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ORIGINAL ARTICLE

Building the taxonomic profile of the Riniaie Marwah hot spring of Kishtwar in Jammu and Kashmir: the first high-throughput sequencing-based metagenome study

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ABSTRACT

Background and Objectives: Rinaie Marwah hot spring Kishtwar (RMHSK) is one of the geothermal springs located at 33°51′51″N 75°32′07″E with an elevation of 2134 meters above sea level in Jammu and Kashmir, India. We aimed to study the microbial diversity of this geothermal spring using metagenomics.

Materials and Methods: In the present study, physiochemical parameters including temperature (65-75°C), pH (6. 9-8. 8), hardness (250 ppm), and mineral content was measured along with the microbial diversity using Illumina MiSeq metagenome-based 16s amplicon sequencing (V3-V4). The sequence reads were classified taxonomically into 31 phyla, 71 classes, 152 orders, 256 families, 410 genus, and 665 species. QIIME 2 (Quantitative Insights into Microbial Ecology), an extensible, powerful, and decentralized analytical tool, was used for taxonomic analysis.

Results: *Bacteroidota* (32. 57%) was the dominant phylum, *Bacteroidia* (32. 51%) the dominant class, *Bacteroidales* (16. 6%) the dominant order, and *Lentimicrobiaceae* (14. 23%) was the dominant family per the abundance analysis. Shannon (2. 28) and Chao 1 (87. 0) diversity indices support the existence of higher microbial diversity in RMHSK (50717 OTUs).

Conclusion: The microbial diversity of RMHSK is reported for the first time through a metagenomic study. Identification of microorganisms with characteristics that are relevant to industries.

Keywords: Hot spring; Metagenome; Taxonomy; High throughput DNA sequencing; Illumina sequencing

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INTRODUCTION

India is home to several geothermal springs (1-3), some of which are very popular for their rejuvenating and disease-curing potentials. Around the world, natural therapeutic techniques such as thermal therapy and balneotherapy are frequently used to treat chronic aches and pains (4). The hot springs located in Jammu and Kashmir are part of North-Western Himalayan region. This region extends to the Himalayan Mountain, to southeast into the Indian states of Uttarakhand and Himachal Pradesh, and on through Tibet and Nepal, and to the northwest Punch, Gilgit, Hunza, Yasin valleys, and Northwest Province of Pakistan (5-7). All the major hot spring locations are clustered near major tectonic features caused by the collision of the Indian and Eurasia plates (8). Every ecological niche, including the tropics (9), the poles (10), glaciers (11), deserts (12) and underwater hydrothermal vents (13), harbor microbes owing to their adaptable nature due to a various molecular and cellular processes (14). Thermal springs are the hubs of microbial diversity that can be used to find new ORFs, genes, metabolites, and hydrolytic enzymes for use in industrial, medical, and agricultural activities (15, 16). Hot springs, the natural reservoirs of microorganisms with temperatures of >55°C and >80°C, are currently the subject of enormous interest. Hot springs were once thought to be sterile, but A landmark discovery of *Thermus aquaticus* from a hydrothermal environment has completely changed our understanding of the microbial richness of hot springs (17, 18). Annual reports on the diversity of microorganisms demonstrate how the microbes have evolved over the course of 3.8 billion years. To better understand microbial diversity, researcher's world over started to examine similar habitats using a culture-dependent methodology. The standard culture-dependent approach has been used for several decades to analyze the microbial diversity, but it has several drawbacks as well (19). The findings, which reveal that many microorganisms had previously gone unnoticed, contribute significantly to the interest in metagenomics. *Emticicia*, a novel genus, was reported from an Indian hot spring by the application of metagenomics (20). A novel species of *Thiomonas bhubaneswarensis,* a moderately thermophilic thiosulfate oxidizer, was discovered in an Atri hot spring in Bhubaneswar, India (21). Depending on the molecular phylogeny of the small-subunit ribosomal RNA gene (16S rRNA), the high-throughput sequencing (HTS), metagenomics (MGs) enables culture-free identification of the microbial diversity (22, 23). This technique has been crucial in recent years for understanding the microbial diversity and their role in the biogeochemical cycles (24, 25). Rupasinghe and his associates used Illumina MiSeq sequencing of the 16S rRNA gene's V3-V4 region to assess the bacterial diversity of six hot water spring clusters in Sri Lanka (26). Utilizing the QIIME2 metagenomics workflow, bacterial abundance measurements and diversity statistics can be evaluated (27-29). QIIME 2 is a platform for microbiome data science. It is an enhanced version of QIIME that offers fresh features for the evaluation of the upcoming microbiome research. Based on a plug-in architecture, QIIME 2 is created with the ability for third parties to contribute. functionality for the most recent tools for sequence quality control from various sequencing platforms, taxonomy assignment, and phylogenetic insertion, QIIME 2 plug-ins are available that significantly outperform QIIME and other tools. The use of paired samples and time series analysis of the microbiome, which is essential for determining how treatments affect the microbiome, as well as machine learning, which includes the application of previously trained models to new data and querying models to find key microbiome features, are other qualitatively new functions supported by plug-ins. Numerous new interactive visualization tools in QIIME 2 make it easier to conduct exploratory analyses and report results. Similar types of studies were carried out by many other research groups at different sites like Borong and Polok hot springs of Sikkim (30), Crater lake of Mexico (31), Manikaran hot spring of Himachal Pradesh India (32), Deulajahri hot spring of Odisha, India (33), Villa Luz caves of Mexico (34), and Lasundra hot spring of Gujarat India (35). In the current study, we carried out the microbial analysis of an Indian hot spring (33°51′51″N 75°32′07″E) situated 2134 meters above the sea-level using culture-independent metagenomic approach. The physiochemical parameters of the hot spring have also been analyzed.

