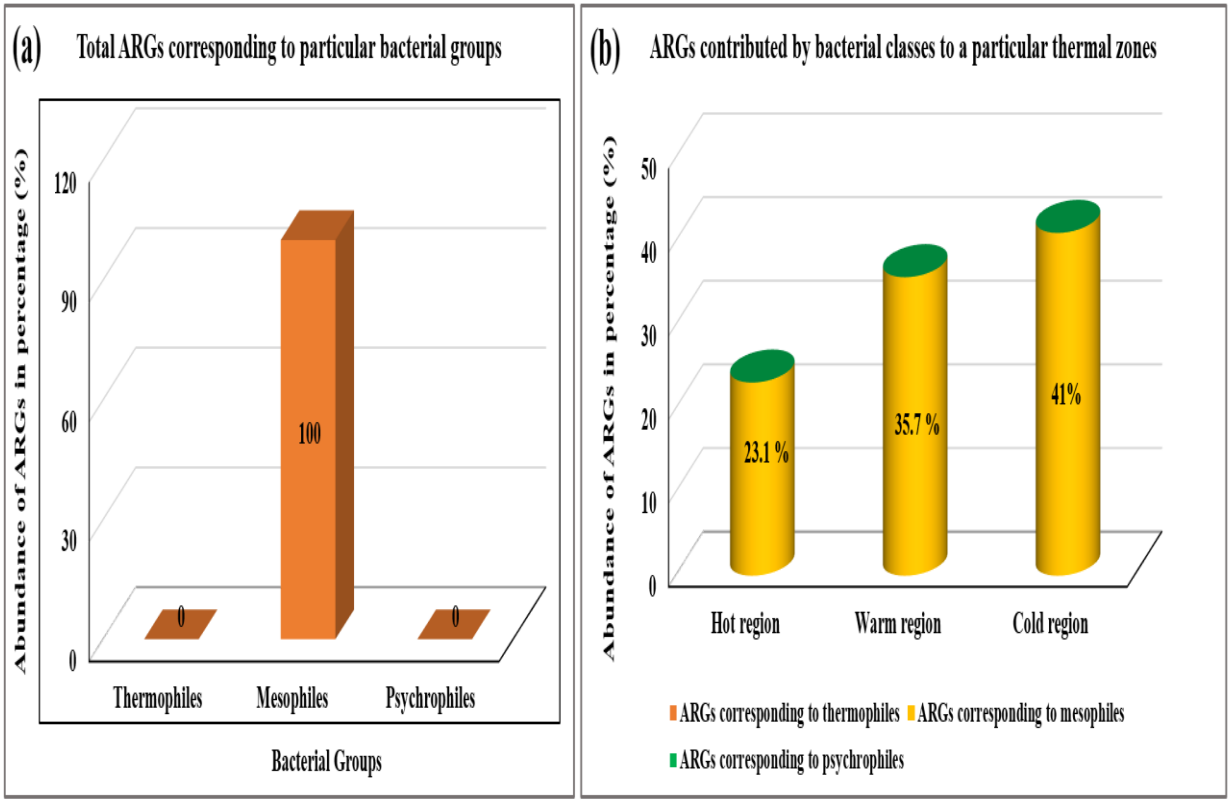


Fig. 35a, b. a-Total Antibiotic Resistant Genes (ARGs) corresponding to a particular bacterial group; **b**- Antibiotic Resistant Genes (ARGs) contributed by bacterial classes to a particular thermal zone.

Table 17. Percentage of Antibiotic Resistance Genes (ARGs per antibiotic class) zone-wise found among metagenomic samples.

Classes of antibiotics	**Percentage of antibiotics resistant genes**			
	Hot region	Warm region	Cold region	Total (hot to cold)
Aminoglycoside	18	31	32	81
Lincosamide	2	5	5	12
Oxazolidinone	3	1	3	7
Macrolides	3	2	4	9
Peptide	2	3	5	10
Beta-lactam	5	5	3	13
Tetracycline	1	2	3	6
Cephalosporin	0	1	0	1
Elfamycin	0	1	3	4
Fluoroquinolone	0	2	0	2
Sulfonamide	0	1	0	1
Diaminopyrimidine	0	1	3	4
Chloramphenicol	0	0	1	1

5.4.4. Correlation analysis between antibiotic resistance and temperature

Regression analysis was done to check how the temperature as a variable effect may determine the antibiotic resistance among isolated bacteria and metagenomic mined ARGs. In the case of ARGs extracted from metagenomic reads the nonlinear regression shows a negative correlation (r^2=0.02, p=0.3) of temperature with various antibiotic-resistant gene classes as shown in **Fig. 36a, b**. Thus, as the temperature increases the antibiotic resistance tends to decrease. These results were also represented by the above data where we have found that the percentage of

antibiotic resistance is higher in cold regions (41 %) as compared to mesophilic and hot regions. Although, all of this resistance is contributed by mesophiles in these zones. PCA also shows a similar pattern, displaying a negative correlation of temperature to various antibiotic gene classes. These results also show that cold region is positively correlated to a large number of antibiotic gene classes followed by mesophilic and hot temperatures as shown in **Fig. 37**. Network analysis was also done between various ARGs and dominant phyla such as Pseudomonadota, Actinomycetota, and Bacillota to check their relationship among themselves. It was shown that among all the thermal zones most of the ARGs were related to Pseudomonadota and Actinomycetota. However, very few ARGs were related to Bacillota as shown in **Fig 38a, b, c.** Thus, these results suggested that Bacillota possess relatively lower antibiotic resistance than Pseudomonadota and Actinomycetota in such pristine high-altitude environments. Similar results have been found when antibiotic resistance among the isolated bacteria correlated with temperature. Antibiotic resistance was found to be maximum in mesophilic bacteria and only a few psychrophilic bacteria. However, when thermal zones were considered, the cold region was found to have higher antibiotic resistance followed by the warm region and hot region, although most of the antibiotic resistance was contributed mainly by the mesophiles in the cold region. Regression analysis showed a decrease in antibiotic resistance with an increase in temperature **Fig. 36c, d.**

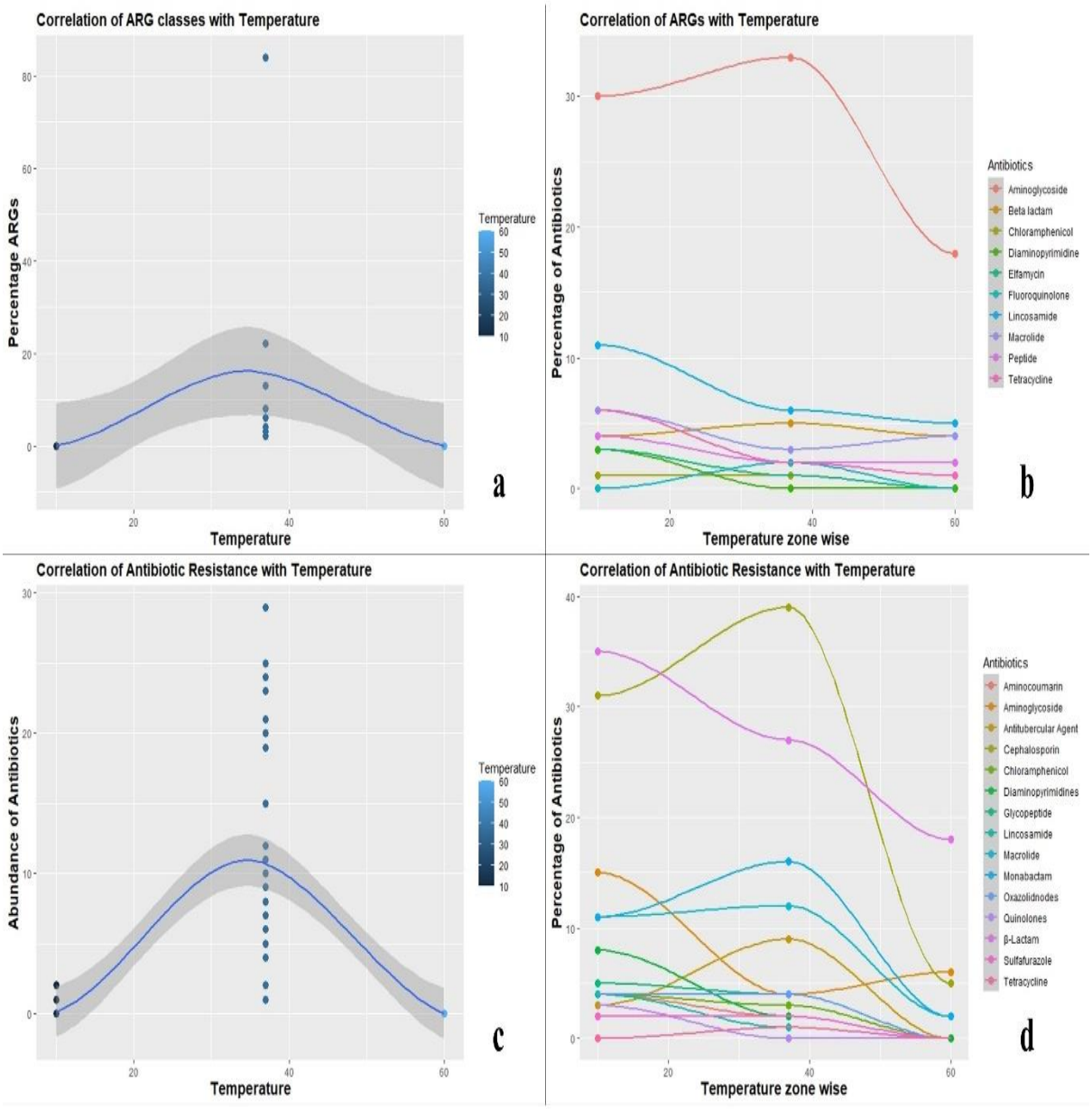


Fig. 36a, b, c, d. a-Correlation of Antibiotic-Resistant Gene (ARG) classes (extracted through metagenomics) with temperature; **b-** Correlation of Antibiotic-Resistant Gene (ARG) classes (extracted through metagenomics) zone-wise with temperature; **c-** Correlation of Antibiotic-Resistant classes (detected through culture-dependent analysis) with temperature; **d-** Correlation of Antibiotic-Resistant classes (detected through culture-dependent analysis) zone wise with temperature.

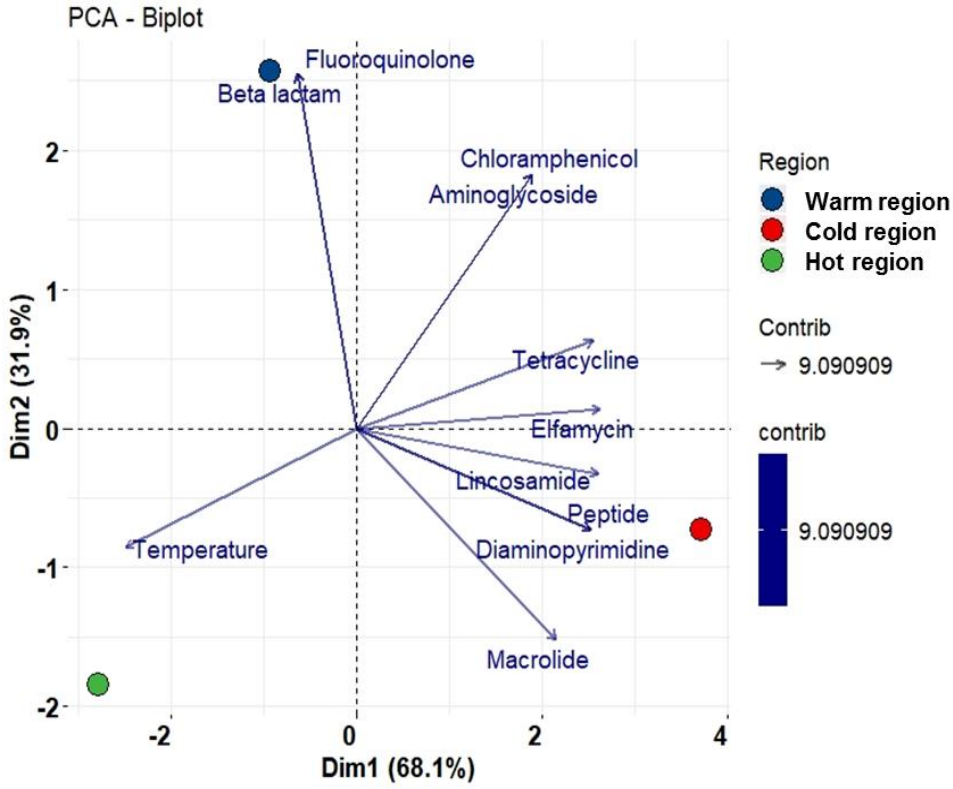


Fig. 37. Principal Component Analysis showing the correlation of various antibiotics in a specific thermal zone with temperature.

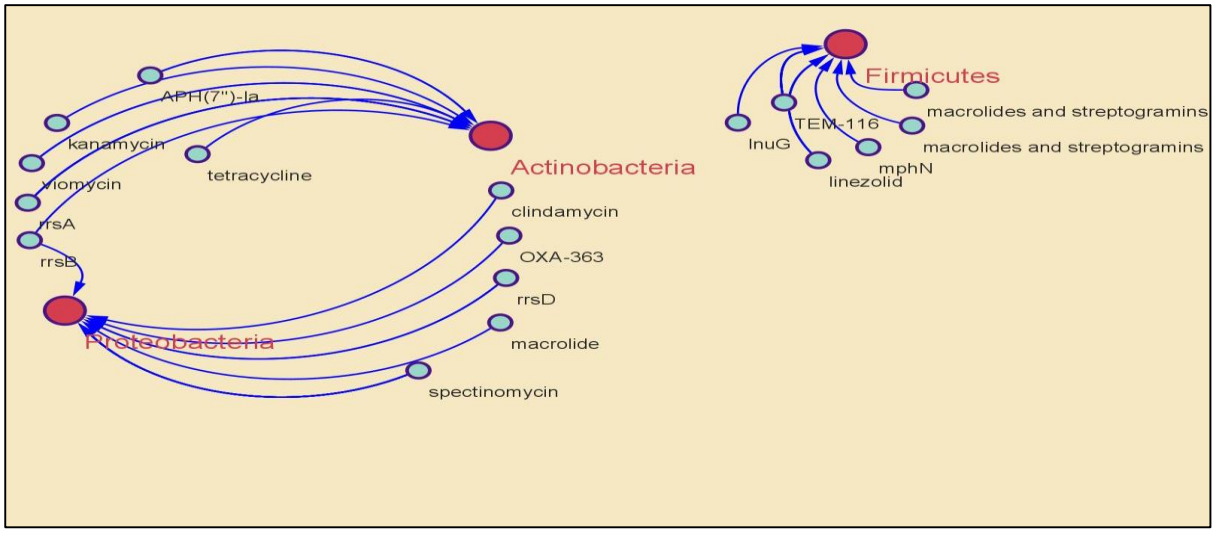


Fig. 38a. Network analysis showing correlation of ARGs with major phyla extracted through metagenomic of the hot region.

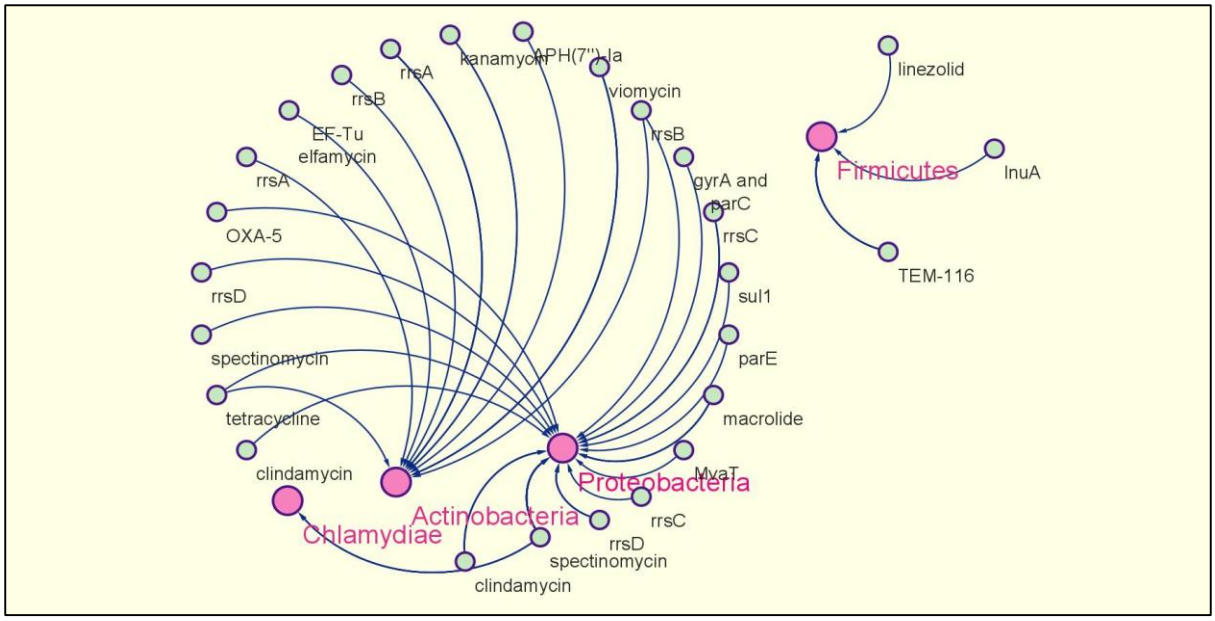


Fig. 38b. Network analysis showing correlation of ARGs with major phyla extracted through metagenomic of the warm region.