MATERIALS AND METHODS

Ethical approval and consent to participate. This article does not contain any studies with human participants or animals performed by any of the authors

and does not require ethical approval and consent to participate.

Sample-site description and sampling. The Rinaie Marwah hot spring Kishtwar (RMHSK) is in India's union territory of J&K. As illustrated in Fig. 1 the Warwan valley, Ladakh, and Zanskar to the southwest, Chatroo and Kishtwar to the south, and Anantnag to the west, all encircle Marwah. The largest tributary of the river Chenab, the Marusudar, drains the region. The Warwan Valley is a Himalayan sub-valley and tehsil in India's union territory of Jammu and Kashmir's Kishtwar District, which is part of the Jammu division. The Valley is 150 miles from Srinagar, the J & K summer capital, and 68 miles northeast of Kishtwar. It is governed by the Kishtwar district in the J & K division of Marwah. In the Indian union territory of J & K, Kishtwar serves as the town, municipality, and administrative center of the Kishtwar District. The districts of Anantnag and Doda border Kishtwar on the west and northwest, the Himachal Pradesh district of Chamba on the south, and the Ladakhi district of Kargil on the east and northeast.

Sample collection and physiochemical parameters. Soil samples were meticulously collected from the RMHSK site and placed into autoclaved oakridge tubes to maintain their sterility. Extreme care was exercised during the sampling process to prevent any contamination from the surrounding environment. Concurrently, critical physiochemical parameters were documented at the precise moment of sample acquisition. These included the measurement of water temperature and pH using a portable digital thermometer and a pH meter, respectively. Additionally, the levels of conductivity and turbidity were assessed using finely calibrated equipment, ensuring precision and accuracy. Alkalinity was determined by employing titration techniques in accordance with established laboratory procedures. Furthermore, an exhaustive analysis of various chemical constituents was conducted. Concentrations of sulphates, bicarbonates, sodium, potassium, silica, and calcium were quantified through rigorous scientific methods. These procedures were executed to maintain the integrity of the samples and provide comprehensive insights into the soil's physicochemical properties, contributing to the overall scientific understanding of the RMHSK site's environmental conditions.

Metagenomic DNA (mDNA) extraction, quantification, and PCR amplification. Following the initial sample collection, careful steps were taken to bring the samples to room temperature, an essential precondition for subsequent molecular processing. Subsequently, the samples were subjected to precise lysis at a controlled temperature of 65°C, with the addition of a specialized lysis buffer, designed to effectively disrupt cellular structures and liberate the DNA of interest from the complex environmental