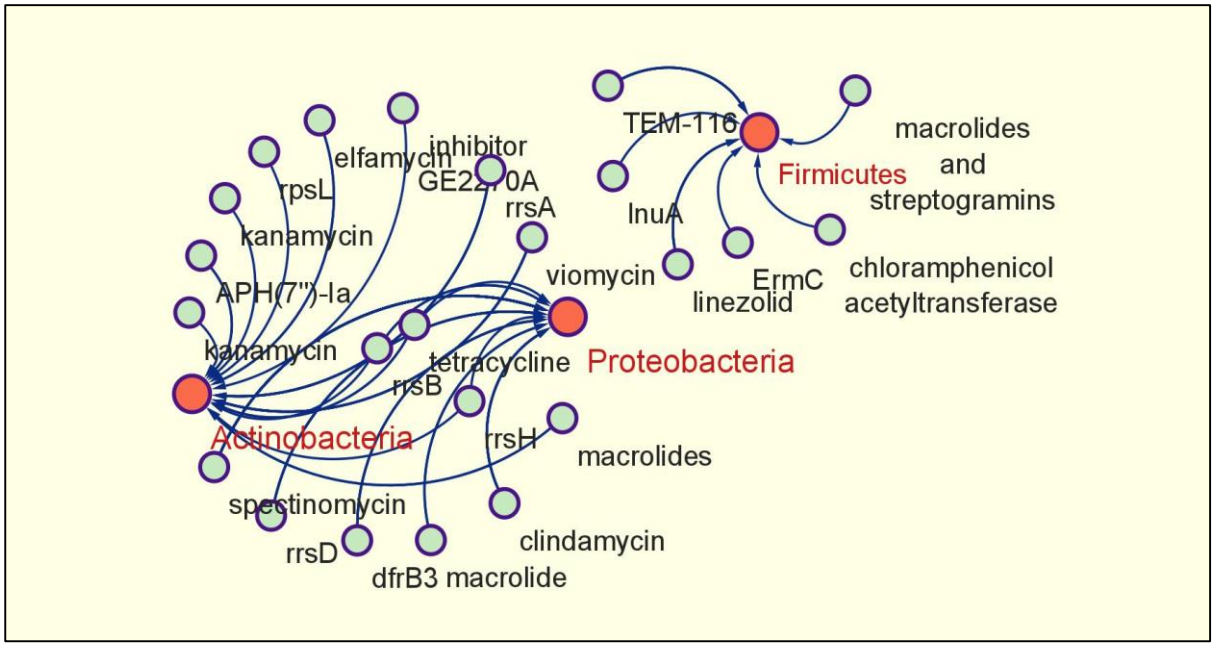


Fig. 38c. Network analysis showing correlation of ARGs with major phyla extracted through metagenomic of the cold region.

5.4.5. Detection of heavy metal resistance genes and gyrase genes through metagenomic

In the hot region, the most prevalent heavy metal resistance gene was for copper (37.16 %), followed by cadmium (27.1 %), nickel (16.80 %), arsenic (10.65 %), lead (7.34 %), and mercury (0.95 %). In the warm region, the dominant heavy metal genes were for copper (36.95 %), cadmium (37.11 %), nickel (15.55 %), arsenic (10.65 %), lead (4.77 %), mercury (0.65 %), and chromium (0.33 %). On the other hand, in the cold region, the dominant heavy metal genes were for copper (37.92 %), cadmium (33.45 %), arsenic (12.88 %), nickel (11.02 %), lead (3.72 %), mercury (0.5 %), and chromium (0.5 %). **Fig. 39a, b, c, d, e, f** illustrates the spatial distribution of genes associated with heavy metal resistance, including arsenic, cadmium, chromium, copper, nickel, lead, and mercury, across distinct gradient thermal regions.

Interestingly, the abundance pattern of these genes in the gradient regions is influenced by temperature. With increasing temperature, the abundance of nickel, lead, and mercury resistance genes also increased, showing a positive correlation. Conversely, as the temperature decreases, the abundance of cadmium, arsenic, and chromium resistance genes increases. However, the abundance of copper resistance genes appears to remain relatively consistent across all thermal regions **Fig. 40**.

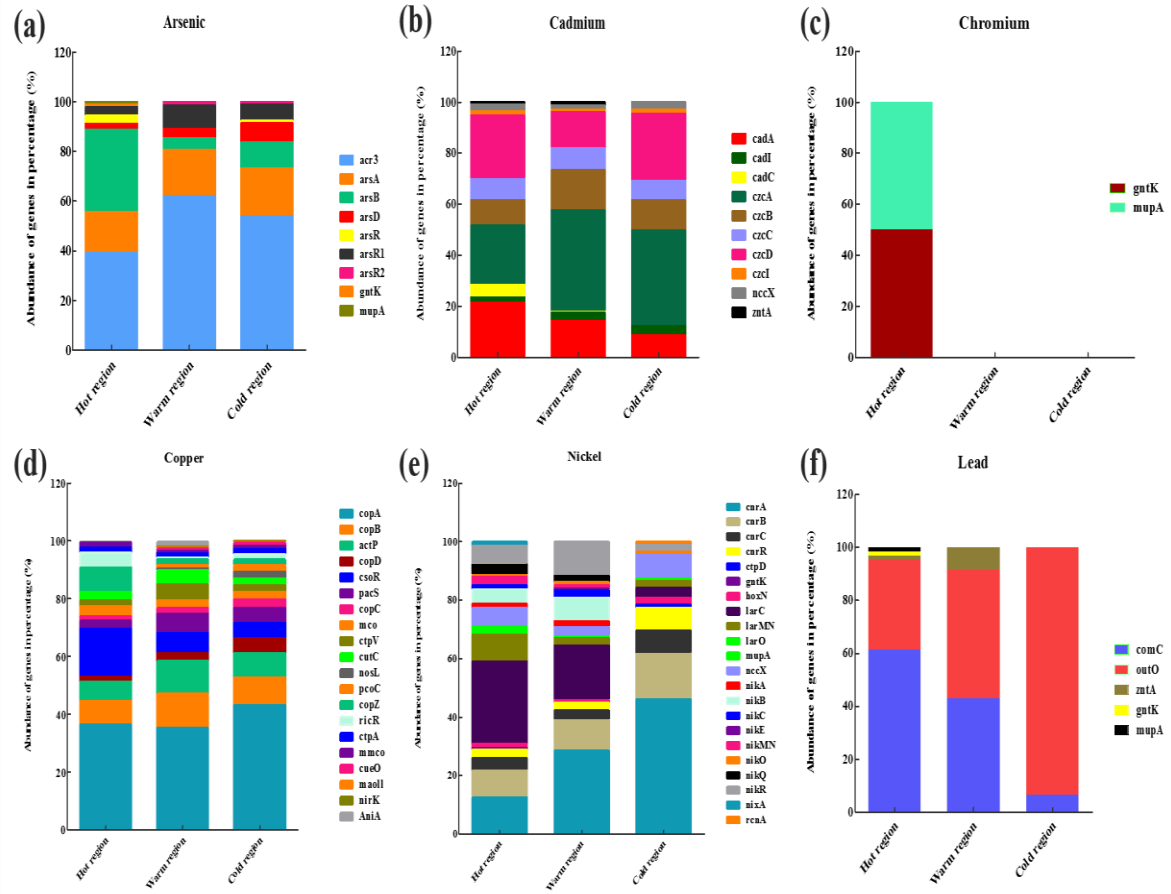


Fig. 39a, b, c, d, e, f a- Spatial Distribution of Arsenic-Resistant Genes across Thermal Gradient Zones. **b**- Spatial Distribution of Cadmium-Resistant Genes across Thermal Gradient Zones. **c**- Spatial Distribution of Chromium-Resistant Genes across Thermal Gradient Zones. **d**- Spatial Distribution of Copper-Resistant Genes across Thermal Gradient Zones**. e**- Spatial Distribution of Nickel-Resistant Genes across Thermal Gradient Zones**. f**- Spatial Distribution of Lead-Resistant Genes across Thermal Gradient Zones.

The prevalence of gyrase genes varied across different regions, with the warm region exhibiting the highest dominance at 46.39 %. Following that, the hot region dominated 28.79 %, while the cold region had the lowest dominance at 24.82 %. Among the gyrase genes, *gyrA* was the most dominant, followed by *gyrB*, *yacG*, *sbmC*, *gyrBR*, and reverse gyrase (*rgy*). Particularly, *gyrBR* was the only gene present in the warm region, whereas only one gene

encoding reverse gyrase was found in the hot region. **Fig. 39h** illustrates the spatial distribution of genes associated with gyrase and reverse gyrase, across distinct gradient thermal regions.

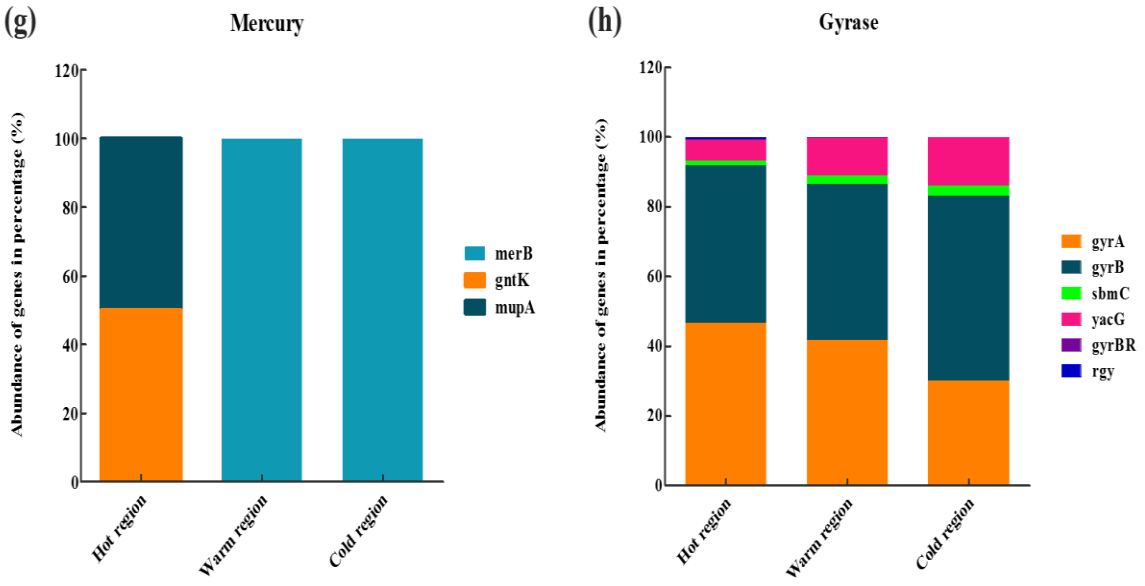


Fig. 39 g- Spatial Distribution of Mercury-Resistant Genes across Thermal Gradient Zones. **h**- Spatial Distribution of Gyrase and Reverse Gyrase Genes across Distinct Thermal Gradient Zones

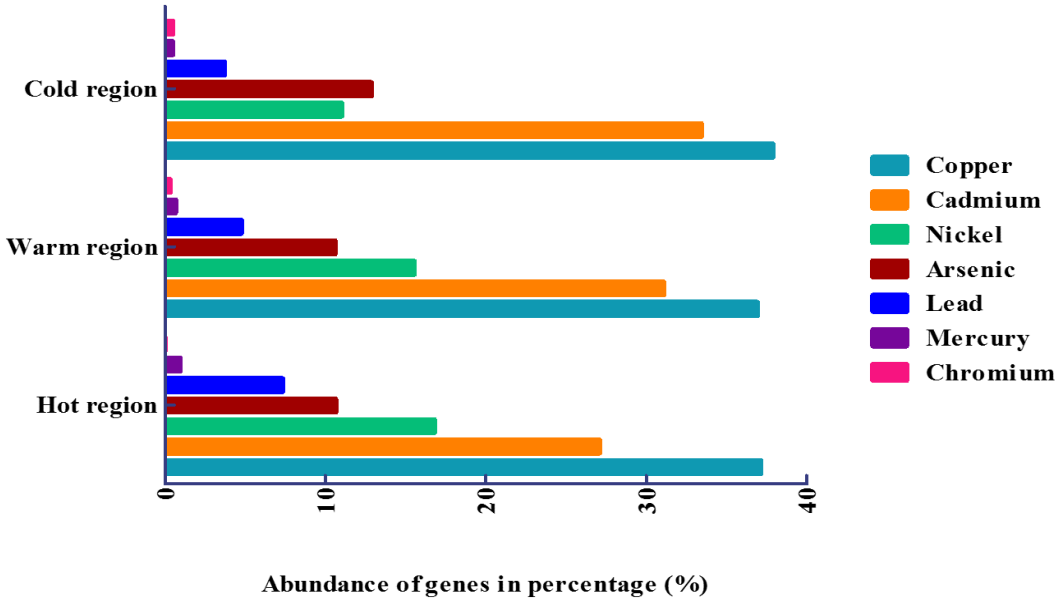


Fig. 40 Displays the abundance of Heavy Metals at distinct thermal gradient zones.

5.4.6. Detection of Virulence Genes

The abundance of virulence genes detected in soil samples suggests the possible existence of pathogenic or virulent organisms within the soil. Virulence genes are responsible for encoding factors that enable microorganisms to initiate infections and induce diseases in their hosts. The presence of these virulence genes indicates the potential presence of bacteria that can colonize and infect hosts. It is essential to emphasize that the mere presence of these genes does not serve as conclusive evidence for the existence of pathogens or disease-causing organisms. In the present study, we checked virulence genes with the help of KBase platform.

We found the virulence genes presence is associated with a temperature-dependent pattern in both sampling sites. As the temperature decreases the virulence genes increase, this indicates the lower temperate has more pathogenic bacteria as compared to the other region. The result revealed that the cold region had the highest occurrences of virulence genes, accounting for 52.94 % of the total. Following the warm region, representing 44.12 % of the total genes as shown in **Fig. 41**. On the other hand, the hot region exhibited the lowest numbers of genes, accounting for 2.94 % of the total occurrences as shown in **Table 18**. Three bacteria, namely *Pseudomonas aeruginosa*, *Brucella melitensis*, and *Legionella pneumophila*, possessed all the genes. *Pseudomonas aeruginosa* exhibited the highest abundance of genes, followed by *Brucella melitensis* and *Legionella pneumophila*, as shown in **Table 19** and illustrated in **Fig. 42**.

Next, we perform heat map analysis to establish correlations between virulence genes and distinct thermal regions. The results reveal significant variations in gene presence within each thermal zone, suggesting that temperature largely influences their abundance. Specifically, we observed higher levels of virulence genes in the warm region, whereas the hot region showed the lowest levels. Only a limited number of genes were found to be common

between these thermal regions. Some genes were shared between the warm and cold regions, while a single gene (*hsiB1/vipA*) was identified as common between the warm and hot regions as depicted in **Fig. 43**.

Table 18 Distribution of virulence gene at distinct thermal gradient region.

Sampling zone	Percentage of Virulence gene
Hot region	2.94
Warm region	44.11
Cold region	52.941

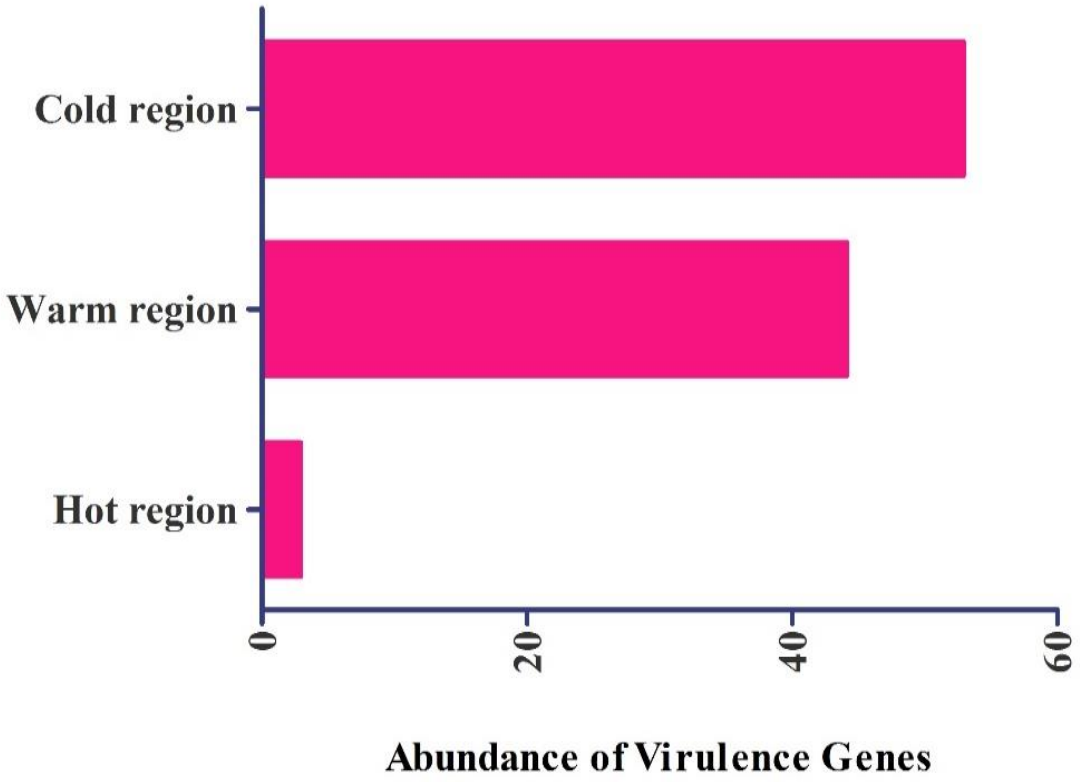


Fig. 41 Displays the abundance of virulence genes at distinct thermal zones.