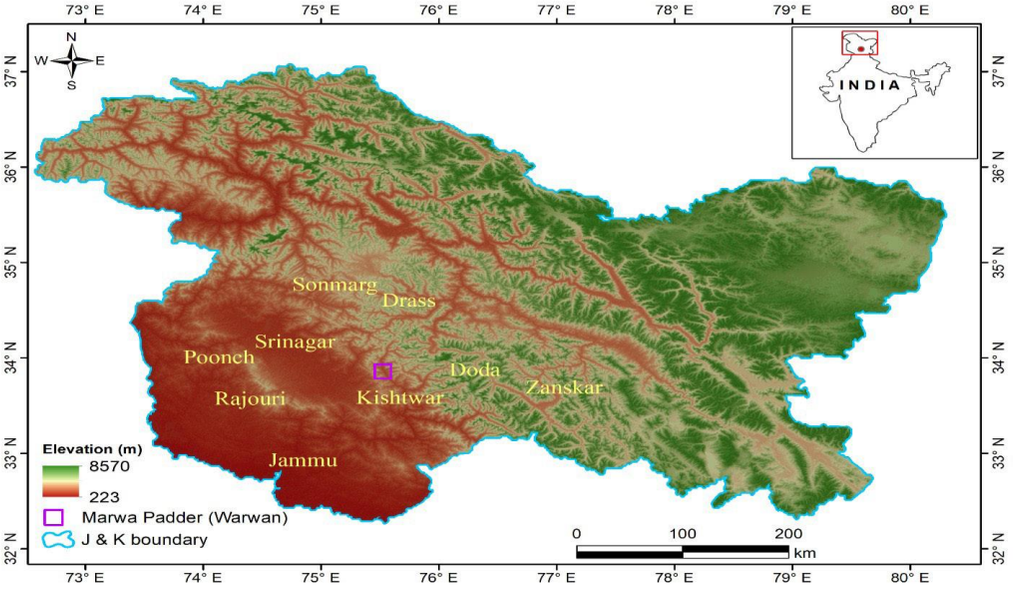

Fig. 1. Geographic location of the Rinaie Marwah hot spring Kishtwar (RMHSK)

matrices. A pivotal step in the protocol involved the utilization of bead beating, a mechanical disruption method that facilitated the efficient release of DNA from the sample, further enhancing the quality of the extracted genetic material. The extracted DNA was then skillfully bound to a dedicated matrix, followed by a series of meticulous washing steps, which served to effectively eliminate impurities, including contaminants and cellular debris, thereby elevating the overall purity of the isolated DNA. Following these critical processing steps, the purified metagenomic DNA was carefully eluted from the matrix, resulting in a high-quality DNA sample, which was subsequently subjected to post-extraction DNA quantification and purity assessment.

The mDNA quantity was measured using Qubit® (Sigma, India) 4. 0 fluorimeter and DNA were amplified with 16S rRNA-specific primers (8F and 1492R). The optimized PCR conditions were, initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 69°C for 30s. The primers used for amplification were 5′TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGAGGCAGCAG3′ (341F-ADA), and 5′GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGATTACCGCGGCTGCTGGC3′ (534R-ADA). PCR products were purified by using Ampure XP (Beckman, USA) beads. The purified amplicons were subjected to index PCR using Nextera XT indices kit (Illumina, USA). The resulting indexed amplicons were purified using AmPure XP beads and checked on Agilent tapeStation. Libraries were quantified using Qubit HS and qPCR mix pooled together for sequencing. The amplified libraries were analyzed on TapeStation 4150 (Agilent Technologies) using High Sensitivity D1000 ScreenTape® (Agilent, India) as per manufacturer's instructions.

Cluster generation and sequencing. The sequencer was strategically loaded with pooled PCR products, which effectively constituted the library of DNA fragments derived from the samples of interest. The objective was to generate distinct clusters of DNA fragments on the surface of the flow cell by facilitating their hybridization with complementary oligonucleotides immobilized on the flow cell's surface. A technique known as bridge amplification was employed. The actual sequencing of the DNA fragments within the established clonal clusters was performed. For this purpose, a PE300 kit (Scientifica, India) was utilized, allowing for paired-end sequencing with a read length of 300 bases. This sequencing process was carried out on the Miseq platform, a renowned and sophisticated sequencing instrument renowned for its accuracy and reliability (36). The sequencing was carried out using a PE300 kit on the Miseq platform, enabling paired-end sequencing with a read length of 300 bases. This comprehensive process is crucial for generating high-quality sequence data, particularly in applications like 16S sequencing and metagenomics research.

Analysis of amplicon reads. The demultiplexed paired end fastq data were imported as QIIME 2 artifacts using demux plug-inplug-in (https://github.com/qiime2/q2-demux). The cutadapt (https://github.com/qiime2/q2-cutadapt) was used for the removal of adapter sequences, primers, and other unwanted sequences from the sequence data. Filtered reads were used as input for further QIIME2 analysis (Table 1). The forward and reverse reads were joined into single sequence using Vsearch (https://github.com/torognes/vsearch). The reads were denoised to get the correct amplicon sequence variants (ASVs) with a 16S reference as positive filter. The reference was used to only assess whether each sequence is like- ly to be 16S alignment using SortMeRNA (https://github.com/sortmerna/sortmerna) with a permissive e-value. However, the reference is not used to characterize the sequences. The quality control of sequences was maintained by using deblur (www.github.com/biocore/deblur/blob/master/README.md). Deblur is a greedy deconvolution algorithm based on Illumina Miseq/Hiseq error profiles for amplicon sequencing.