Table 19 The table demonstrates that various thermal zones contain all the specific bacteria's virulence genes.

Bacteria	Abundance of Genes (%)
Pseudomonas aeruginosa PAO1	85.29412
Brucella melitensis 1 str. 16M	11.76471
Legionella pneumophila subsp. pneumophila str. Philadelphia 1	2.941176

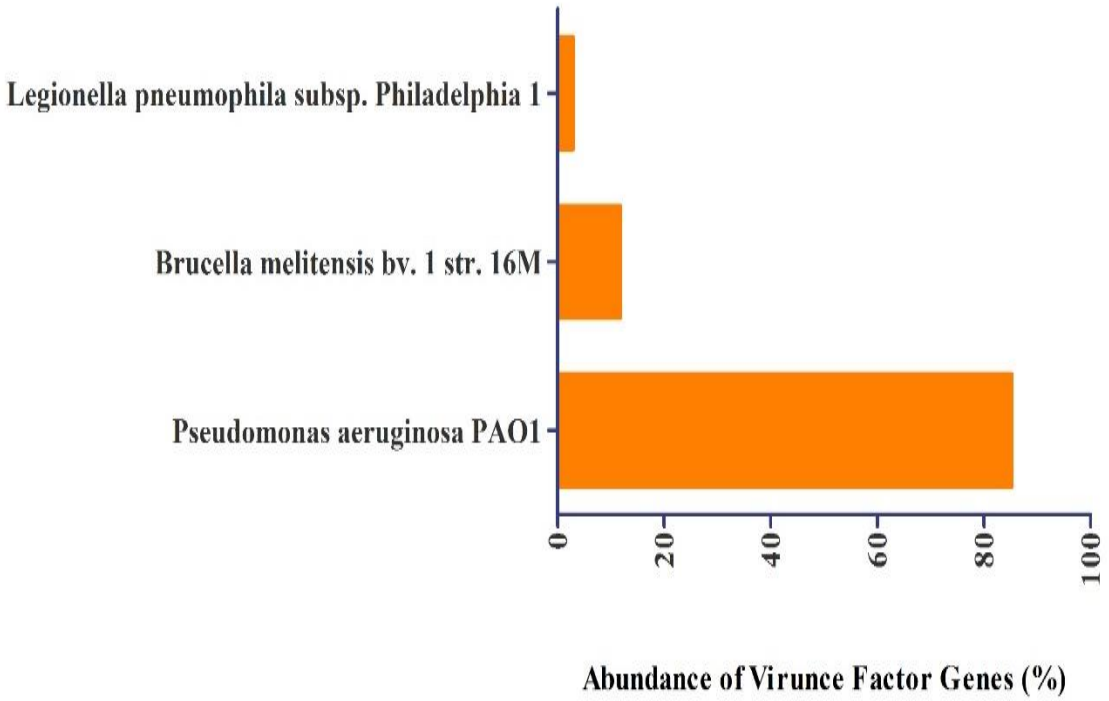


Fig. 42 Displays the virulence genes along with their corresponding bacteria.

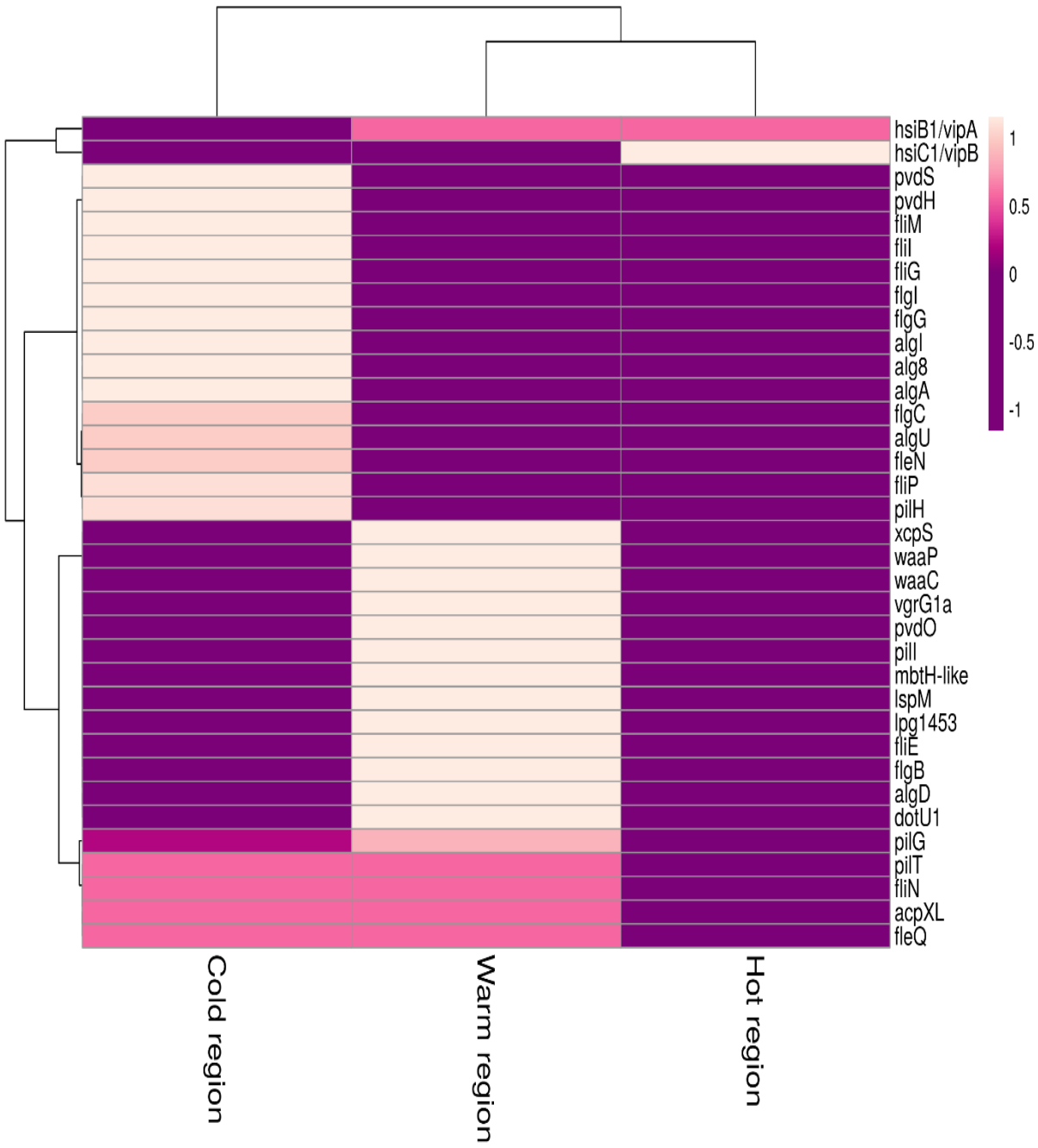


Fig. 43 Displays the heat map of virulence genes, shared between distinct thermal regions.

Discussion

Chapter 6

The discovery of extreme environments around the world has augmented the extent of exploring the limitations in life. The word “extremes” has been classified into physical extremes (e.g., temperature, radiation, or pressure) and geochemical extremes (e.g., desiccation, salinity, pH, oxygen species, or redox potential) (Rothschild and Mancinelli, 2001). The major domain that spans these extreme environments is usually archaea. But due limited studies on the archaea and successful isolation of pure culture has been the major drawback on understanding their mechanisms to survive at such harsh conditions. Thus, immediate domain that follows it, is prokaryotes. They can be hyper-thermophiles, thermophiles, thermo-tolerant, psychrophiles and psychro-tolerant depending on their optimal growth temperature requirements. They possess distinct adaptive features or genotypic attributes to survive in such extreme environments. Psychrophiles growing at lower temperatures has increased percentage of unsaturated membrane lipids, and a decreased percentage of branched chain lipids on comparison to mesophiles and thermophiles (Nedwell, 1999). At low temperature, cold shock proteins (csp) help the psychrophiles to adapt and survive in the cold conditions (Russell, 1990; Kuhn, 2012; Koh *et al.*, 2017; Singh, 2022). Similarly, there are various adaptations by thermophilic microbes also in order to survive at the higher temperatures. There are changes in the protein folding such as the presence of higher number of disulfide bonds than the mesophiles which supports the protein stabilization (Mallick *et al.*, 2002). Also, the proteins of thermophiles form super complexes and the number of heat shock proteins (hsp) increases in these complexes as temperature increases (Farhoud *et al.*, 2005; Wang *et al.*, 2015). In contrast to mesophiles, which contain ester lipids, the membranes of all the hyper-thermophiles contain ether lipids, unveiling an extraordinary resistance against hydrolysis at high temperature and acidic pH (Stetter, 1999). Thus, there are a large number of distinct adaptations among both psychrophiles and thermophiles with respect to their cellular components like proteins, nucleic acids and membranes. Moreover, there are

also adaptation in extremophiles related to their nutritional requirements and affinity for substrates. For instance, in case of thermophiles residing in many extreme hot ecosystems, they usually exhibit a chemolitho-autotrophic mode of nutrition with CO_2 as a sole carbon source (Stetter, 1999). However, psychrophiles possess a wide variety of photosynthetic, heterotrophic, and autotrophic nutritional abilities (Scherer and Neuhaus, 2006). It has also been known that uptake affinity for substrates usually decreases with decrease in temperature (Moran *et al.*, 2022).

Generally, it has been hypothesized that macro-climate (large scale factors) has distinct effects on the biodiversity than that of the micro-climate (local factors, resource availability etc.) (Cockell *et al.*, 2015; Bässler *et al.*, 2016). However, the effect of different micro-climates (within the macro-climate) on bacterial diversity with respect to high temperature gradients has been very less studied. The present study was aimed to evaluate the bacterial diversity changes within the three micro-environments with extreme temperature distribution such as from cold to warm to hot environment or vice versa. The sampling area chosen for this study was a high altitude Indian Himalayan Geothermal Belt that in its geological topographical settings has three distinct micro-environments co-habiting each other in the same locale. Thus, the study area provided three distinct micro-environment regions studied presently, viz., hot spring (hot region, 60 °C), dissipation areas i.e., the area between the geothermal zone and river basin (warm region, 37 °C) and the river basin (cold region, 10 °C) depicted in **Fig. 1**.

Here, mainly three questions have been discussed - a) which microbes reside in these environments, how many are distributed in these regions and how are their community population increasing or decreasing with respect to abiotic stress factors b) which is the major physicochemical variable affecting the bacterial diversity in such micro-environments, and c) how antibiotic resistance is affected, contributed and evolved in these micro-environments with respect to temperature.

The culture dependent studies suggested the presence of more diverse species at warm region followed by cold region and hot region as shown in **Fig. 12**. Thus, these temperature-dependent bacteria contribute to the bacterial abundance and diversity according to their specific thermal regions that supports their optimal growth incubation. The hot springs contained the greatest number of cultivated Gram-positive bacteria, similar to Najar *et al.*, (2018a) and Das *et al.*, (2021). By using partial 16s rRNA gene sequencing, 63 representative bacterial isolates were purportedly identified. Among them 27 isolates were thermophiles (*Bacillus*, *Geobacillus*, *Anoxybacillus* and *Aeribacillus* species); 19 isolates were mesophiles (*Bacillus*, *Cronobacter*, *Exiguobacterium*, *Lysinibacillus*, *Citrobacter*, *Brevundimonas*, and *Staphylococcus*) and 17 isolates were psychrophiles (*Exiguobacterium*, *Janthinobacterium*, *Aeromonas*, *Arthrobacter*, and *Acinetobacter*). The discovered microorganisms from each zone matched the temperature variations in those regions. The existence of three phyla —Bacillota, Pseudomonadota, and Actinomycetota—across several temperature gradient zones, including hot, warm, and cold, was demonstrated by the findings of culture-dependent phylum level diversity. Bacillota, one of these phyla, had the highest abundance and were found in all temperate zones. Pseudomonadota, on the other hand, were found in both warm and cold zones. Surprisingly, among all phyla, the cold zone had the most diversity.

Notably, when temperatures fluctuated, the phylum abundances followed separate patterns. Bacillota had a progressive decline in dominance as the temperature dropped. The fact that this pattern persisted in every place suggests that Bacillota prefer hotter conditions and that when the temperature drops, so do their populations. Pseudomonadota, on the other hand, showed the reverse tendency, with their numbers rising as temperatures dropped. This shows that Pseudomonadotal populations increase and flourish in cooler settings. However, Actinomycetota were only discovered in the cold zones, showing that they favor lower temperatures and had a restricted distribution to this particular habitat.

The variety of bacteria at the genus level showed that different types of bacteria were present in various temperature zones. Following the cold and hot zones in terms of bacterial diversity was the warm region. The two main bacterial species in the warm zone were *Anoxybacillus* and *Bacillus*. The *Bacillus* group of bacteria were the most numerous in the warm zone, whereas *Exiguobacterium* dominated in the cold. This pattern of bacterial diversity at the genus level also shown a relationship with temperature variations. The cold zone has the highest concentration of *Exiguobacterium* bacteria, followed by the warm region. *Exiguobacterium* bacteria, on the other hand, were less prevalent as the temperature increased, demonstrating the impact of temperature on this species.

In the present study, the culture-independent bacterial diversity has shown a distinct pattern among each micro-environment at both phylum and genus levels. It was found that the bacterial diversity is higher in the warm region than those of the hot and cold regions **(Table 3)**. Also, different phyla showed increasing and decreasing patterns of abundance during the thermal transitional phases. Pseudomonadota and Bacteroidota displayed a linear increase in abundance from hot region to cold region. Similarly, Bacillota and Chloroflexota displayed a linear decrease in abundance from hot to cold region. Although, the warm region possessed higher bacterial diversity but only few phyla contributed to the bacterial abundance. On the other hand, there is an increase of certain phyla in both the hot and cold regions and thus may have similar alpha diversity and Shannon indices as shown already. Similarly, culture-dependent studies showed the dominance of Bacillota at hot region whereas the dominance of Pseudomonadota in cold region followed by warm region as shown in **Fig. 11**. This may be due to the fact that the increment of the bacterial diversity and population in these regions are temperature dependent. For instance, in hot region the abundance of Bacillota, Chloroflexota, Deinococcota increased and contributed to the bacterial communities. Similarly, Bacteroidota

and Cyanobacteriota tends to increase and contribute towards the abundance of bacterial diversity in the cold region.

This trend was also observed in the bacterial genus level diversity. At genus level, temperature- dependent bacteria have been detected in their respective thermal regions. It has been found that thermophilic genera such as *Geobacillus*, *Meiothermus*, *Deinococcus*, *Thermus*, *Bacillus*, *Anoxybacillus* etc. mainly resides in hot region. Similarly, cold region is dominant with psychrophilic genera such as *Bacteroides*, *Flavobacterium*, *Nitrosomonas*, *Burkholderia*, *Acidovorax*, *Comamonas*, *Pseudomonas*, and *Yersinia* etc.

Different studies have also shown that mainly thermophilic bacteria dominate in hot conditions and psychrophilic bacteria tend to evolve under cold conditions. Studies conducted on the thermal hot springs located in the Tibetan Plateau of Northwest China has made predictions regarding the distribution of various thermophilic phyla (Wang *et al.*, 2013). It is anticipated that the phylum Aquificota (Aquificae) will be prevalent at higher temperatures, while the phyla Deinococcota, Cyanobacteriota (Cyanobacteriota), and Chloroflexota are expected to exhibit higher abundances at temperatures slightly lower but still within the hot range (Wang *et al.*, 2013). A similar study done on streamers, found the dominance of *Aquificae*, *Thermodesulfobacteria*, and *Thermos* at higher temperatures (>60 °C) and the dominance of Synechococcus, *Chloroflexi*, *Rhodothermus* and *Armatimonadota* at relatively lower temperatures (Nakagawa and Fukui, 2002). A study done on the sub-arctic glacier fore-fields of Styggedalsbreen (Norway) showed that at lower temperatures (2-20 °C) the abundant bacteria were psychrophiles such as *Methylibium*, *Polaromonas* and *Janthinobacterium* (Mateos-Rivera *et al.*, 2016).

Besides these results, we have found community mutualism of certain bacteria between two different thermic regions such as warm and cold region. This community mutualism of bacteria between these thermal regions were found in both culture-dependent and culture-

independent approaches. However, culture-dependent results represented a clear picture about this increase in diverse bacteria among these gradient thermal regions. It has been shown that only the thermophilic bacteria solely thrives in hot regions, while there is an absence of mesophilic or psychrophilic bacteria in these environments. The reason for this phenomenon might be attributed to higher temperatures, which result in a decrease in non-thermophilic organisms. Only specialized cells equipped with adaptive phylogenetic traits are capable of surviving and enduring such challenging environmental conditions. Also, the higher temperature causes bacteria to form filaments or abnormal cells and mainly acts on the synthesis of the wall and the septation (Thanbichler *et al.*, 2005; Egan and Vollmer, 2013).