Taxonomy analysis. Raw sequence quality was checked based on GC distribution, average base content, and base quality score distributions. Singletons, the OTUs that did not cluster with other sequences, were eliminated because they could lead to errors and produce spurious OTUs. With the aid of the clustering program UCLUST, the pre-processed consensus V3 sequences were organized into OTUs, at a similarity threshold of 0. 97, and chimaeras were also eliminated using UCHIME (www.drive5.com/usearch/manual/uchime_algo.html). Using the QIIME programme to create a representative sequence for each OTU, all the pre-processed reads were used to identify the OTUs. Using the PyNAST (https://github.com/topics/microbiology) program, the representative sequence was finally aligned

Table 1. QIIME2 plug-ins used for analysis

Plug-in	Function
Demux	Demultiplexing and viewing sequence quality
Cutadapt	Removing adapter sequences, primers, and other unwanted sequence from sequence data
Vsearch	Joining paired-end clustering
Quality-filter	Phred-based filtering and trimming
Deblur	Sequence quality control
Feature-table	Working with sample by feature tables
Feature-classifier	Taxonomic classification Extending
Fragment insertion	Phylogenies
Diversity	Exploring community diversity
Q2-Krona	Plotting Krona graph

with the reference (88% OTUs from Greengenes 13_8) (37). The "classify-sklearn", a pre-fitted sklearn-based taxonomy classifier, was used to assign taxonomy to ASVs against V3-V4 region of SILVA 132 database (27). The stacked bar chart of taxa relative abundance was generated. This was followed by OTU heat map generation at genus level, determination of rarefaction curves, calculation of diversity metrics (α-diversity), and plotting of Krona graph using feature table and taxa information (38).

RESULTS

Physiochemical analysis. The physiochemical parameters of RMHSK are mentioned in Table 2. The recorded temperature was found to be in the range of 65-75°C. The pH, electrical conductivity (EC), turbidity, dissolved oxygen (DO), and hardness observed were in accordance with the permissible values set by Central Pollution Control Board (India), and WHO.

mDNA extraction, quantification, and sequencing. The concentration of isolated mDNA from RMHSK was found to be 57. 8 ng/µl with a yield of 2. 312 µg and subsequently used for PCR amplification. The libraries prepared from the mDNA using Nextera XT Indices kit were of good quality and was labeled as KKMG. The V3-V4 region amplicons of the 16S rDNA segment were used to create the metagenomic sequencing libraries, which contained 611 bp for KKMG. The 16S metagenome sequencing libraries were sequenced on Illumina Miseq platform (2 × 300 bp chemistry) to generate ~60-100 MB data/Sample. The sequencer flash assembler generated 201, 902 flash reads out of total 403, 804 reads.

Taxonomy distribution. The results for phylum abundance indicated that *Bacteroidota* were prevalent in the amplicon library. In KKMG, 50717 OTUs represented distinct phyla dominated by *Bacteroidota* (32. 57%), *Firmicutes* (31. 33%), *Proteobacteria* (8. 28%), *Campilobacterota* (6. 34%), *Spirochaetota* (6. 21%), and *Bdellovibrionota* (3. 91%). The class is dominated by *Bacteroidia* (32. 51%), *Clostridia* (22. 28%), *Campylobacteria* (6. 34%), *Spirochaetia* (5. 76%), *Gammaproteobacteria* (5. 48%), *Limnochordia* (4. 34%), *Alphaproteobacteria* (2. 79%), and *Oligoflexia* (2. 58%). The taxonomic order is dominated by *Bacteroidales* (16. 6%), and *Sphingobacteriales* (14. 55%). The top ten phyla, class, and order are represented in Fig. 2.

The dominant families in RMHSK under the order of *Bacteroidales* and *Sphingobacteriales* based on 16S amplicon sequencing was found to be *Lentimicrobiaceae* (14. 23%), and *Clostridia_vandinBB60* (13. 31%). The results are also supported by previous 16s studies conducted on geothermal springs. As shown in Fig. 3. plenty of the species belonging to dominant genus were either part of metagenome data-sets, uniden-

Table 2. Physiochemical parameters of RMHSK

Parameter	Temperature air (ºC)	Temperature water (ºC)	pH	Alkalinity (ppm)	EC (µS/cm)	Hardness (ppm)	DO (ppm)	$SO_{42}-$ (ppm)	HCO_3- (ppm)	Na^+ (ppm)	K^+ (ppm)	Ca2+ Ppm
Value	20	65-75	6. 9-8. 8	500-550	500-520	250	5. 3	75-80	253	10-12	3. 5-4	75-80