Psychrophiles were also unable to grow in hot region, which signifies the temperature specificity of these psychrophilic bacteria. On the other hand, there was presence of thermophilic bacteria in warm region. Similarly, the presence of mesophilic bacteria into cold region. The most probable origin or source of these thermophilic and mesophilic bacteria residing in the warm region is hot springs as there is a constant distribution of hot water via the fissures and mixing with the groundwater of the river basin. Moreover, as the hot springs are located in slightly elevated areas as compared to the mean river basin level, the outlet of the hot spring water flows from the hot springs towards the river basin. This intermediate path or the distance between the hot springs and river basin provides an ideal mesophilic growth incubator for the mesophiles to thrive upon. Thus, it increases the chances of getting these thermophilic and mesophilic bacteria in cold region. Similar, results have been found by Podar *et al.*, (2020), as they have isolated numerous thermophilic and hyperthermophilic archaea and bacteria in the lagoon (e.g., Pyrobaculum, Aquificae, Thermi) (Podar *et al.*, 2020). They have discussed it by inferring that the lagoon receives a constant stream of high temperature hot spring water, thus the possibility of getting such thermophilic bacteria was higher in the lagoon. However, there arises a question that how these thermophilic or mesophilic bacteria survive in

the cold environment. The possible reason may be the production of spores by these thermophilic and mesophilic bacteria in these low temperature environments (Corwin, 2002). Moreover, the biomass productivity in the cold areas is less and more competitive. By performing the high-temperature (50 °C) incubation tests, thermophilic endospore-forming bacteria have been found in cold marine sediments (Hubert *et al.*, 2009; DeRezende *et al.*, 2013; Müller *et el.*, 2014; Volpi *et al.*, 2017; Bell *et al.*, 2018; Chakraborty *et al.*, 2018; Cramm *et al.*, 2019; Hanson *et al.*, 2019). Also, according to Kormas *et al.*, (2003), the thermophile-containing samples from hot settings can stay dormant for extended periods of time at low temperatures without losing viability (Kormas *et al.*, 2003).

In comparison to bacteria, fungi have a greater level of morphological complexity but share similar physiological characteristics (Xia *et al.*, 2020). Many fungi require aerobic conditions and rely on organic matter as their source of carbon and energy for growth and reproduction (Xia *et al.*, 2020). As a result, competition within the fungal community can be more intense. Fungi have the ability to obtain and distribute nutrients over long distances through hyphal elongation and branching, particularly through larger pores (Otten *et al.*, 2001; Cairney, 2005; Guhr *et al.*, 2015). Certain fungi are well-suited to extreme temperatures, as found in thermal vents, while others exhibit more temperate thermal ranges. Temperature stands as a significant determinant of fungal growth and metabolism, as most species manifest an optimum temperature spectrum for their development and reproductive activities. In colder temperatures, fungal growth and metabolism decelerate, possibly leading to a dormant state (Mensah-Attipoe and Toyinbo, 2019). Consequently, techniques like refrigeration are efficacious for food preservation, retarding fungal growth and prolonging shelf life to a certain extent. Conversely, heightened temperatures can stimulate fungal growth and metabolism; however, excessive heat (Maheshwari *et al.*, 2000) can induce cellular harm and demise. Certain fungi possess the capacity to adapt to temperature shifts by modulating their genetic

expression or physiological functions. For instance, when exposed to cold, specific fungi yield cold-shock proteins that safeguard cell membranes, while others produce heat-shock proteins under elevated temperatures, thereby shielding cells from heat stress (Bakar *et al.*, 2020).

All soil samples collected across distinct thermal zones contained two prominent fungal phyla, Ascomycota and Basidiomycota. Ascomycota, recognized by the presence of asci and ascospores, and Basidiomycota, typified by septate hyphae and basidium-produced spores, play pivotal roles in soil ecosystem vitality (Frąc *et al.*, 2018). Their capacity to degrade organic matter into simpler compounds facilitates the carbon cycle. Both Ascomycota and Basidiomycota are experts at breaking down plant material, particularly lignin found in woody substances (Purohit *et al.*, 2018). Furthermore, they engage in mutually beneficial relationships with plants, forging symbiotic ties with far-reaching ecological ramifications. Their ubiquity across soil samples attests to their resilience and adaptability in diverse thermal contexts. Fungi prevailing in high-temperature environs, like thermophilic habitats, have evolved distinctive adaptations to ensure survival (Newsham *et al.*, 2016). They boast heat-resistant enzymes, proficient nutrient absorption mechanisms, and safeguards against temperature-induced cellular impairments. Their stable enzymes proficiently degrade complex organic matter for sustenance, alongside protective attributes such as heat shock proteins that preserve proper protein folding and deter cellular harm. Competition from other organisms often dwindles in high-temperature settings, affording thermophilic fungi a competitive edge. Variations in competitive prowess are observed among species; some exhibit traits that confer advantages, facilitating the ousting of rivals and establishment in distinct niches or habitats (Lyu and Alexander, 2022).

In a study done by Liu *et al.*, (2018) the fungal communities residing in Rehai hot springs were classified into five phyla and 67 orders. Notably, the Ascomycota phylum was the dominant group, closely followed by Basidiomycota. However, Chytridiomycota,

Glomeromycota, and Zygomycota constituted only a small portion of the total communities (Liu *et al.*, 2018).

The highest fungal diversity surfaced in thermophilic zones, encompassing 36.83 % Ascomycota and 8.52 % Basidiomycota. This trend aligns with a recent metagenomic investigation by Das *et al.*, (2021) in North Sikkim, India, where analogous patterns were detected in hot springs. These locales were predominantly populated by Ascomycota genera (Das *et al.*, 2021). Similar trends were observed by Huang *et al.*, (2023), Liu *et al.*, (2023), Morgenstern *et al.*, (2012), and Powell *et al.*, (2012), highlighting Ascomycota and Basidiomycota as prevalent phyla (Morgenstern *et al.*, 2012; Powell *et al.*, 2012; Huang *et al.*, 2023; Liu *et al.*, 2023). A study by Salano *et al.*, (2017) in Kenya also noted Ascomycota and Basidiomycota as dominant in hot spring fungal communities, suggesting their adaptation to high temperatures (Salano *et al.*, 2017). The consistent presence of Ascomycota and Basidiomycota in thermal environments suggests their potential heat tolerance or thermophilic nature. The findings also reveal a significant pattern at the phylum level: as temperatures transition from hot to cold through the intermediate warm range, there is a conspicuous decline in the fungal population. This observation suggests that temperature also plays a significant role in shaping fungal diversity, akin to its influence on bacterial diversity.

The examination of fungal diversity was expanded to the genus level in various thermal zones, revealing a broad spectrum of prevalent and diverse genera. *Ricinus*, *Hydra*, *Homo*, and *Neosartorya* emerged as the most frequently encountered genera across all these zones. In the hot region, dominance was observed with *Neosartorya*, *Aspergillus*, *Malassezia*, *Gibberella*, and *Penicillium*. Meanwhile, the warm region exhibited a rich diversity featuring *Gibberella*, *Neosartorya*, *Schizosaccharomyces*, *Aspergillus*, and *Ustilago*. Whereas in the cold region, the prevalent genera included *Aspergillus*, *Gibberella*, *Magnaporthe*, *Neosartorya*, and *Yarrowia*. Our examination of metagenomic data, which underscores the presence of fungal genera,

reveals variations among thermal zones. This finding aligns with prior studies that have established a connection between temperature and disparities in microbial communities. Notably, hot springs are home to a wide array of fungal communities, with certain genera standing out prominently. Kambura *et al.*, (2016) and Salano *et al.*, (2017) identified predominant genera such as *Penicillium*, *Cladosporium*, *Aspergillus*, and others in these environments (Anne *et al.*, 2016; Salano *et al.*, 2017). Similarly, Li *et al.*, (2012) found *Penicillium*, *Aspergillus*, *Davidiella*, and *Fusarium* in Tibetan Plateau geothermal regions (Li *et al.*, 2012). These results highlight the abundant presence of specific fungal genera within extreme environments, underscoring the diversity of fungal life in such conditions. The presence of *Aspergillus*, *Gibberella*, and *Neosartorya* across all zones suggests their adaptability to varying thermal conditions.

In our study, we observed significant variations in the dominant fungal species found within different temperate regions. In the high-temperature zone, *Neosartorya fumigata* was the prominent species, whereas in both the warm and cold regions, the dominance was shared between *Gibberella zeae* and *Neosartorya fumigata*. This finding aligns with other studies highlighting temperature-driven distribution patterns. In a research conducted by Newsham and colleagues in 2016, they investigated soil fungal diversity by examining 22 predictor variables. Their findings highlighted the significant role of surface air temperature in influencing the composition and diversity of fungal communities in maritime Antarctic soil (Newsham *et al.*, 2016). The study revealed that higher temperatures played a crucial role in fostering fungal diversity in warmer habitats. This effect was attributed to increased water accessibility, which, when coupled with elevated temperatures, led to heightened metabolic activity. Consequently, this extended the timeframe during which fungi remained active throughout the year and facilitated a transition from survival-oriented to growth and dispersal strategies.

This study emphasizes the utility of employing rarefaction curves and principal component analysis for the examination of species richness and fungal diversity in various environments. The findings indicated that the hotter region exhibited greater diversity as shown in Fig. 28, suggesting temperature's influence on fungal diversity. Temperature and pH have been recognized as crucial factors shaping microbial communities (Rousk *et al.*, 2010; Zhang *et al.*, 2016; Liu *et al.*, 2018), a notion supported by our findings. The correlation between temperature and fungal community emphasizes the intricate balance between organisms and their environment. The results also highlight PCA's role in dimensionality reduction while retaining data variation, enabling a specialized exploration of fungal diversity.

Aerobic bacteria depend on oxygen for their metabolic processes, which involve the oxidation of organic substances. In contrast, algae containing chlorophyll generate oxygen via photosynthesis. This oxygen production is advantageous for adjacent bacteria as it facilitates their ability to metabolize organic matter and ammonia through oxidation. Consequently, this mutualistic association between algae and bacteria fosters the proliferation of both species, contributes to ecosystem stability, and provides protection against the infiltration of detrimental bacteria (Anand *et al.*, 2023). Microbial community composition is widely acknowledged to be influenced by abiotic environmental factors like salinity, temperature, water availability, and pH levels. As environmental conditions become increasingly harsh, there is a noticeable decrease in both the diversity and metabolic activity of the microbial cells (Oren, 2001; Fierer and Jackson, 2006; Sharp *et al.*, 2014).

The study evaluated how various physicochemical factors, including temperature, pH levels, and elemental composition, influence bacterial diversity. The findings suggest that temperature exerts a notable influence on the microbial diversity within these specific habitats. Linear regression analysis uncovered a significant positive association between temperature and Bacillota and Chloroflexota, while an inverse correlation was detected with

Pseudomonadota and Bacteroidota. Multiple studies have also established a connection between temperature and bacterial diversity (Wang *et al.*, 2013; Li *et al.*, 2015; Mateos-Rivera *et al.*, 2016; Podar *et al.*, 2020; Ma *et al.*, 2022; Ruhl *et al.*, 2022) but for the first time ever, a wide thermal range has been introduced, creating distinct microenvironments.

A study by Podar *et al.*, (2020) has revealed that temperature had the most significant impact on bacterial diversity in Iceland's hot springs. It was found that temperature was inversely correlated with the number of detected taxa Amplicon sequence variant (ASVs) and phylogenetic diversity (with p-values of 0.006 and 0.000, respectively) (Podar *et al.*, 2020). In another study, it was found that the species richness and diversity indices were significantly correlated with temperature, with r2 values reaching up to 0.62 for neutral-alkaline springs (such as Humboldt's spa). The distributions showed a unimodal pattern, with the highest diversity observed at 24 °C and the lowest diversity at the two temperature extremes (Sharp *et al.*, 2014). Our results also indicate a similar trend, where warmer regions support greater bacterial communities with higher alpha diversity. Many research investigations have conclusively established that temperature stands as the predominant factor that shapes bacterial diversity (Cole *et al.*, 2013; Cuecas *et al.*, 2014; Sharp *et al.*, 2014; Power *et al.*, 2018). pH emerges as a notable factor associated with microbial diversity, as indicated by various research findings. Multiple studies have underscored the role of pH in shaping the diversity of bacterial communities (Rousk *et al.*, 2010; Sharp *et al.*, 2014; Power *et al.*, 2018). However, in the current study, we found a minimal correlation between pH and bacterial diversity. Nonetheless, there is a slight positive correlation observed between Pseudomonadota and pH. This limited correlation could be attributed to the small variation in pH ranges within the studied microenvironments, which were between pH 8.5-9.1, indicating an alkaline composition.

Several elements are also known to affect the microbial diversity in soils (Chu, 2018). Various studies have shown varying effects on microbial diversity due to different heavy

metals. One study found that heavy metals had a minor impact on bacterial diversity, whereas rare earth elements had a significant influence. These studies also revealed a decrease in richness and diversity in soils contaminated with pollutants, especially heavy metals, compared to uncontaminated soils (Luo *et al.*, 2021). In the present study we have also found negligible effects of various elements on bacterial diversity. However, Cl was showing positive and negative correlations with bacterial diversity. The reason for this anomalous behavior might be the similar concentration of elements among the three thermal microenvironments. Based on various elements the Piper plot analysis suggests that all the microenvironments have calcium-sulfate type soil.

Modern microbiology has embraced the notion that relying solely on pure cultures is insufficient. Consequently, contemporary microbiologists have undertaken the task of investigating and characterizing the vast array of uncultured microbial diversity. Various unique and isolated environments, including soil, hot springs, deep-sea vents, rivers, human gut, and the animal rumens have been thoroughly examined to unravel the intricacies of microbial diversity (Vakhlu *et al.*, 2012; Sushmitha *et al.*, 2020). Another significant challenge has been elucidating the functional attributes of both cultured and uncultured microbial diversity (Torsvik and Øvreås, 2002). To address these challenges, innovative techniques such as metagenomics have emerged, enabling microbiologists to cleverly employ phylogenetic and functional metagenomic approaches to uncover concealed, untapped, or previously uncultivable microbial diversity. One of the fundamental aspects of the functional metagenomic approach revolves around the discovery and understanding of genes associated with antibiotic and metal resistance, along with their respective functions

Antibiotic resistance is a term that describes the capacity of bacteria and other microorganisms to endure the impact of antibiotics (Sarkar *et al.*, 2023). Antibiotic resistance stands as a prominent and urgent public health concern, representing an escalating global

dilemma (Roca *et al.*, 2015). The culture-independent analysis has emerged as a pivotal, method for assessing the complete diversity and the abundance of antibiotic resistance genes (ARGs) and metal resistance genes (MRGs) across various environmental contexts. These contexts encompass soil (Knapp *et al.*, 2010), sediment (Yang *et al.*, 2016), water (Fernando *et al.*, 2016), and activated sludge (Peltier *et al.*, 2010).

Antibiotic resistance emerges when these pathogens adapt and acquire mechanisms that diminish the effectiveness of antibiotics, complicating the treatment of infections (Garvey, 2023). This resilience undermines the potency of antibiotics, making them less effective or entirely futile in addressing bacterial infections. Heavy metals were employed in the treatment of diseases long before the advent of antibiotics, spanning centuries in their historical medicinal usage (Medici *et al.*, 2015). Prior to the widespread proliferation of pharmaceuticals, reflective research indicated the existence of resistance genes within bacteria that did not produce antibiotics. It is plausible that the utilization of heavy metals for treating diseases, coupled with the prevalent presence of heavy metals in various ecosystems, may have favored the development of genes that confer resistance to both antibiotics, and heavy metals (Bartoloni *et al.*, 2004; Allen *et al.*, 2010). Antibiotic resistance factors have likely existed naturally and been capable of horizontal transfer well before the antibiotic era, possibly aiding the rapid emergence of resistance in pathogenic bacteria. These resistance genes spread primarily through vertical gene transfer during bacterial reproduction and horizontal gene transfer between bacterial cells using mobile elements.

It has been observed that both the fraction and the overall number of bacterial infections that exhibit multidrug resistance to antibacterial drugs have dramatically increased during the past decade (Colpan *et al.*, 2013). A key cause of antimicrobial resistance is the extensive use of antibiotics in animals and the food chain (Marshall and Levy, 2011). A recent research paper published in The Lancet has highlighted a concerning trend in India, where a significant

increase in antibiotic resistance, exceeding 50 %, has been documented for a range of antibiotics, including methicillin, cephalosporin, carbapenem, and fluoroquinolone (Murray *et al.*, 2022). Nonetheless, the Indian Himalayas and coastal regions boast numerous unique and ecologically pristine ecosystems that offer valuable opportunities for investigating the development and integration of antibiotic resistance. A prior investigation conducted on specific thermal springs in Sikkim, for instance, revealed the absence of antibiotic resistance in water samples (Najar *et al.*, 2020a; Najar *et al.*, 2022a). The primary objective of this study was to investigate the occurrence of antibiotic resistance within various thermal environments and to explore whether temperature influences the development of antibiotic resistance.

We employed both culture-dependent and culture-independent methods to investigate antibiotic resistance in these thermal ecosystems. The results revealed a notable absence of antibiotic resistance in cultivable thermophilic bacteria across all three thermal regions. In contrast, mesophilic bacteria exhibited the highest levels of antibiotic resistance in these areas, while psychrophiles displayed significantly lower antibiotic resistance. Likewise, within the metagenomes, antibiotic-resistant genes (ARGs) were associated with mesophilic organisms. There were no identified ARGs originating from thermophiles or psychrophiles. While antibiotic resistance was more prevalent in cold regions compared to warmer regions, it's worth noting that the majority of ARGs were attributed to mesophiles. These results are consistent with earlier studies in the field, which also reported a limited presence of Antibiotic Resistance Genes (ARGs) in metagenomic research. In most instances, these genetic elements displayed a significant similarity, surpassing the 97 % threshold, to Gram-negative and mesophilic bacteria, corroborating previous observations (Najar *et al.*, 2020a; Najar *et al.*, 2022a). Hence, we can deduce that mesophiles play a significant role in the emergence of antibiotic resistance within these untouched ecosystems. The observed antibiotic resistance patterns are likely a consequence of the temperature variations in the researched region. The results of regression

analysis indicate a negative association between temperature and antibiotic resistance, meaning that as temperature rises, antibiotic resistance tends to decrease.