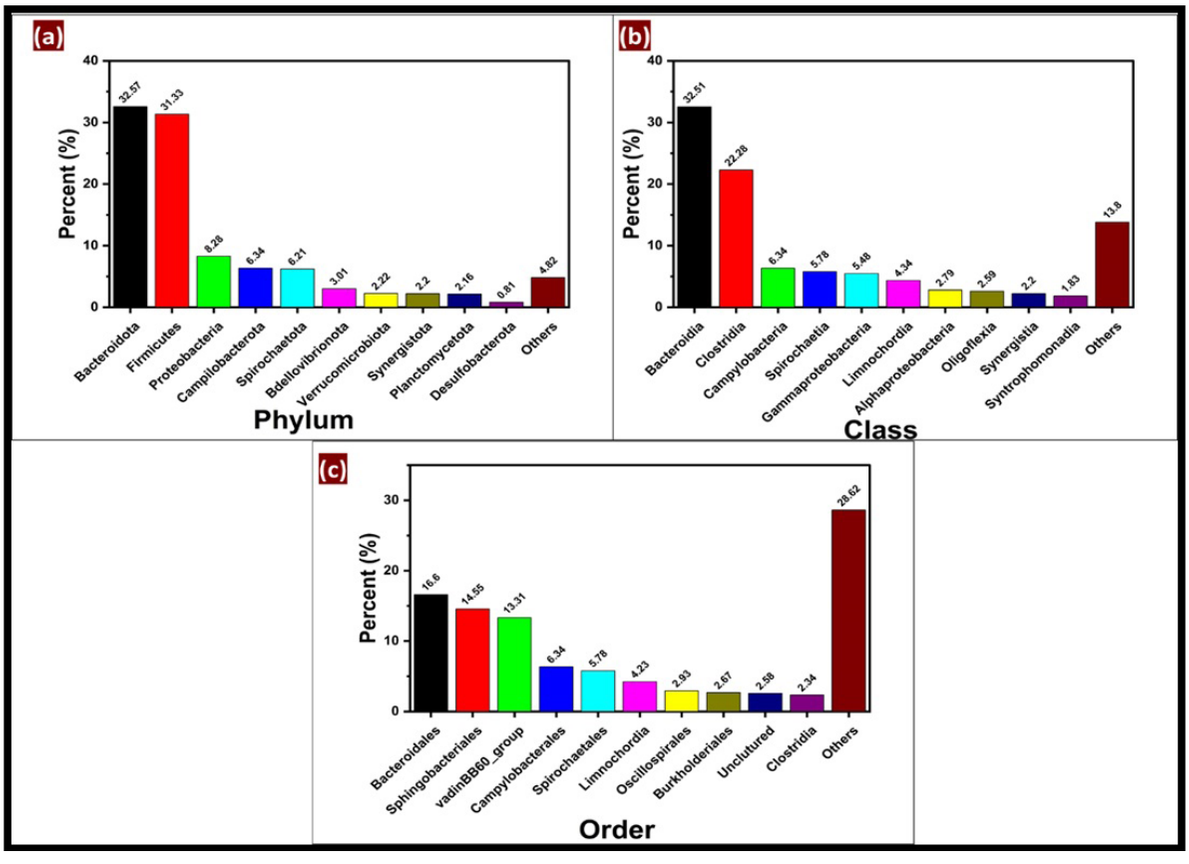

Fig. 2. Top ten Phyla, class, and order in RMHSK on the basis of 16s amplicon sequencing

tified, and/or uncultured. Feature-table plug-in having heat map module was used for generation of feature heat map. For this, feature table was first collapsed at genus level. Heatmap representation of a feature table with clustering on features and samples axes was done after normalizing the feature Table by adding a pseudo-count of 1 and then taking the log10 of the Table.

Alpha-diversity, and rarefaction plot. A feature table is used to calculate diversity within a sample and provides information on species richness. Using various metrics, α-diversity uses a single number to represent the diversity of organisms in a sample. Different indices of α-diversity including Shannon index entropy and chao1 index were calculated and found to be 2. 28728086 and 87. 0 respectively.

Based on the creation of rarefaction curves, rarefaction enables the calculation of species richness for a given number of individual samples. The curve presented in (Fig. 4) showed the number of species is plotted as a function of samples. The steep slope suggests that there is still much to learn about the diversity of species. The community's diversity is shown on the vertical axis, and the number of sequences taken into account for the diversity calculation is shown on the horizontal axis.

Krona graph. Within a Web browser, Krona is an interactive visualization tool for investigating the make-up of metagenomes. Through the use of zoomable, multi-layered pie charts, Krona enables the exploration of hierarchical metagenome data. Krona graph was generated from feature table and taxonomy assigned by qiime2. Krona for sample KKMG of RMHSK is given in (Fig. 5).

DISCUSSION

The hot spring refers to a groundwater body that is mineral-rich and with temperature above 50°C. Although hot spring water is typically clear, it contains a variety of minerals that are dissolved from the rocks as it travels to the surface, including calcium, chloride, magnesium, sodium, silica, and sulfates (39). Hot springs have a relatively higher con-