Numerous research investigations have consistently revealed a correlation between elevated temperatures and the development of antibiotic resistance. For instance, research has demonstrated that warmer temperatures are associated with an escalation in the prevalence of antibiotic-resistant bacteria and genes (Pärnänen *et al.*, 2019) and relatively decrease at higher temperatures (Sun *et al.*, 2016; Dunivin and Shade, 2018; Jang *et al.*, 2018; Gong *et al.*, 2021; Najar *et al.*, 2022a). However, antibiotic resistance significantly increases under moderate (optimal, warm) temperature conditions (25–37 °C) (Sorn *et al.*, 2022). In a separate investigation, it was demonstrated that the fitness penalties in terms of growth rate were more pronounced at 32 °C and 42 °C when compared to their ideal growth temperature of 37 °C. This suggests that bacteria displaying resistance are less adept in competitive terms when exposed to new environmental conditions (Beceiro *et al.*, 2013) . The earlier metagenomic study provided clear evidence of the susceptibility of thermophiles, revealing that there were no instances of antibiotic resistance genes (ARGs) present in this particular category (Najar *et al.*, 2020a; Najar *et al.*, 2022a). Furthermore, there is a suggestion that these hot springs might not possess consistent antiseptic characteristics when subjected to high temperatures exceeding 60 °C. This is due to their unique ecosystems that experience less human intervention. These thermophilic microorganisms could potentially face less pressure or competition to acquire Antibiotic Resistance Genes (ARGs) due to the absence of antibiotics in their environment (Najar *et al.*, 2020a; Najar *et al.*, 2022a). Hence, it could be suggested that the exclusive presence of antibiotic resistance among mesophiles in these untarnished regions or microenvironments may be attributed to this factor. Consequently, these investigations support the hypothesis that antibiotic resistance is most prevalent within the range of normal temperatures (25–37 °C) and diminishes as temperatures rise above their optimum values.

The results obtained from the Minimum Inhibitory Concentration (MIC) assay shed light on the susceptibility of mesophilic bacterial isolates to various antibiotics. These findings indicate that these mesophilic bacteria exhibited a notable level of resistance when subjected to the antibiotics tested. In order to effectively inhibit the growth of these mesophilic bacteria, it was essential to employ higher antibiotic concentrations.

Specifically, three antibiotics; Ceftazidime, Erythromycin, and Streptomycin- stood out as being notably ineffective in controlling the growth of the mesophilic bacterial isolates. These antibiotics demonstrated a reduced ability to hinder the proliferation of the tested mesophilic bacteria, highlighting a concerning level of resistance that may have implications for the efficacy of these antibiotics in clinical or environmental settings.

Antibiotics possess the capacity to create robust coordination complexes with metal ions, leading to diverse outcomes that may influence the efficacy of these drugs (Božić Cvijan *et al.*, 2023). When an antibiotic resistance gene (ARGs) and a heavy metal resistance gene (HMRGs) are present on the same genetic element, bacterial resistance to antimicrobial agents can occur. This resistance can manifest even when only one of the factors, either the antibiotic or the heavy metal, is present (Pal *et al.*, 2015; Wales and Davies, 2015; Nguyen *et al.*, 2019). Consequently, the presence of heavy metals in the environment can induce antibiotic resistance in bacteria, even in the absence of antibiotics (Komijani *et al.*, 2022). Studies have identified co-selection plasmids that contain both ARG and HMRG (Pal *et al.*, 2017; Chen *et al.*, 2019; Nguyen *et al.*, 2019). Another mechanism for co-selection is cross-resistance, where a single mechanism, such as an efflux pump, confers resistance to multiple antimicrobial agents, including both antibiotics and heavy metals. Some studies have reported a correlation between the emergence of multi-drug resistant pathogens and resistance to several heavy metals (Nguyen *et al.*, 2019; Komijani *et al.*, 2022).

In this study, a positive correlation has been established between temperature and the presence of nickel, lead, and mercury genes, indicating that as temperature increases, the abundance of these genes also increases. Pavlic *et al.*, (2021) observed that low concentrations of nickel had minimal effects on bacterial adhesion and biofilm formation, but they might enhance bacterial resistance to antibiotics. Conversely, higher concentrations of nickel made bacteria more susceptible to antimicrobial treatments (Pavlic *et al.*, 2021). This finding suggests that hotter regions predominantly harbor more susceptible bacteria compared to warmer and colder regions.

Our findings demonstrated that as temperatures drop, cadmium, arsenic, and chromium genes become more prevalent. This is due to the adaptation of microbes to survive and resist heavy metals, whether they are present naturally in environments or as a result of anthropogenic activity. Certain genes produce proteins that alter or eliminate certain metals from cells, shielding bacteria from their harmful effects. It's interesting to note that the mechanisms of heavy metal resistance can also produce antibiotic resistance. Antibiotics can be pumped out by proteins involved in heavy metal detoxification, decreasing their efficacy, and antibiotics can be broken down by certain enzymes. Therefore, a larger potential for antibiotic resistance may also be caused by the higher number of these genes in colder conditions.

Copper (Cu) is widely present in the environment, with numerous sources of this heavy metal in environmental samples. This metal has long been associated with the development of heavy metal tolerance in the environmental microflora (Wales and Davies, 2015; Poole, 2017). Cu are consistently found together with antibiotic-resistant microbial populations in environmental samples (Knapp *et al.*, 2011; Becerra-Castro *et al.*, 2015). As the concentration of Cu increases, Cu-dependent antibiotic resistance genes (ARGs) have a higher likelihood of transferring through mobile genetic elements (MGEs), indicating that Cu-dependent ARGs are more mobile and accessible (Hu *et al.*, 2016). Interestingly, there have been intriguing findings

suggesting that the presence of Cu at low levels, such as sub-toxic concentrations, can positively impact specific ARGs (Knapp *et al.,* 2017). Copper has also been shown to bind to penicillin and promote the hydrolysis of this beta-lactam antibiotics (Longhi *et al.,* 2022). Copper can bind to various antibiotics, including aminoglycosides, tetracyclines, chloramphenicol, novobiocin, macrolides, isoniazid, quinolones, vancomycin, and a range of beta-lactams such as cephalosporins, penicillins, streptomycin, and neomycin (Poole, 2017).

Hyperthermophilic microorganisms rely on the presence of gyrase activity for their successful adaptation to the challenging and extreme environmental conditions (Dadwal *et al.*, 2021). While reverse gyrase is essential for the survival of hyperthermophilic bacteria and archaea in harsh environments (Pati *et al.*, 2023). Gyrase and topoisomerase IV represent two distinct variations of type II topoisomerases that can be located within different bacterial and archaeal species (Gibson *et al.*, 2018). Gyrase is a unique enzyme that can add negative supercoils to DNA by using ATP hydrolysis. Gyrase is created through the dimerization of a fusion protein composed of *gyrB* and *gyrA*. During transcription and replication processes, it primarily controls DNA supercoiling and reduces topological stress (Schmutz *et al.*, 2003). Significant differences had been found when our study compared the frequency of gyrase genes in various geographic areas. Gyrase genes were most prevalent in the warm region, then in the hot region with a moderate frequency, and least prevalent in the cold zone. *GyrA* was the most prevalent gyrase gene according to assessment, suggesting that it was present throughout the examined locations. Reverse gyrase (*rgy*), *gyrB*, *yacG*, *sbmC*, and *gyrBR* all shown variable levels of occurrence. It's interesting that *gyrBR* was only found in the warm region, indicating that this gene may have specialized or adapted to that climate.

After successfully conducting an extensive examination of microbial diversity, antibiotic resistance, metal resistance, gyrase, and reverse gyrase genes our investigation delved into the impact of temperature. Additionally, we explored the presence of virulence

genes as a means to establish a potential correlation between temperature and the presence of these genes. The results unveiled a promising connection between the existence of virulence genes within soil samples and the potential presence of pathogenic or virulent microorganisms in the soil ecosystem. Virulence genes, responsible for encoding factors that empower microorganisms to initiate infections and cause diseases in their respective hosts, underwent thorough analysis in this study. The outcomes illuminated a temperature-dependent pattern in the prevalence of these virulence genes. Specifically, as temperatures decreased, there was an observable increase in the frequency of virulence gene occurrences. This data highlighted significant disparities in the presence of these genes across different thermal zones, underscoring the pivotal role played by temperature in shaping their abundance. To elucidate further, the warm region exhibited notably higher levels of virulence genes, while the hot region exhibited the lowest levels among the studied thermal zones. Notably, three specific bacteria- *Pseudomonas aeruginosa*, *Brucella melitensis*, and *Legionella pneumophila* were found to possess all the virulence genes. Moreover, the commonality of these genes varied between thermal regions, with certain genes shared between the warm and cold regions, and a singular gene, *hsiB1*/*vipA*, identified as common between the warm and hot regions.

Summary

Chapter 7

Microbial diversity is a fascinating aspect of our planet, encompassing bacteria, archaea, and eukarya thriving in almost all the habitats of earth (Singh, 2017). These ecosystems are influenced by a range of abiotic factors, including temperature, pH, pressure, radiation, acidity, and salinity, all of which affects the diversity of microbial life forms (Reysenbach and Shock, 2002; Wang *et al.*, 2013). Among these abiotic factors, temperature and pH have been extensively studied in relation to microbial diversity within a particular temperate regions (Cole *et al.*, 2013; Cuecas *et al.*, 2014; Sharp *et al.*, 2014; Power *et al.*, 2018). The present study was conducted in the geothermal zone having three different thermal zones, i.e., hot, warm and cold to address the role of temperature in determination of microbial diversity and antibiotic resistance.

Hot springs have proven to be invaluable model systems for understanding how organisms adapt to varying environmental conditions, especially temperature gradients, which create diverse ecological niches (Ward *et al.*, 2012). Geothermal habitats offer a unique opportunity to explore microbial adaptation across fluctuating temperature ranges, leading to the development of distinct microbial communities, each suited to its optimal niche (Li *et al.*, 2015). Examining biological diversity patterns along temperature gradients is crucial for understanding how ecosystems respond to global climate change, as temperature fluctuations significantly impact microbial diversity, causing fluctuations in bacterial and archaeal populations. Research has shown that the high temperatures, as found in thermophilic environments, can lead to reduced microbial cell counts due to challenges posed by prolonged heat and desiccation, particularly for non-spore-forming thermophilic bacteria (Castenholz, 1969; Beblo *et al.*, 2009). Similarly, studies of temperature changes along sub-arctic glacier fore-fields have revealed that soil surface temperature and exposure duration significantly shape microbial community richness and composition (Mateos-Rivera *et al.*, 2016). Despite these insights, the influence of temperature gradients on microbial diversity remains

underexplored. A recent study by Podar *et al.*, (2020) examined the consequences of a temperature gradient ranging from warm (38 °C) to boiling and found a significant nonlinear reduction in microbial taxa as temperatures increased (Podar *et al.*, 2020). However, a comprehensive exploration of bacterial diversity within distinct temperature gradient zones is still needed. In this context, Bendia *et al.*, (2018) research in Sao Paulo, Deception Island, is noteworthy. Their culture-dependent approach identified both thermophilic and psychrophilic isolates across a wide range of thermal gradients, demonstrating the resilience of extremophilic bacteria even in unfavourable conditions (Bendia *et al.*, 2018). However, this study primarily focused on bacteria survival in thermophilic and psychrophilic environments only.

Studying microbial diversity across different thermal regions, such as hot springs and its surrounding area with cold conditions, offers valuable insights into the interplay between temperature gradients and microbial communities. Although temperature is widely acknowledged as a crucial factor impacting microbial diversity, there is an increasing demand for thorough research aimed at unravelling the intricate consequences of temperature fluctuations within diverse ecological habitats. Our study delves into the realm of microbial diversity within various thermal (temperate) regions, including hot, warm, and cold soil environments. We also hypothesize that antibiotic resistance among bacteria will vary according to temperature due to distinct fitness costs, contact rates, or horizontal gene transfer. The study area we have chosen, have three distinct thermal zones coexist in close proximity and thus provides a perfect environment to examine the microbial diversity and prevalence of antibiotic resistance with varying temperatures. The hot water oozing out from the hot spring represents the hot zone, and as this water flows towards the river, the temperature decreases, creating warm and cold zones. The presence of three distinct thermal zones in the close pxoimity, we hypothesize that abiotic factors remain consistent across these regions, and the

primary determinant of bacterial diversity distribution and antibiotic resistance patterns in each thermal zone is temperature.

Our study was designed to work simultaneously on the three temperature zones (hot, warm, and cold), ranging from 4 °C to 60 °C, with three major goals: a) Assess microbial diversity in the three thermal zones: hot, warm, and cold. b) Determine the relative abundance of thermophilic, thermotolerant, mesophilic, psychrotolerant, and psychrophilic bacteria in different thermal zones and, c) Investigate the occurrence of antibiotic resistance among bacteria in different thermal zones. Here we have done both the traditional (culture-dependent) and Next Generation Sequencing (NGS)-shotgun metagenomic (culture-independent) sequencing to access the microbial diversity and the antibiotics resistance profile.

The first aim of this study was to investigate the bacterial diversity in three distinct natural thermal regions (hot, warm, and cold). In total, we obtained 263 bacterial isolates from these thermal regions, with 104 originating from the hot region, 80 from the warm region, and 77 from the cold region. Our findings unveiled intriguing mutualistic relationships among different bacterial communities inhabiting these diverse thermal zones. Specifically, we observed mutualistic interactions between thermophilic-mesophilic and mesophilic-psychrophilic zones, signifying an expansion of bacterial diversity across these thermal gradients. The thermophilic bacteria were found in the mesophilic zone, and mesophilic bacteria were found in the psychrophilic zone, indicating their adaptability to varying temperature environments beyond their optimal growth conditions.

The second objective to identify the bacterial isolates using both phenotypic (biochemical) and genotypic (16S rRNA sequencing) methods. Initially, we selected 63 isolates for further identification using 16S rRNA sequencing, considering their morphological and biochemical traits. The majority of these bacterial isolates exhibited characteristics such as rod shape, Gram-positive, aerobic metabolism, and endospore formation. Furthermore, all tested

isolates showed positive results in the catalase test. In addition, more than 90 % of the isolates demonstrated positive reactions in carbohydrate fermentation, casein hydrolysis, gelatin hydrolysis, and lipid hydrolysis tests, indicating their ability to ferment carbohydrates and hydrolyze casein, gelatin, and lipids. Similarly, over 90 % of the isolates yielded positive results in citrate utilization and starch hydrolysis tests. However, upon closer examination of starch activity zones, only a limited number of isolates exhibited larger zones, suggesting higher starch hydrolysis activity. The majority of the dominant bacterial isolates belonged to the Gram-positive group. H_2S testing revealed that only a minority of isolates tested positive.

After conducting biochemical grouping, 63 bacterial isolates were identified through partial 16s rRNA gene sequencing. These isolates included 27 from hot region (*Bacillus*, *Geobacillus*, *Anoxybacillus*, and *Aeribacillus*), 19 warms region (*Bacillus*, *Cronobacter*, *Exiguobacterium*, *Lysinibacillus*, *Citrobacter*, *Brevundimonas*, and *Staphylococcus*), and 17 cold region (*Exiguobacterium*, *Janthinobacterium*, *Aeromonas*, *Arthrobacter*, and *Acinetobacter*), indicating common soil bacteria. Each bacterial isolate formed distinct clades with their respective temperatures, affirming their thermophilic, mesophilic, or psychrophilic nature. The study revealed three phyla (Bacillota, Pseudomonadota, and Actinomycetota) across various thermal gradient regions (hot, warm, and cold). Bacillota were abundant in temperate regions and its numbers decreased as temperature dropped. Pseudomonadota increased in colder regions, while Actinomycetota were exclusive found in the cold environments.

Genus-level diversity showed varying bacterial presence across temperature regions. Warm regions had diverse bacteria, hot regions were dominated by *Anoxybacillus* and *Bacillus*, warm regions by *Bacillus*, and cold regions by *Exiguobacterium*. *Exiguobacterium* prevalence decreased with rising temperatures, while *Bacillus* increased. *Geobacillus* was found only in higher temperature regions. Temperature strongly correlated with genus-level bacterial

diversity. Based on our culture-dependent analysis, thermophilic bacteria were most prevalent in the hot region (70.37 %), followed by thermotolerant bacteria (22.22 %), and mesophilic bacteria (7.4 %). No psychrophilic or psychrotolerant bacteria were found there. In the warm region, mesophiles were dominant (68.42 %), followed by psychrophiles (15.78 %), thermophiles (10.52 %), and thermotolerant bacteria (5.26 %). No psychrotolerant bacteria were detected. In the cold region, psychrophiles were the most common (70.58 %), followed by mesophiles (23.52 %), and psychrotolerant bacteria (5.88 %). Thermophiles were absent in the cold region.

The third objective of my research is to assess antibiotic resistance in selected bacterial isolates. In this study, we used 28 antibiotics from 16 different classes. Our findings revealed varying levels of antibiotic resistance across thermal zones, with the warm region showing the highest resistance (36.84 %), followed by the cold region (23.52 %) and the hot region (7.4 %). Among specific bacterial types, mesophiles exhibited the highest antibiotic resistance (92.3 %), while psychrophiles had lower resistance, mainly in cold regions. Notably, thermophiles displayed no antibiotic resistance in any thermal zone. In terms of cumulative antibiotic class abundance across thermal zones, monobactams (19.12 %), cephalosporin (17.65 %), β-Lactams (13.24 %), rifamycin (8.82 %), and macrolide (7.35 %) were the most prevalent antibiotics resistant bacteria.

The final goal of this research aimed to assess the Minimum Inhibitory Concentration (MIC) assay for specific bacterial isolates. The findings indicated that the mesophilic bacterial isolates exhibited significantly higher resistance to the tested antibiotics. To effectively inhibit the proliferation of these mesophilic bacteria, it was essential to administer higher antibiotic doses. Notably, ceftazidime, rrythromycin, and streptomycin were identified as notably ineffective when tested against the mesophilic isolates.