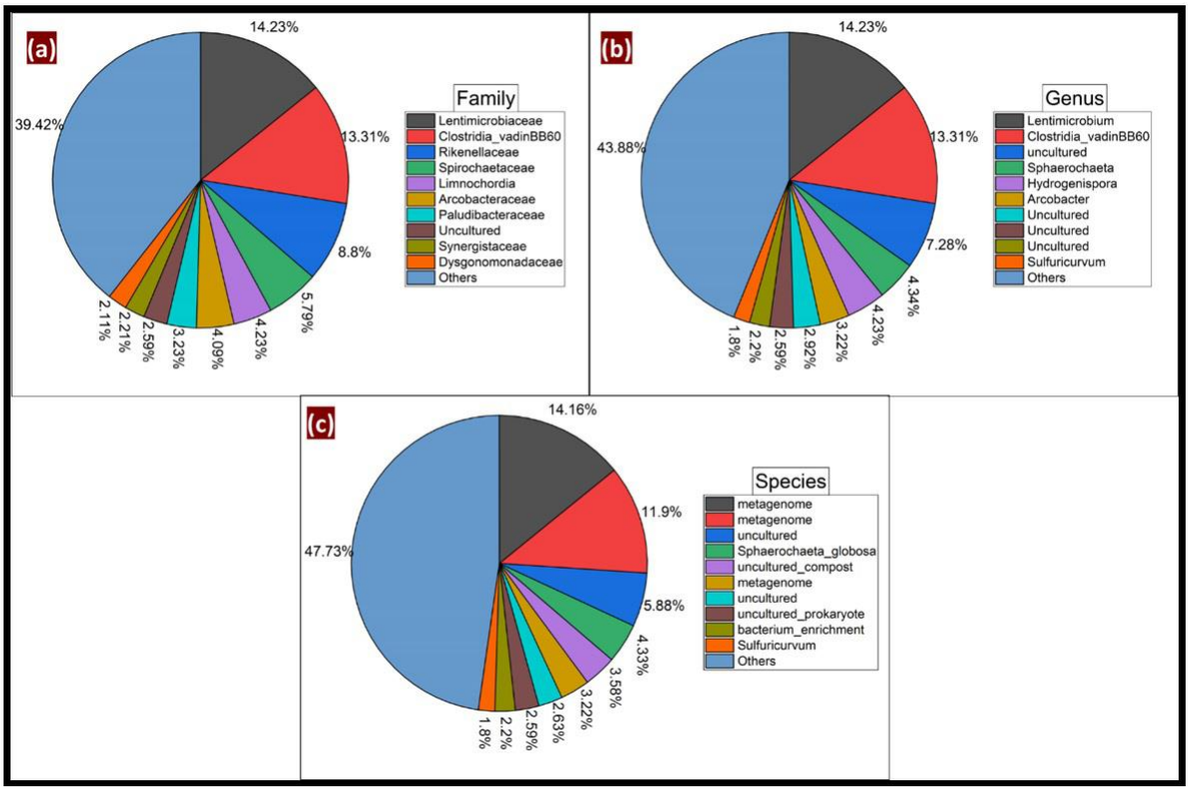

Fig. 3. Dominant family, genus, and species in RMHSK based on 16s amplicon sequencing.

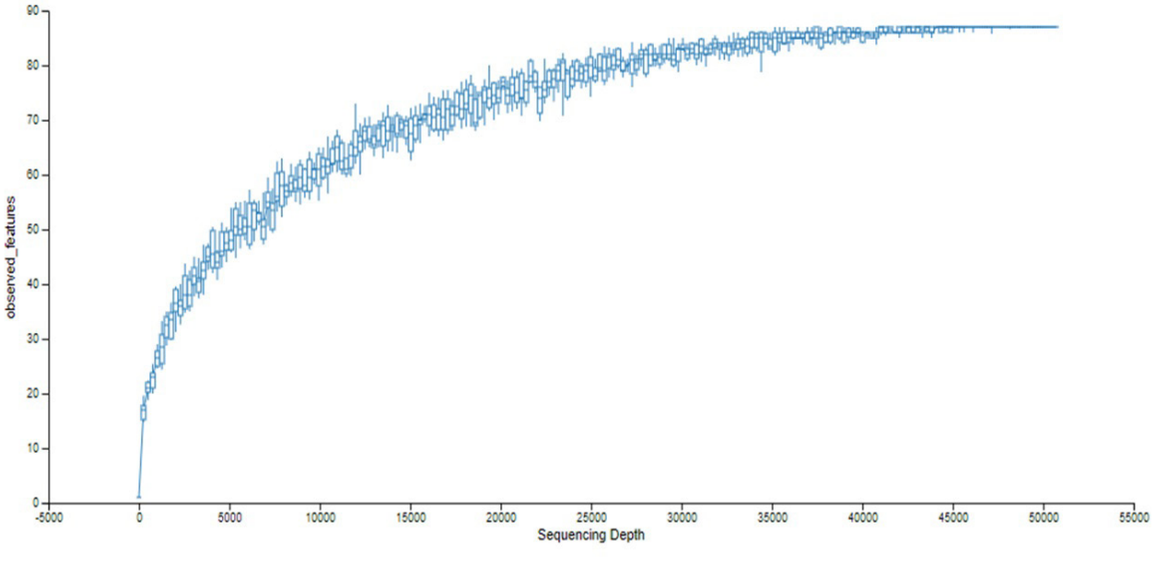

Fig. 4. OTU observation through rarefaction analysis of RMKHS

centration of these minerals than non-geothermal groundwater. The main ions are calcium, magnesium, potassium, fluorides, and bicarbonates, which have been found to be beneficial for the digestive system, blood acid-base balance, bone mineralization, vein diseases, arthritis, and cardiovascular diseases (40). Third-generation technologies are now widely available, making high-throughput sequencing of the entire 16S gene feasible. A gene can now be distinguished between millions of sequence reads that