In addition to our primary goals, we conducted the culture-independent analysis of all the collected soil samples. First, we check the elemental analysis using ICP-MS. This analytical technique was utilized to determine the concentration of 19 different elements in soil samples. Our findings indicated that the samples from both sampling sites exhibited three distinct zones: hot, warm, and cold regions. Notably, these zones displayed significant higher concentrations of some elements Mg, K, Ca, Mn, Fe, and Li. Furthermore, it's worth mentioning that the concentrations of these elements remained relatively uniform across multiple samples collected from different zones.

We conducted shotgun metagenomic sequencing on 12 soil samples, with 4 samples representing each of the hot, warm, and cold regions across two sites. For analysis, we averaged results from two samples in each zone at both Site-I and Site-II, resulting in six metagenomic samples. Our diversity and rarefaction curve analysis indicate that the warm region (10 to 40℃) is ideal for bacterial growth and community composition. Temperature strongly influences bacterial communities, with a positive correlation observed between temperature and Bacillota and Chloroflexota, and a negative correlation with Pseudomonadota and Bacteroidota. Thermophilic bacteria thrive in extreme heat, while mesophilic bacteria cluster in the warm region, displaying higher diversity compared to the cold region.

At the phylum level, Pseudomonadota were dominate in the hot region, followed by Bacillota, Chloroflexota, Bacteroidota, and Deinococcota. In the warm region, Pseudomonadota are still prevalent but with more Bacteroidota, while Bacillota are present in lower percentages. The warm region also exhibits higher biodiversity with additional phyla like Acidobacteriota, Actinomycetota, and Verrucomicrobiota. In the cold region, Pseudomonadota and Bacteroidota were dominant, with an increase in Cyanobacteriota and a reduction in other phyla.

At the genus level, hot regions were rich in thermophilic bacteria like *Meiothermus*, *Deinococcus*, *Geobacillus*, *Thermus*, *Bacillus*, *Anoxybacillus*, and *Acinetobacter*. In the warm region, mesophilic bacteria like *Geobacter*, *Thiobacillus*, *Nitrobacter*, *Candidatus Solibacter*, *Acidobacterium*, *Nitrococcus*, *Rhodobacter*, *Roseobacter*, *Nitrospira*, and *Marivirga* are prevalent. The cold region exhibited the presence of psychrophilic bacteria, including *Bacteroides*, *Gramella*, *Flavobacterium*, *Nitrosomonas*, *Burkholderia*, *Acidovorax*, *Comamonas*, *Pseudomonas*, *Yersinia*, and *Sphingomonas*.

Regression analysis was conducted to assess the influence of various abiotic factors on bacterial diversity across three distinct temperature zones. The study considered major phyla, including Pseudomonadota, Bacillota, Bacteroidota, and Chloroflexota, as well as elements with significant concentrations and potential roles in shaping microbial diversity (Mg, K, Ca, Na, P, and Cl). However, the findings indicated that only Chlorine (Cl) exhibited some correlation with bacterial diversity ($r2 \leq 0.5$, $p<0.05$). Chlorine displayed a positive correlation with Pseudomonadota ($r2 \leq 0.8$, $p=0.01$) and Bacteroidota ($r2 \leq 0.2$, $p=0.2$), and a negative correlation with Bacillota ($r2 \leq 0.7$, $p=0.03$) and Chloroflexota ($r2 \leq 0.7$, $p=0.01$). In summary, the overall impact of various elements on bacterial diversity in these thermal zones appeared to be negligible. Additionally, the study investigated the role of temperature and pH in shaping bacterial diversity using regression analysis. pH showed no significant correlation with dominant phyla (Pseudomonadota, Bacillota, Bacteroidota, and Chloroflexota) (mean $r2 \leq 0.6$, $p<0.05$). Conversely, temperature exhibited a notable negative correlation with Pseudomonadota and Bacteroidota ($r2 \geq 0.8$, $p<0.05$), and a positive correlation with Bacillota and Chloroflexota ($r2 \geq 0.8$, $p<0.05$). Principal Component Analysis (PCA) further confirmed temperature as the primary factor influencing bacterial diversity in extreme thermal conditions.

We examined fungal diversity across different regions and found distinct patterns. The Simpson_1-D index indicated higher biodiversity in both warm and cold regions, with the

lowest values in hot regions. Rarefaction curves revealed that the hot region had the highest species-level diversity, while the cold region had the lowest. All soil samples contained Ascomycota and Basidiomycota phyla, with the highest proportions in the hot region. The cold region had the lowest percentages, indicating temperature's influence on fungal diversity.

At the phylum level, fungi were most abundant in the hot region, followed by the warm and cold regions, consistent with genus-level observations (*Ricinus*, *Hydra*, *Homo*, and *Neosartorya*). Principal Component Analysis (PCA) showed a significant correlation among fungal communities in all three regions, with a distinct pattern in the hot region.

The analysis of soil metagenomic sequences involved the identification of antibiotic resistance genes (ARGs) using multiple databases. Across the 12 metagenomic sequences, with two from each of the three distinct zones, a total of 151 resistant genes were anticipated. Notably, all these resistant genes exhibited a resemblance to mesophilic bacteria. This implies that antibiotic resistance in these thermal zones can be attributed solely to mesophiles. Specifically, mesophiles contributed antibiotic resistance in the proportions of 23.1 % to hot regions, 35.7 % to warm regions, and 41 % to cold regions. It is noteworthy that the antibiotic resistance genes to various antibiotic classes were predominantly observed against aminoglycosides (81 %), followed by beta-lactams (13 %), lincosamides (12 %), and peptides (10 %).

To examine the potential impact of temperature on antibiotic resistance, regression analysis was performed on both isolated bacteria and ARGs extracted from metagenomic reads. The results indicated a negative correlation (r^2=0.02, p=0.3) between temperature and various antibiotic-resistant gene classes in the case of ARGs. This suggests that as temperature rises, antibiotic resistance tends to decrease. This aligns with the previous finding that cold regions had the highest percentage of antibiotic resistance (41 °°%) compared to mesophilic and hot regions, despite the primary contribution coming from mesophiles in cold regions. Principal

Component Analysis (PCA) reinforced this trend, showing a negative correlation between temperature and various antibiotic gene classes.

Further exploration involved network analysis, focusing on the relationship between various ARGs and dominant phyla such as Pseudomonadota, Actinomycetota, and Bacillota. The results indicated that in most thermal zones, the majority of ARGs were associated with Pseudomonadota and Actinomycetota, while Bacillota exhibited relatively lower antibiotic resistance levels in pristine high-altitude environments.

Similarly, when considering antibiotic resistance among isolated bacteria, it was highest in mesophilic bacteria, with only a few psychrophilic bacteria displaying antibiotic resistance. However, when evaluating different thermal zones, the cold region displayed the highest antibiotic resistance, followed by the warm and hot regions, although the primary contribution was from mesophiles in the cold region. Regression analysis in this context also demonstrated a decrease in antibiotic resistance with increasing temperature.

When considering heavy metal resistance genes, copper resistance genes prevailed in all regions, with temperature playing a significant role in influencing resistance to nickel, lead, and mercury. Cadmium, arsenic, and chromium resistance genes increased with decreasing temperature. Similarly, gyrase genes varied, with the warm region having the highest dominance. *GyrA* was the most prevalent, and some genes were region-specific. The effect of temperature was also seen on the virulence genes, which displayed a temperature-dependent pattern, with the cold region having the highest occurrence. *Pseudomonas aeruginosa*, *Brucella melitensis*, and *Legionella pneumophila* possessed all virulence genes, with *Pseudomonas aeruginosa* having the highest abundance of virulence genes. Heat map analysis showed temperature influencing the virulence gene abundance, with higher levels in the warm region and the lowest in the hot region. Some genes were common between regions, but mostly they were specific to each thermal zone.

Conclusion

Chapter 8

Temperature is a critical abiotic factor which determines the microbial diversity in several spheres of earth. Based on the microbial preference for their optimal range of temperature, microorganisms are classified in thermophiles, mesophiles and psychrophiles. However, in an environmental niche with gradient temperature i.e., across hot via warm to cold temperate region with other similar abiotic factors like pH and elemental compositions etc., how the microbial diversity changes with respect to temperature is largely unknown? Similarly, the abundance of various types of temperature-dependent bacteria like thermophiles, thermo-tolerant, mesophiles, psychro-tolerant and psychrophilic bacteria in a wide range of thermal gradient having antibiotic resistance and ARGs are also unknown.

Here in this study, we have found that only specific bacteria tolerant to specific temperature grows optimally in their respective micro-environments. However, there were some commonly shared microbial communities among the mesophiles and psychrophiles but they did not contribute to bacterial abundance. Metagenomic analysis shows the prevalence of Pseudomonadota (Proteobacteria), Bacillota (Firmicutes), Bacteroidota (Bacteroidetes) and these phyla showed a linear increase or decrease in their abundance with respect to temperature. Temperature was the governing factor in shaping the bacterial diversity that was statistically significant by regression models in these microenvironments. Other factors such as pH and various elements, possessed insignificant effect on bacterial diversity. The abundance of mesophilic ARGs were predominant in the distinct thermal microenvironments which suggested that the antibiotic resistance was conferred by mesophiles at large and very few psychrophiles might also play a role in it. Presence of gyrase genes showcased the adaptability of the thermophilic bacteria across the thermal gradients. However, the minimal percentage of reverse gyrase also confirmed that the hot regions were devoid of any hyper-thermophilic microbes.

Thermophilic bacteria were found to be devoid of antibiotic resistance. Regression analysis showed the inverse correlation of temperature with antibiotic resistance, i.e., antibiotic resistance decreases with increase in temperature. Antibiotic resistance is maximum at moderate temperatures and usually decreases with the increase and decrease in temperature from the optimum. These studies were carried out in pristine ecosystems with less anthropogenic activities. However, these decisive arguments could be also highlighted by further studying such environments. Furthermore, there is a great scope to perform such studies in contaminated environments that habits such thermal gradients.

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Chapter 9

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Appendix

Appendix

Luria Bertani Agar, Miller (M1151)	
Ingredients	**Gms / Litre**
Tryptone	10.000 gm.
Yeast extract	5.000 gm.
Sodium chloride	10.000 gm.
Agar	25.000 gm.
Final pH	7.5±0.2
Distilled water	1.000 litre
Sterilized by autoclaving at 15 lbs pressure (121 °C) for 15 minutes.	
R-2A Broth (M1687)	
Casein acid hydrolysate	0.500 gm.
Yeast extract	0.500 gm.
Proteose peptone	0.500 gm.
Dextrose	0.500 gm.
Starch, soluble	0.500 gm.
Dipotassium phosphate	0.300 gm.
Magnesium sulphate	0.024 gm.
Sodium pyruvate	0.300 gm.
Final pH	7.2±0.2
Distilled water	1.000 litre
Sterilized by autoclaving at 15 lbs pressure (121 °C) for 15 minutes	
Carbohydrate fermentation broth	
Sugar	5.000 gm.
Peptone	10.000 gm.
Sodium chloride	5.000 gm.
Phenol Red	0.018 gm.
Distilled water	1.000 litre
Mueller Hinton Agar (M173)	
HM Beef infusion	300.000 gm.

Acicase (Casein acid hydrolysate)	17.500 gm.
Starch	1.500 gm.
Agar	17.000 gm.
Final pH	7.3±0.1
Distilled water	1.000 litre
Sterilized by autoclaving at 15 lbs pressure (121 °C) for 15 minutes.	
SOC Broth (M1379)	
Casein enzymic hydrolysate	20.000 gm.
Yeast extract	5.000 gm.
Sodium chloride	0.500 gm.
Magnesium sulphate	2.400 gm.
Potassium chloride	0.186 gm.
Final pH	7.0±0.2
Distilled water	980 ml
Sterilized by autoclaving at 15 lbs pressure (121 °C) for 15 minutes. Cool to 45-50 °C and aseptically added 20 ml of sterilized filter glucose solution (20 %) and well mixed.	
Nutrient Broth (M002)	
Peptone	5.000 gm.
Sodium chloride	5.000 gm.
Beef extract	1.500 gm.
Yeast extract	1.500 gm.
Final pH	7.4±0.2
Distilled water	1.000 litre
Sterilized by autoclaving at 15 lbs pressure (121 °C) for 15 minutes.	
Plate Count Agar (M091A)	
Tryptone	5.000 gm.
Yeast extract	2.500 gm.
Dextrose (Glucose)	1.000 gm.
Agar	9.000 gm.

Final pH	7.0±0.2
Distilled water	1.000 litre
Sterilized by autoclaving at 15 lbs pressure (121 °C) for 15 minutes.	
Thermus Agar (TA)	
Peptone	8.000 gm.
Yeast Extract	4.000 gm.
Sodium chloride	2.000 gm.
Agar	25.000 gm.
Distilled water	1.000 litre
Sterilized by autoclaving at 15 lbs pressure (121 °C) for 15 minutes.	
Starch Agar	
Peptone	5.000 gm.
Yeast Extract	1.500 gm.
Beef extract	1.500 gm.
Starch soluble	2.000 gm.
Sodium chloride	5.000 gm.
Agar	25.000 gm.
Distilled water	1.000 litre
Sterilized by autoclaving at 15 lbs pressure (121 °C) for 15 minutes.	
Urea Agar	
Urea	20.000 gm.
Peptone	1.000 gm.
Dextrose	1.000 gm.
Phenol Red	0.010 gm.
Monopotassium Phosphate	2.000 gm.
Sodium chloride	5.000 gm.
Agar	25.000 gm.
Distilled water	1.000 litre
Sterilized by autoclaving at 15 lbs pressure (121 °C) for 15 minutes.	

Journal/Articles

List of Research Articles as First Author
Kumar, S., Najar, I. N., Sharma, P., Tamang, S., Mondal, K., Das, S., and Thakur, N. (2023) Temperature–A critical abiotic paradigm that governs bacterial heterogeneity in natural ecological system. ***Environmental Research*** 234: 116547. (**Impact Factor: 8.3**)
Kumar, S., Das, R., Sharma, P., Tamang, S., Ranjan, R.K., and Thakur, N. (2023) Evaluation of Fungal Diversity in High Altitude Soils in Different Temperature Conditions. ***Journal of Climate Change*** 9: 65–75. (**UGC-Listed Journal**)

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Temperature – A critical abiotic paradigm that governs bacterial heterogeneity in natural ecological system

Santosh Kumar [a,1], Ishfaq Nabi Najar [a,1], Prayatna Sharma [a], Sonia Tamang [a], Krishnendu Mondal [b], Sayak Das [c], Mingma Thundu Sherpa [a], Nagendra Thakur [a,*]

[a] Department of Microbiology, School of Life Sciences, Sikkim University, Gangtok, 737102, Sikkim, India
[b] Department of Microbiology, Vidyasagar University, Midnapore, 721102, India
[c] Department of Life Science & Bioinformatics, HK School of Life Sciences, Assam University, Silchar, 788011, Assam, India

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ABSTRACT

A baseline data has been presented here to prove that among the abiotic factors, temperature is the most critical factor that regulates and governs the bacterial diversity in a natural ecosystem. Present study in Yumesamdong hot springs riverine vicinity (Sikkim), parades a gamut of bacterial communities in it and hosts them from semi-frigid region (- 4–10 °C) to fervid region (50–60 °C) via an intermediate region (25–37 °C) within the same ecosystem. This is an extremely rare intriguing natural ecosystem that has no anthropogenic disturbances nor any artificial regulation of temperature. We scanned the bacterial flora through both the culture-dependent and culture-independent techniques in this naturally complex thermally graded habitat. High-throughput sequencing gave bacterial and archaeal phyla representatives of over 2000 species showcasing their biodiversity. Proteobacteria, Firmicutes, Bacteroidetes and Chloroflexi were the predominant phyla. A concave down-curve significance was found in temperature-abundance correlation as the number of microbial taxa decreased when the temperature increased from warm (35 °C) to hot (60 °C). Firmicutes showed significant linear increase from cold to hot environment whereas Proteobacteria followed the opposite trend. No significant correlation was observed for physicochemical parameters against the bacterial diversity. However, only temperature has shown significant positive correlation to the predominant phyla at their respective thermal gradients. The antibiotic resistance patterns correlated with temperature gradient where the prevalence of antibiotic resistance was higher in case of mesophiles than that of psychrophiles and there was no resistance in thermophiles. The antibiotic resistant genes obtained were solely from mesophiles as it conferred high resistance at mesophilic conditions enabling them to adapt and metabolically compete for survival. Our study concludes that the temperature is a major factor that plays a significant contribution in shaping the bacterial community structure in any thermal gradient edifice.

1. Introduction

Different spheres of Earth are inhabited by a diverse domain of Bacteria, Archaea and Eukarya (Singh, 2017). These spheres possess many distinct abiotic factors which governs this domain diversity such as pH, temperature, pressure, radiation, acidity and salinity etc. (Reysenbach and Shock, 2002; Wang et al., 2013). However, temperature and pH are the two main parameters which have been widely studied with respect to microbial diversity patterns (Cole et al., 2013; Cuecas et al., 2014; Power et al., 2018; Sharp et al., 2014). Ward et al. (2012) suggested that hot springs can be used as model systems to show how sympatric adaptation to variables that vary along well-known environmental gradients leads to ecological diversity (Ward et al., 2012). Temperature gradients possess a great influence on microbial diversity as bacterial and archaeal abundance gets fluctuated significantly with temperature variations (Li et al., 2015). It has also been shown that the number of microbial cells is usually lower at high temperatures (90.8 °C). The thermophilic bacteria that do not produce endospore are not able to disperse over wide geographical areas because they cannot endure prolonged heat desiccation (Beblo et al., 2009; Castenholz, 1969). Similarly, the effect of temperature change on the microbial diversity and community structure along the chronosequence of the

* Corresponding author.
E-mail address: nthakur@cus.ac.in (N. Thakur).
[1] Mr. Santosh Kumar and Dr. Ishfaq Nabi Najar contributed equally.