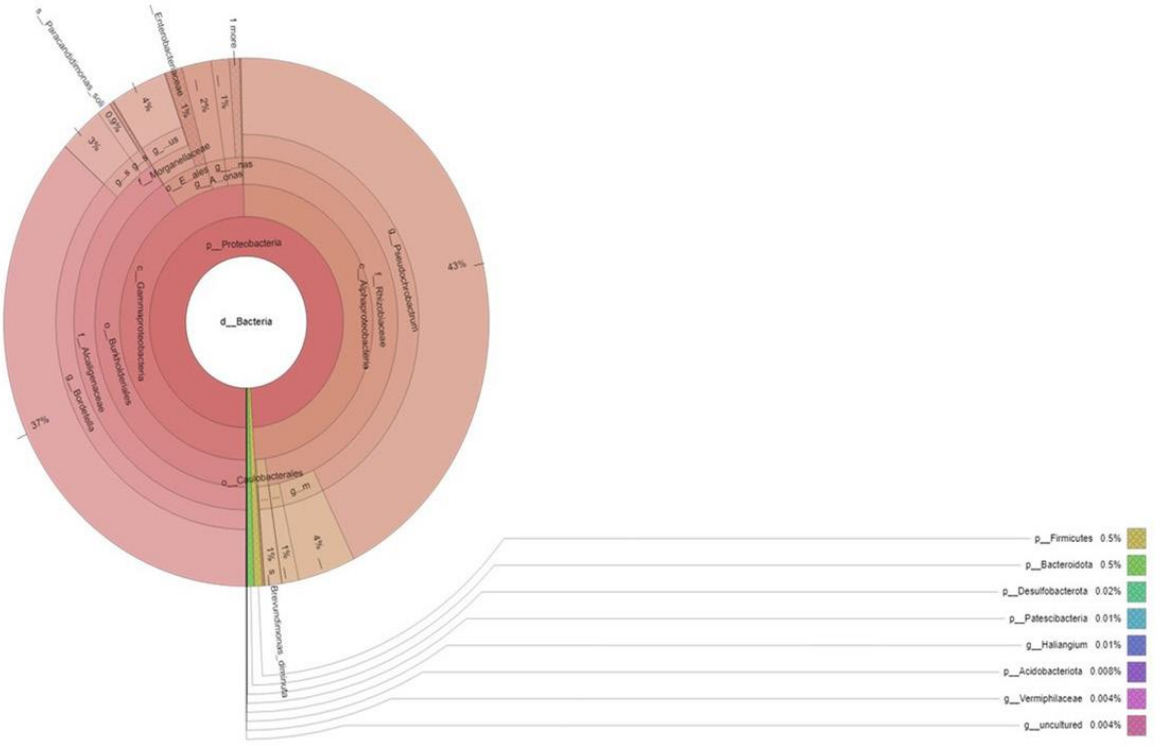

Fig. 5. Microbial community composition of RMHSK by 16S amplicon library sequencing

differ by as little as one nucleotide thanks to circular consensus sequencing and sophisticated denoising algorithms that eliminate PCR and sequencing error. Together, these methodological and technological advancements make it possible to fully utilize 16S distinguishing potential in a high-throughput manner time. This study explored the microbial diversity associated with RMHSK using culture-independent 16S amplicon sequencing. Taxonomical composition revealed that the predominant taxa were *Bacteroidota*, *Firmicutes*, *Proteobacteria*, and *Campilobacterota*. Their prevalence has also been documented in the past from a variety of harsh environments using both culture-dependent and independent techniques (41, 42). *Firmicutes* and *Proteobacteria* potentially exhibit a plausible positive correlation with elevated temperatures owing to their well-established thermophilic characteristics. The pH levels within the hot spring are recognized as influential determinants impacting the distribution of *Campilobacterota* and *Spirochaetota*, with the former demonstrating an affinity for acidic conditions and the latter for alkaline environments. *Bacteroidota's* association with nutrient-rich habitats suggests a potential correlation between the abundance of this taxon and nutrient availability. Furthermore, the prevalence of *Bdellovibrionota* appears to be linked to the availability of potential prey organisms, which is a pertinent factor governing its distribution. The geochemical composition and microenvironmental heterogeneity play pivotal roles in shaping the microbial assemblage, with specific taxa exhibiting preferences for particular ions or ecological niches. Additionally, the consideration of microbial interaction networks and the evaluation of seasonal variations contribute significantly to our comprehensive comprehension of microbial adaptation strategies and the multifaceted ecological roles enacted within the dynamic milieu of the Riniaie Marwah Hot Spring ecosystem. The bacterial families with the greatest relative abundances within these dominant phyla were *Lentimicrobiaceae*, *Clostridia_vandinBB60*, *Rikinellaceae*, and *Spirochaetaceae*. In general, the most common bacterial genus observed in this study was found to be *Lentimicrobium*. The presence of *Lentimicrobium* has been frequently reported from the other hot springs around the world (43, 44). In this study, we examined the distributions of microbial communities in soil samples from RMHSK using a practical method. We used the MoBio kit to analyze the hy-

pervariable regions of the 16S rRNA to ascertain the taxonomic designation, abundance, and distribution of the microbial communities in RMHSK. The identification of metagenome sequences belonging to the genera *Lentimicrobium, Clostridia, Hydrogenispora,* and *Arcobacter* in a metagenomic study signifies the presence of diverse microbial communities within the studied environment. These genera have distinct metabolic capabilities and potential ecological roles. *Lentimicrobium* and *Clostridia* are likely involved in organic matter decomposition, impacting carbon and nutrient cycling. *Hydrogenispora* may contribute to hydrogen gas production, affecting redox reactions and energy metabolism. *Arcobacter,* with both pathogenic and non-pathogenic species, warrants further investigation for its role in water quality and ecosystem health. These findings highlight the ecosystem's microbial diversity and functional potential, calling for deeper analyses to understand their specific roles and interactions within the environment.

The QIIME2 package was used to predict the bacterial functionalities based on 16S rRNA profiles to highlight any potentially intriguing bacterial functionalities that open perspectives for in-depth future soil microbiome. Several microorganisms reported in the study remain unknown owing to the limitations in 16S amplicon sequencing. The structure of complex microbial communities can be better understood through network analysis, and this knowledge is especially useful in environments where many microbial taxa's basic ecology and life history strategies are unknown. Furthermore, it helps to identify the niches that community members share, direct symbioses between community members, potential biotic interactions, habitat affinities, or shared physiologies that may help focus research or experimental settings. The improved taxonomic resolution required to reflect species cannot be obtained by finer clustering of the sub-regions. Although some sub-regions approximate 16S diversity well, the majority lack the sequence diversity to distinguish between closely related taxa. Studies on hot springs in the past have mainly concentrated on microbial enrichment and isolation with regard to the microbiology of the studied springs. Numerous thermophilic bacteria were later found in numerous springs. Most of the bacteria that have been isolated from the hot springs are members of the genera *Bacillus, Geobacillus,* and *Anoxybacillus* (45-47). However, as predicted, the culture-independent, metagenomics approach revealed a higher diversity of microorganisms. A significant number of bacterial phyla that had not previously been described in the studied springs were found in the current RMHSK metagenomic analysis. The observation that rarefaction curve is not saturated suggests several significant implications. Firstly, it indicates the presence of unexplored microbial diversity within the hot spring, potentially comprising rare or low-abundance taxa that were not adequately sampled in the initial sequencing effort. This highlights the need for deeper sequencing to uncover hidden microbial species, thus providing a more comprehensive understanding of the ecosystem's complexity, ecological roles, and potential biotechnological applications. Additionally, it underscores the scientific rigor of the study, acknowledging the potential for further investigation to refine our knowledge of this unique and diverse microbial community.

CONCLUSION

The RMHSK of the North-Western Himalayas is home to many possibly unknown and novel microbes as indicated by the presence of many unknown OTUs. The primary goal of the current metagenomic study was to examine the community structure of the microflora connected to a hot spring in the North-Western Himalayas. The unidentified microbes were also revealed using 16S amplicon based metagenomic study. The RMHSK, an ecologically rich niche, possesses higher proportions of ecologically significant *Firmicutes* and *Bacteroidota*. As 16S rDNA amplicon sequencing has its limitations, in-depth metagenomic studies are necessary to clarify the true extent of variations caused by seasonal change and anthropogenic pressures. However, the results of this study can be used as a case study in the future to support the findings of the hot spring microbiome research. The taxonomic analysis clearly reflects the diversity of microorganisms, and it is crucial to investigate how these microbes function. Thus, recommending the preservation of such ecologically and functionally diverse ecosystems and offering a wide range of opportunities for exploring secondary metabolites and bioremediation agents that are significant for industry. Consequently, based on these presumptions, it can be said that climate change will undoubtedly have an impact on the microbial diversity of such ecologically diverse environments.

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