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Evaluation of Fungal Diversity in High Altitude Soils in Different Temperature Conditions

Santosh Kumar[1], Rohit Das[1], Prayatna Sharma[1], Sonia Tamang[1] Rakesh Kumar Ranjan[2] and Nagendra Thakur[1]*

[1]Department of Microbiology, School of Life Sciences, Sikkim University, Gangtok – 737102, Sikkim, India
[2]Department of Geology, School of Physical Sciences, Sikkim University, Gangtok –737102, Sikkim, India
✉ nthakur@cus.ac.in

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Abstract: Hot springs are like nature's spas, which include warm and humid aquatic habitats that serve as a sanctuary for a diverse range of microorganisms, including bacteria, archaea, viruses, and eukaryotes fungi. Among these, fungi are one of the most important microorganisms, carrying out essential functions that often go unnoticed but are crucial in accelerating biological processes. These organisms have adapted to survive under a wide range of thermal conditions. While thermophilic fungi can withstand the scorching heat of deserts and hyper-saline conditions, other variants like mesophilic and psychrophilic fungi prefer more moderate and colder temperatures, respectively. The study employs shotgun metagenomic sequencing to obtain a fine-grained taxonomic classification of lesser-known species and microbial eukaryotes. This study aims to explore the relationship between fungal diversity and temperature in three distinct thermal zones; thermophilic (hot spring), mesophilic (plain field), and psychrophilic (semi-frigid zone) zones. The findings of our study demonstrated that there is a notable and positive association between the diversity of fungi and temperature, suggesting that temperature is a key factor in moulding fungal communities. However, it is important to note that further research is necessary to elucidate the underlying causal mechanisms of this relationship. Overall, our study adds to the knowledge of how environmental factors influence microbial diversity and can aid in the development of strategies for the conservation and management of fungal communities. Fungi are essential for many industrial applications, and their role in causing illnesses is also significant, making this research valuable for both scientific and practical purposes.

Keywords: Metagenomic study; Fungal diversity; Thermophilic; Mesophilic; Psychrophilic.

Introduction

Microbial communities in natural and host-associated environments commonly harbour a mix of bacteria, archaea, viruses and microbial eukaryotes (Marcelino et al., 2020). Among these, bacteria can be easily identified at the species and strain levels (Scholz et al., 2016; Marcelino et al., 2020), however, it remains challenging to obtain a fine-grained taxonomic classification of lesser-known species and microbial eukaryotes (Nilsson et al., 2019). Eukaryotic microorganisms exhibit immense diversity, encompassing species with varying life cycles, morphological adaptations, and nutritional requirements. While viruses and bacteria are responsible for a larger number of diseases, certain microscopic eukaryotes, such as fungi, protists and algae, are associated with significant public health concerns. Fungi, in particular, play a crucial role in performing essential functions that are often invisible but vital for facilitating biological processes (El-Gendi et al., 2022).

*Corresponding Author

Book Chapters as First Author
Kumar, S., Thakur, N., Singh, A. K., Gudade, B. A., Ghimire, D., and Das, S. (2022). Microbes-assisted phytoremediation of contaminated environment: global status, progress, challenges, and future prospects. *In Phytoremediation technology for the removal of heavy metals and other contaminants from soil and water*, 1, 555-570. ***Elsevier***.
Kumar, S., Thakur, N., Singh, A. K., Gudade, B. A., Ghimire, D., and Das, S. (2022). Aquatic macrophytes for environmental pollution control. *In Phytoremediation Technology for the Removal of Heavy Metals and Other Contaminants from Soil and Water*, 1, 291-308. ***Elsevier***.
Kumar, S., Abedin, M. M., Singh, A. K., & Das, S. (2020). Role of phenolic compounds in plant-defensive mechanisms. *In Plant Phenolics in Sustainable Agriculture*. 1, 517-532. ***Springer***.

CHAPTER

26 Microbes-assisted phytoremediation of contaminated environment: Global status, progress, challenges, and future prospects

Santosh Kumar[a], Nagendra Thakur[a], Ashish K. Singh[a], Bharat Arjun Gudade[b], Deepak Ghimire[c] and Saurav Das[c]

[a] *Department of Microbiology, School of Life Sciences, Sikkim University, Gangtok, Sikkim, India,* [b] *Spice Park, Spices Board, Chhindwara, Madhya Pradesh, India,* [c] *Department of Agronomy and Horticulture, University of Nebraska-Lincoln, Lincoln, NE, United States*

26.1 Introduction

Industrialization, exponential population growth, agricultural practices, and anthropogenic activities are continually contaminating the natural environment (air, water, and soil). Toxic materials added through different anthropogenic activities are altering the inherent biotic community ofenvironment, including microorganisms, plants, and animals (Ashraf et al., 2017). Environmental contamination is becoming a concern throughout the world for the negative health impact associated with it and subsequent alteration of natural biodiversity and process. Heavy metals like lead (Pb), arsenic (As), cadmium (Cd), chromium (Cr), and mercury (Hg) can cause health hazards like chronic anemia, damage to nervous system, skin, teeth, bones, cardiovascular diseases, cognitive impairment, cancer, and much more. Organic compounds like polyaromatic hydrocarbons (PAHs), phenols, polychlorinated biphenyls (PCBs), toluene, trinitrotoluene, benzene, and different pesticides, herbicides can be persistent and enter the food chain causing mutagenicity and carcinogenicity in animals and human. In the past, several strategies to clean-up pollutants especially from soil are being used,which can bebroadly categorized as biological, chemical, and physical approaches. Most used physicochemical remediation process is like electro reclamation, leaching, landfill, thermal treatment, excavation are not very cost-efficient, and also produces large secondary pollution. While biological process like using microbes and plant for remediation has been popular over chemical and physical processesas a natural process (Fig. 26.1). However, individual process often lacks the efficiency

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CHAPTER

Aquatic macrophytes for environmental pollution control

14

Santosh Kumar[a], Nagendra Thakur[a], Ashish K. Singh[a], Bharat Arjun Gudade[b], Deepak Ghimire[c] and Saurav Das[c]

[a] *Department of Microbiology, School of Life Sciences, Sikkim University, Gangtok, Sikkim, India,* [b] *Spice Park, Spices Board, Chhindwara, Madhya Pradesh, India,* [c] *Department of Agronomy and Horticulture, University of Nebraska-Lincoln, Lincoln, NE, United States*

14.1 Introduction

Water is an essential medium and necessary for the survival of all life forms. The global water crisis is one of the severe problems in today's world. Water is continuously contaminated due to various anthropogenic activities, creating a threat to all living organisms' survival and wellbeing. Population growth, industrialization, agricultural expansion, and urbanization have degraded the quality of natural water resources. A contaminated aquatic environment shifts the natural diversity of aquatic ecosystems by altering the community composition of animals, plants, and microorganisms. Contaminations by inorganic and organic pollutants to water bodies are of serious concern. It is not easy to degrade contaminants using natural phenomena; however, biologically, the pollutants can be transformed from high to low toxic form (Ansari et al., 2020). In developing countries, water bodies serve as the disposing ground for the pollutants, directly or indirectly from households and industries. Water bodies are also at the receiving end of stormwater, runoff from agricultural land, which makes it at risk for different contamination (Dhote and Dixit, 2009).

Every year millions of people die due to fatal waterborne diseases worldwide, especially in developing countries like India (Singh et al., 2020). The water quality monitoring results obtained during 1995–2007 around the globe indicated that organic and microbial contamination is an acritical concern. Discharge of untreated domestic wastewater from the country's urban centers into water bodies is the primary reason. With the increasing population and expansion of cities, municipal corporations are having difficulty treating the growing load of sewage flowing into water bodies. Secondly, the collecting water bodies also do not have adequate water for dilution. A survey carried out by the Central Pollution Control Board (2008) reported that groundwater in 40 districts from 13 states of India, namely, Andhra Pradesh, Assam, Bihar, Haryana, Himachal Pradesh, Karnataka, Madhya Pradesh, Orissa, Punjab, Rajasthan, Tamil Nadu, Uttar Pradesh, West Bengal, and five blocks of Delhi is

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Role of Phenolic Compounds in Plant-Defensive Mechanisms

22

Santosh Kumar, Md. Minhajul Abedin, Ashish Kumar Singh, and Saurav Das

Abstract

Phenolics are ubiquitous secondary metabolites found in plant. They are aromatic compounds synthesized by phenylpropanoid pathway. Phenolics have been in the focus of many findings on plant-defenses mechanisms to pathogens, including bacteria, fungi, and viruses, and major abiotic stresses like drought, salinity, and UV. Phenolic compound exhibits antimicrobial and antioxidant properties which helps plant to evade pathogenic infections as well as protect the major tissues from toxic effect of reactive oxygen species. Rapid upregulation of genes in the phenylpropanoid pathway and the accumulation of phenolics can be observed in response to environmental stress. Phenolic compounds also play an important role in protecting the plant from insect herbivory. Phenolic compounds are diverse and classified based on the number of carbons. Structural diversity of the phenolic compound defines its functional properties and distribution in different plant species. Beside defensive mechanisms, phenolic compounds are also important in cross-talk or plant-microbe interaction and communications. Structural diversity of flavonoids from the leguminous plant is important in species-specific symbiotic relationships.

Keywords

Phenolics · Ubiquitous · Drought · Salinity · Antioxidant · Stress

S. Kumar · M. M. Abedin · A. K. Singh
Department of Microbiology, School of Life Sciences, Sikkim University, Gangtok, Sikkim, India

S. Das (✉)
Department of Agronomy and Horticulture, University of Nebraska – Lincoln, Lincoln, NE, USA
e-mail: sdas4@unl.edu

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List of Journal Articles as Co-author

Das, S., Najar, I. N., Sherpa, M. T., **Kumar, S.**, Sharma, P., Mondal, K., and Thakur, N. (2023). Baseline metagenome-assembled genome (MAG) data of Sikkim hot springs from Indian Himalayan geothermal belt (IHGB) showcasing its potential CAZymes, and sulfur-nitrogen metabolic activity. ***World Journal of Microbiology and Biotechnology***, 39(7), 1-21. (**Impact Factor:4.1**)

Sharma, P., Mondal, K., **Kumar, S.**, Tamang, S., Najar, I. N., Das, S., and Thakur, N. (2022). RNA thermometers in bacteria: Role in thermoregulation. ***Biochimica et Biophysica Acta (BBA)-Gene Regulatory Mechanisms***, 194871. (**Impact Factor:4.7**)

Najar, I. N., Das, S., **Kumar, S.**, Sharma, P., Mondal, K., Sherpa, M. T., and Thakur, N. (2022). Coexistence of heavy metal tolerance and antibiotic resistance in thermophilic Bacteria belonging to Genus Geobacillus. ***Frontiers in microbiology***, 13, 914037. (**Impact Factor:5.2**)

Najar, I. N., Sharma, P., Das, S., Sherpa, M. T., **Kumar, S.**, and Thakur, N. (2022). Bacterial diversity, physicochemical and geothermometry of South Asian hot springs. ***Current research in microbial sciences***, 3, 100125. (Impact Factor: NA)

Singh, A. K., Das, S., **Kumar, S.**, Gajamer, V. R., Najar, I. N., Lepcha, Y. D., and Singh, S. (2020). Distribution of antibiotic-resistant Enterobacteriaceae pathogens in potable spring water of eastern Indian Himalayas: emphasis on virulence gene and antibiotic resistance genes in Escherichia coli. ***Frontiers in Microbiology***, 11, 581072. (**Impact Factor:5.2**)

Singh, A. K., Das, S., Singh, S., Pradhan, N., Gajamer, V. R., **Kumar, S.**, and Tiwari, H. K. (2019). Physicochemical parameters and alarming coliform count of the potable water of Eastern Himalayan state Sikkim: An indication of severe fecal contamination and immediate health risk. ***Frontiers in public health***, 7, 174. (**Impact Factor:5.2**)

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RESEARCH

Baseline metagenome-assembled genome (MAG) data of Sikkim hot springs from Indian Himalayan geothermal belt (IHGB) showcasing its potential CAZymes, and sulfur-nitrogen metabolic activity

Sayak Das[1,2] · Ishfaq Nabi Najar[1] · Mingma Thundu Sherpa[1] · Santosh Kumar[1] · Prayatna Sharma[1] · Krishnendu Mondal[3] · Sonia Tamang[1] · Nagendra Thakur[1]

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Abstract
Here we present the construction and characterization of metagenome assembled genomes (MAGs) from two hot springs residing in the vicinity of Indian Himalayan Geothermal Belt (IHGB). A total of 78 and 7 taxonomic bins were obtained for Old Yume Samdong (OYS) and New Yume Samdong (NYS) hot springs respectively. After passing all the criteria only 21 and 4 MAGs were further studied based on the successful prediction of their 16 S rRNA. Various databases were used such as GTDB, Kaiju, EzTaxon, BLAST XY Plot and NCBI BLAST to get the taxonomic classification of various 16 S rRNA predicted MAGs. The bacterial genomes found were from both thermophilic and mesophilic bacteria among which Proteobacteria, Chloroflexi, Bacteroidetes and Firmicutes were the abundant phyla. However, in case of OYS, two genomes belonged to archaeal *Methanobacterium* and *Methanocaldococcus*. Functional characterization revealed the richness of CAZymes such as Glycosyl Transferase (GT) (56.7%), Glycoside Hydrolase (GH) (37.4%), Carbohydrate Esterase family (CE) (8.2%), and Polysaccharide Lyase (PL) (1.9%). There were negligible antibiotic resistance genes in the MAGs however, a significant heavy metal tolerance gene was found in the MAGs. Thus, it may be assumed that there is no coexistence of antibiotic and heavy metal resistance genes in these hot spring microbiomes. Since the selected hot springs possess good sulfur content thus, we also checked the presence of genes for sulfur and nitrogen metabolism. It was found that MAGs from both the hot springs possess significant number of genes related to sulfur and nitrogen metabolism.

Keywords Metagenome-assembled genome · Sikkim · Data binning · CAZymes · Uncultured microbiota

Introduction

The microbial communities residing in various spheres of the Earth are continually deciphered by sophisticated deep throughput sequencing techniques vis-a-vis microbiome analysis (Deb and Das 2022; Bennett et al. 2022; Zhong et al. 2022). Their diversification and ecological role are magnanimous and revealing their taxonomy is itself an intrigue concern for biologists even today. Prokaryotes are diverse and known to dominate almost all the spheres of life (Whitman et al. 1998). They play crucial roles in biogeochemical processes that is a pre-requisite for life (Falkowski et al. 2008). They also can reside in extreme environments such as thermophiles living at hot temperatures, psychrophiles living in cold habitats, halophiles living in high saline regions or barophiles dwelling in the varying depths of oceans. One of the important extreme environmental habitats are the

Sayak Das and Ishfaq Nabi Najar contributed equally to this work.

✉ Nagendra Thakur
nthakur@cus.ac.in

[1] Department of Microbiology, School of Life Sciences, Sikkim University, 6th Mile, Samdur, Gangtok, Sikkim 737102, India

[2] Department of Life Science and Bioinformatics, Hargobind Khurana School of Life Sciences, Assam University, Silchar, Assam 788011, India

[3] Department of Microbiology, Vidyasagar University, Midnapore, West Bengal 721102, India

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Review

RNA thermometers in bacteria: Role in thermoregulation

Prayatna Sharma [a], Krishnendu Mondal [b], Santosh Kumar [a], Sonia Tamang [a], Ishfaq Nabi Najar [a], Sayak Das [a], Nagendra Thakur [a,*]

[a] Department of Microbiology, School of Life Sciences, Sikkim University, 6th Mile, Samdur, Gangtok, 737102, Sikkim, India
[b] Department of Microbiology, Vidyasagar University, Midnapore, West Bengal 721102, India

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ABSTRACT

An array of external factors, an important one being temperature, decide the fate of survival in a microbe. The ability of microbes to sense external cues and to regulate the expression of genes accordingly is critical for its likely survival. Among a myriad of cellular defence mechanisms, a strategy to recuperate stress involves RNA regulatory elements. RNAs own a repertoire of functions in a cell as messengers, for transfer or as a component of ribosomes. A shift from its indigenous role is as regulators of gene expression, where in the cis-encoded RNA termed as "RNA Thermometers" play a pivotal role in translational level of gene expression. In this paper, we review the occurrence, the different types and molecular mechanism of gene regulation by RNATs, with a special focus limited to the domain Bacteria. We discuss the role of RNATs in mediating expression of temperature-responsive genes like heat shock/cold attributing in heat/cold shock response and a cascade of virulence genes to evade host defence mechanisms.

1. Introduction

A majority of the prokaryotic genes organise in transcriptional units or operon: a cluster of genes operating under the command of same promoter [1]. The occurrence of operon allows genes to be *co*-transcribed into polycistronic mRNA that encode multiple proteins from a single mRNA [2]. Regardless of clustering of multiple genes in a single mRNA, the individual genes within an operon can be subjected to differential expression. This differential expression of genes may be controlled at transcriptional and translational level: the former mechanism entails promoters or terminators which govern the transcriptional-level of differential regulation [3]. Translational level of regulation is mainly determined by its initiation and elongation steps [4]. While elongation primarily depends upon usage of codons and availability of tRNA, initiation depends on structural accessibility of Shine-Dalgarno sequence (SDS) or ribosome binding site [5] This reliance of translational efficiency prominently on the ampleness of ribosome binding site widens the opportunity for cis-regulatory elements (Riboswitches and RNA thermometers) to control gene expression.

It is well acclaimed that proteins anticipate major functions in cell signalling, most commonly *via* activating regulatory pathways (that may either activate or repress gene expression at transcriptional, translational or post translational level) [6]. Bacterial RNA elements possessing regulatory phenomenon have gained a considerable attention for the past two decades now [7]. Not all RNAs are prominently known for encoding proteins-some RNAs persisting in intergenic regions of open reading frames also attribute regulatory mechanisms assigning control of gene expression. Such RNA regulatory elements are cis-acting (referred to as cis-encoded RNAs or antisense RNAs present at upstream of genes to be regulated and are complementary to their target genes) or trans-acting (partially complement to their target genes and encoded elsewhere in the genome) [8]. Examples of cis-acting regulatory elements include RNA thermometers and riboswitches, which regulate expression of mRNA transcripts at transcriptional or translational level without participation of any other factors. Both these regulatory RNAS are present within the untranslated region of bacterial mRNA [9,10]. To put in simple terms, these elements attribute the phenomenon of thermoregulation *i.e.*, modulate their mRNA structures without the aid of any other external factors, in such a manner that under ambient temperature, the mRNA remains in their native folded form. However, a rise in temperature induces structural changes in its secondary or tertiary structures, thereby exposing the Shine Dalgarno Sequence (SDS) for initiation of heat shock, cold shock or virulence genes [11]. Unlike the riboswitches, RNATs employ a ligand-independent regulatory mechanism. With ability to detect a prompt change in temperature as low as 1 °C, they confer regulation of gene expression by controlling translation

* Corresponding author at: Department of Microbiology, School of Life Sciences, Sikkim University, 6th Mile, Samdur, Tadong, Angtok 737102, Sikkim, India.
E-mail address: nthakur@cus.ac.in (N. Thakur).

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Coexistence of Heavy Metal Tolerance and Antibiotic Resistance in Thermophilic Bacteria Belonging to Genus *Geobacillus*

*Ishfaq Nabi Najar[1], Sayak Das[1], Santosh Kumar[1], Prayatna Sharma[1], Krishnendu Mondal[2], Mingma Thundu Sherpa[1] and Nagendra Thakur[1]**

[1] Department of Microbiology, Sikkim University, Gangtok, India, [2] Department of Microbiology, Vidyasagar University, Midnapore, India

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Laura Zucconi,
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South Dakota School of Mines and Technology, United States
Avinash Sharma,
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***Correspondence:**
Nagendra Thakur
nthakur@cus.ac.in

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Hot springs are thought to be potential repositories for opportunistic infections, such as antibiotic-resistant strains. However, there is a scarcity of information on the mechanisms of antibiotic resistance gene (ARG) uptake, occurrence, and expression in thermophilic bacteria. Furthermore, because the genesis and proliferation of ARGs in environmental microorganisms are unknown, the research on antibiotic resistance profiles and probable mechanisms in thermophilic bacteria will become increasingly important. The goals of this study are to explore bacterial diversity, antibiotic and heavy metal resistance, and the prevalence and presence of ARG and metal resistance gene (MRG) in *Geobacillus* species. The 16S rRNA sequencing was used to determine the culturable bacterium diversity of 124 isolates. Standard Kirby Bauer Disc Diffusion and tube dilution procedures were used to determine antibiotic sensitivity and minimum inhibitory concentration (MIC). The tube dilution method was also used to check metal tolerance. To detect ARG and heavy MRG (HMRG), whole genome sequencing studies of the type species of the genus *Geobacillus* and five randomly selected *Geobacillus* species were performed. Graph Pad Prism and XLSTAT were used to perform statistical analyses such as ANOVA, EC50 analysis, and principal component analysis (PCA). The phylum Firmicutes and the genus *Geobacillus* dominated the culture-dependent bacterial diversity. Surprisingly, all thermophilic isolates, i.e., *Geobacillus* species, were sensitive to at least 10 different antibiotics, as evidenced by the lack of ARGs in whole genome sequencing analysis of numerous *Geobacillus* species. However, some of these isolates were resistant to at least five different heavy metals, and whole genome sequencing revealed the presence of MRGs in these thermophilic bacteria. The thermophilic genus *Geobacillus* is generally antibiotic sensitive, according to this study. In contrast, heavy metal is tolerated by them. As a result, it is possible that ARGs and MRGs do not coexist in these bacteria living in hot springs.

Keywords: thermophilic microbes, antibiotics resistance, heavy metal tolerance, *Geobacillus*, Sikkim Himalaya

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Bacterial diversity, physicochemical and geothermometry of South Asian hot springs

Ishfaq Nabi Najar, Prayatna Sharma, Sayak Das, Mingma Thundu Sherpa, Santosh Kumar, Nagendra Thakur *

Department of Microbiology, School of Life Sciences, Sikkim University, 6th Mile, Samdur, Gangtok, 737102 Sikkim, India

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ABSTRACT

Extreme ecosystems with enormous arrays of physicochemical or biological physiognomies serve as an important indicator of various processes occurred and/or occurring in and on the Earth. Among extreme habitats, hot springs represent geothermal features which are complex systems with a well-defined plumbing system. Besides geological tectonic based hypsography and orology annotations, the hot springs have served as hot spots for ages where there is an amalgamation of nature, religion, faith, health, and science. Thus, there remains an escalating scope to study these hot springs all over the world. The Himalayan Geothermal Belt (HGB) banquets three densely demographic countries i.e. Pakistan, India and China, that hosts numerous hot springs. Studies on the hot springs distributed over these countries reveal Proteobacteria, Firmicutes, Bacteroidetes and Actinobacteria as the predominant bacterial phyla. The bacterial diversity shows a significant positive correlation with physico-chemical parameters like temperature, pH, Na^{+}, HCO_3^{-}, etc. Physicochemical analyses of these hot springs indicate the water mainly as Na-Cl, Na-HCO_3, SO_4-Cl, and mixed type, with temperature ranging approximately between 100-250°C as predicted by various geothermometers. Numerous studies although done, not much of a comprehensive database of the analysis are provided on the hot springs harboured by the HGB. This review aims to give a cumulative illustration on comparative facets of various characteristic features of hot springs distributed over the HGB. These are found to be of great importance with respect to the exploitation of geothermal energy and microflora in various sectors of industries and biotechnology. They are also important sources in terms of socio-economic perspective, and routes to eco-medical tourism.

1. Introduction

The earth hosts various unique, unknown and extreme niches. Among them the hot springs are the hot spots for ages where there is an amalgamation of nature, religion, faith, health, and science. Hot springs also provides the information on geological tectonic based hypsography and orology annotations. Although many of these hot springs have been studied, however there is lot to be done. A lot of data has been published and research is still going on to be pursued all around world in these areas of extreme environments. The data is however haphazardly being represented. There is neither an accumulation of data at one place nor any extensive analysis done on this precious data which may correlate many facts with the past, present and future of the microbial science. Therefore, it is important to co-integrate the data and to analyze it with respect to bacterial diversity and hydro-geochemistry to give basic ideas of geothermal areas and features which are hardly being represented and discussed in such a way.

In this review, we have extensively analyzed the data based on bacterial diversity and hydro-geochemistry of studied hot springs residing in Himalayan Geothermal areas covering India, China and Northern Pakistan. As the hot springs can be both orogenic and non-orogenic in nature, here interestingly in the HGB both these types of geothermal points can be found. Thermal springs have been in use for religious and/or balneotherapeutic purposes since ages. Bathing at hot springs helps in efficient and rapid increase in blood circulation and helps improve metabolic activities and also from various other ailments. Hence, it is significant to study the hot springs from balneotherapeutic stratagem and its chemical ecology to understand the geothermometry

* Corresponding Author at: Assistant Professor Dr. Nagendra Thakur; Department of Microbiology, School of Life Sciences, Sikkim University, 6th Mile, Samdur, Tadong, Gangtok – 737102, Sikkim, India.

E-mail addresses: urooj.ishfaq@gmail.com (I.N. Najar), psharma.su@gmail.com (P. Sharma), sayakdas2002@gmail.com (S. Das), mingmasherpa8@gmail.com (M.T. Sherpa), krsantosh228@gmail.com (S. Kumar), nthakur@cus.ac.in (N. Thakur).

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Distribution of Antibiotic-Resistant *Enterobacteriaceae* Pathogens in Potable Spring Water of Eastern Indian Himalayas: Emphasis on Virulence Gene and Antibiotic Resistance Genes in *Escherichia coli*

*Ashish Kumar Singh[1], Saurav Das[2], Santosh Kumar[1], Varsha Rani Gajamer[1], Ishfaq Nabi Najar[1], Yangchen D. Lepcha[3], Hare Krishna Tiwari[1] and Samer Singh[4]**

[1] Department of Microbiology, School of Life Sciences, Sikkim University, Gangtok, India, [2] Department of Agronomy and Horticulture, University of Nebraska–Lincoln, Lincoln, NE, United States, [3] State Institute of Rural Development (SIRD), Government of Sikkim, Gangtok, India, [4] Centre of Experimental Medicine and Surgery (CEMS), Institute of Medical Sciences, Banaras Hindu University, Varanasi, India

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Edited by:
Santi M. Mandal,
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Bangladesh Agricultural University, Bangladesh
Farhad Safarpoor Dehkordi,
University of Tehran, Iran

***Correspondence:**
Samer Singh
samer.singh10@bhu.ac.in

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Every year millions of people die due to fatal waterborne diseases around the world especially in developing countries like India. Sikkim, a northeastern state of India, greatly depends on natural water sources. About 80% of the population of Sikkim depends on natural spring water for domestic as well as agricultural use. Recent waterborne disease outbreaks in the state raises a concerning question on water quality. In this study, we analyzed water quality especially for the detection of *Enterobacteriaceae* members from four districts of the state. Isolation with selective culture media techniques and taxonomic characterization of *Enterobacteriaceae* bacteria with 16S rRNA gene showed the prevalence of *Escherichia coli* (37.50%), *Escherichia fergusonii* (29.41%), *Klebsiella oxytoca* (36.93%), *Citrobacter freundii* (37.92%), *Citrobacter amalonaticus* (43.82%), *Enterobacter sp.* (43.82%), *Morganella morganii* (43.82%), *Hafnia alvei* (32.42%), *Hafnia paralvei* (38.74%), and *Shigella flexneri* (30.47%) in the spring water of Sikkim. Antibiotic susceptibility test (AST) showed resistance of the isolates to common antibiotics like ampicillin, amoxicillin as well as to third generation antibiotics like ceftazidime and carbapenem. None of the isolates showed resistance to chloramphenicol. *E. coli* isolated from spring water of Sikkim showed presence of different virulence genes such as *stx1* (81.81%), *elt* (86.66%), and *eae* (66.66%) along with resistance gene for ampicillin (*CITM*) (80%), quinolones (*qnrB*) (44.44%), tetracycline (*tetO*) (66.66%), and streptomycin (*aadA1*) (66.66%). The data indicates a high incidence rate of multiple antibiotic resistant enteric bacteria in the spring water of Sikkim. Additionally, the presence of enteric bacteria in the water samples indicates widespread fecal contamination of the spring water.

Keywords: spring, *Enterobacteriaceae*, virulence gene, antibiotic resistance gene, multidrug resistant (MDR), water quality, waterborne pathogens

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Physicochemical Parameters and Alarming Coliform Count of the Potable Water of Eastern Himalayan State Sikkim: An Indication of Severe Fecal Contamination and Immediate Health Risk

*Ashish Kumar Singh[1], Saurav Das[1], Samer Singh[2,3], Nilu Pradhan[1], Varsha Rani Gajamer[1], Santosh Kumar[1], Yangchen D. Lepcha[4] and Hare K. Tiwari[1]**

[1] Department of Microbiology, School of Life Sciences, Sikkim University, Gangtok, India, [2] Department of Microbial Biotechnology, Panjab University, Chandigarh, India, [3] Centre of Experimental Medicine & Surgery, Institute of Medical Sciences, Banaras Hindu University, Varanasi, India, [4] State Institute of Rural Development, Government of Sikkim, Gangtok, India

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Mohiuddin Md. Taimur Khan, Washington State University Tri-Cities, United States

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Peiyue Li, Chang'an University, China

***Correspondence:**
Hare K. Tiwari
hktiwari_2005@rediffmail.com

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Continuous decline in potable water sources has raised serious concerns over human health. Developing countries are the most affected in this regard due to a lack of proper hygiene maintenance. Sikkim, an Eastern Himalayan state with mountains as the predominant topological features, harbors several perennial natural springs. Spring water is the primary source of potable water for the population in four districts of the state viz. East, West, North and South. Recent outbreaks of water-borne diseases and the relative lack of scientific studies on its potential correlation with the water quality of the area have educed this study. Physicochemical parameters of springs, community reservoirs, and household water were analyzed by ICP-MS and multi probe meter. Using the membrane filtration method, the microbial quality of the water samples during different seasons was assessed, primarily evaluating the presence of fecal indicators viz. *Escherichia coli*, total coliform and *Enterococcus*. The seasonal risk category of the water sources was also determined. Most of the physicochemical parameters of the spring water were within the permissible limits of WHO standards. However, water from four districts was recorded with traces of toxic heavy metals like mercury (0.001–0.007 mg/l), lead (0.001–0.007 mg/l), and selenium (0.526–0.644 mg/l), which are above the permissible limits of WHO. All the spring water samples were categorized as Mg-HCO_3^- type and can be predicted as shallow fresh ground water based on the piper analysis. Microbial confirmatory testing indicated severe fecal contamination of water sources with high counts of total coliform (TC), *Escherichia coli* (EC) and *Enterococcus* (EN). The highest level of TC was recorded from West Sikkim (37.26 cfu/100 ml) and the lowest in North Sikkim (22.13 cfu/100 ml). The highest level of contamination of *E. coli* and *Enterococcus* was found in East Sikkim (EC = 8.7 cfu/100 ml; EN = 2.08 cfu/100 ml) followed by South Sikkim (EC = 8.4 cfu/100 ml; EN = 2.05 cfu/100 ml). There was a significant positive correlation between

National/International Conferences

SL No.	National/International Seminar	Year
1	Delivered an ***Oral Presentation*** at a National conference on "Emerging Challenges and Prospects in Biological Sciences" (22/02/2023 to 24/02/2023) on "***Culture independent study of microbial diversity in different Natural thermal zones of Sikkim Himalaya***". Organized by Department of Zoology, Sikkim University, Gangtok, Sikkim, India.	2023
2	Delivered an ***Oral Presentation*** in the International Conference on " Innovative Sustainable Practices in Science and Technology (ISPISAT-2022) on 26th November with titled "***Comperative study of microbial diversity and antibiotic resistance profile at various natural thermal zones of Sikkim Himalaya***". Organised by Shirish Madhukarrao Chaudhari College, Jalgaon, Maharashtra, India.	2022
3	Delivered an ***Oral Presentation*** at the 2nd International E-Conference on "Recent Advancement of Bio-molecule Inventions In Life Sciences" (RABILS-2020) on "***An assessment of drinking water quality: A Survey and Microbiological study of spring water of Sikkim Himalayan region***", held on 26th and 27th June 2020, Organized by Foundation for Innovative Research in Science and Technology, Nagercoil, Tamil Nadu, India.	2022
4	Presented a **Poster** at International web conference on Global Research Initiative for Sustainable Agriculture & Allied Sciences organized by GRISAAS-2020 from 20th-30th December 2020 with titled: "***Antibiotic Susceptibility Pattern in bacteria isolated from different natural thermal zones of Sikkim Himalaya***. Organised by Astha Foundation, M.P., India.	2020

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This is to Certify that Prof./Dr./Mr./Mrs. Santoshkumar

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has attended/presented a paper/ poster/ chaired a session in the International Conference on "Innovative Sustainable Practices in Science and Technology (ISPISAT-2022)" held at Jalgaon (M.S.) on 26th Nov. 2022. He / She has presented a paper entitled

Comparative study of microbial diversity and antibiotic resistance profile at various natural thermal zones of Sikkim, Himalaya.

Dr. Sandeep S. Joshi
(Convener)

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(RABILS-2020)

Certificate of Presentation

This is to certify that **Mr. Santosh Kumar** From *Sikkim university,* has presented a paper entitled ***An assessment of drinking water quality: A Survey and Microbiological study of spring water of Sikkim Himalayan region*** In the **2nd International E-Conference on "RecentAdvancement Of Biomolecule Inventions In Life Sciences" (RABILS-2020)** held on 26 th and 27 th June 2020 Organized by Foundation for Innovative Research in Science and Technology, Nagercoil.

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Programme Coordinator
Foundation for Innovative Research In Science and Technology ,Nagercoil .

Awards/Achievements

SL No.	Name of the Awards	Year
1	***Scientist Associate Award***, at the occasion of International web conference on "Global Research Initiative for sustainable Agriculture & Allied Sciences (GRISAAS-2020), Organised by Astha Foundation, M.P, India.	2020
2	***Young Scientists Award***, for the year 2019-2020, honored by Microbiologists Society India (Reg. No. MAH/4814/SAT)	2020
3	***Best Poster Presentation Award*** at International web conference on Global Research Initiative for Sustainable Agriculture & Allied Sciences organized by GRISAAS-2020 from 28th-30th December 2020.	2020

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& Allied Sciences (GRISAAS-2020)

during 28-30 December, 2020

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This is to Certify that

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East Champaran, Bihar.

Has secured State Level Young Scientist Award during Academic Year 2019-20

Dr. S. D. Patil
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GRISAAS-2020

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Certificate of Merit

This is to certify that the poster presented by **Santosh Kumar and Nagendra Thakur** entitled **Antibiotic susceptibility patterns in bacteria isolated from different natural thermal zones of Sikkim Himalaya** ***Best Poster Presentation Award*** in International web Conference (GRISAAS -2020) held during 28-30th December, 2020.

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