Title: Microbial diversity associated with the natural spring water of Western Himalayas

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Abstract

Springs in the Himalayan landscape are vital sources of clean, potable water for millions of inhabitants who use it for domestic and farming purposes. The rise in urbanization, pilgrimage and various other anthropogenic activities invariably results in microbial contamination. In this

study, we conducted a microbiological examination of water samples collected from four distinct Himalayan Mountain springs located in the Rudraprayag district. We employed targeted metagenomics, using 16S rRNA and Internal Transcribed Spacer (ITS) gene analysis, to elucidate the bacterial and fungal composition. Cumulatively, the high-throughput sequencing data from all four springs revealed the abundance of *Proteobacteria* (35.38%), followed by *Firmicutes* (22.47%), *Acidobacteriota* (14.73%), and *Actinobacteriota* (5.35%). Genus level analysis of bacterial communities revealed the prevalence of *Tumebacillus* (13.85%) and *Massilia* (12.97%). Mycobiome analysis demonstrated the dominance of the fungal phyla *Ascomycota* (72.11%), followed by *Basidiomycota* (16.04%), *Mortierellomycota* (9.21%), and *Chytridiomycota* (1.2%). We further analysed the functional composition of microbial communities using marker gene (16S rRNA) data through PICRUSt and the KEGG database, identifying the gene abundance associated with carbon metabolism.

Keywords: Western Himalayas, Water springs, Targeted metagenomics, 16S rRNA gene, Fungal communities

Introduction

Natural water springs represent intricate ecosystems formed by groundwater surfacing at the terrestrial landscape interface. They are vital as they fulfil a crucial role in addressing the mounting demand for potable water among millions of people, particularly in rural areas lacking water supply infrastructure (Springer et al., 2009; Sharma et al., 2022; Chhimwal et al., 2022). High-altitude water springs are home to diverse flora and fauna and represent unique ecosystems for various groups of microorganisms. The Indian Western Himalayan (IWH) region encompasses approximately 1.69% of the total area of the country, constituting 10.54% of the Himalayan landscape. The elevation in the IWH region ranges from 90 meters to over 7000 meters above sea level (Kumar et al., 2021). It is known as one of the biodiversity hotspots, with a wide array of ecosystems such as hot springs, glaciers, forests, wetlands, and pristine rivers (Sharma et al., 2014; Rambia et al., 2023; Chaudhari et al., 2020). Springs in the Himalayas are an important source of perennial high-quality freshwater for remote communities. The majority of the population (60-70%) living in the Himalayan region heavily depends on these natural water sources (Verma et al., 2022).

The intensification of water scarcity, driven by climate change and growing global water requirements, presents an escalating concern. Freshwater shortage is acknowledged as a substantial risk to worldwide public health. According to the United Nations, population growth is outpacing water supply, resulting in almost half of the population being deprived of water for at least one month each year, which contributes to the rise in global temperatures due to climate change (Martin, 2023). A recent study has reported that 8-10 major cities and 500 expanding townships in the Himalayas have compromised these essential water resources, creating an extensive water issue (Verma et al., 2022). The water quality of Himalayan rivers and springs

has been deteriorating over time due to several anthropogenic activities, including contamination from untreated and treated effluents, poorly managed drainage systems, agricultural runoff, tourism, pilgrimage, and dilapidated sewerage systems (Sharma et al., 2023; Gupta et al., 2023; Li et al., 2020). Due to the lack of adequate sewage treatment facilities, there is not only an increase in the levels of coliforms but also a rise in antimicrobial-resistant bacteria along downstream reaches, especially those within the lower Jhelum and Dara watershed. This could jeopardize water quality and public health (Qayoom et al., 2023; Sharma et al., 2023; Jani et al., 2021). Given the factors contributing to water quality deterioration and the influx of contaminants, understanding the microbial diversity in natural springs becomes imperative. It is crucial to identify potential pathogens and assess the overall microbial diversity in these ecosystems to mitigate associated risks.

Nowadays, with the emergence of high-throughput sequencing technology, it has become easy to assess the complete microbial diversity structure with unprecedented depth and accuracy. This method not only reveals the richness of microbial diversity but also sheds light on the functional roles of these organisms in the ecosystem (Das et al., 2023). The advancement, cost-effectiveness, and expansive coverage of high-throughput sequencing techniques have significantly facilitated detailed investigations of microbial communities (Devi et al., 2021). Leveraging these advancements, we conducted a targeted metagenomics study to analyze the microbial diversity of four springs in the Indian Western Himalayas. This approach aimed to unveil their overall microbial composition, highlight the presence of indigenous microflora, and assess whether the observed diversity poses potential risks to human health and the environment.

Materials and Methods

Sampling points

Water samples were collected in triplicate from four distinct springs at different altitudes. These samples were maintained within a cold chain until transported to the laboratory for community DNA extraction. The details of the samples, along with their geographical locations, are provided in Table 1.

Community DNA extraction and amplicon sequencing.

To perform community DNA extraction of the water samples, 500 mL of collected spring water was filtered through 0.22-µm sterile filter paper (Jani et al., 2021). These filters were then used for community DNA extraction using the DNeasy PowerSoil Kit (Qiagen) according to the manufacturer's guidelines. The V4 region of the 16S rRNA gene was amplified using the 515F/806R primer set, as used in the study by Jani et al. (2022). The ITS1 region was amplified using the ITS1F/ITS2R primer set, as mentioned by Jiya et al. (2023). Template and library preparation were performed following Illumina 16S and ITS-targeted amplicon sequencing protocols. Subsequently, barcoded libraries were equimolarly pooled and sequenced on the

Illumina MiSeq platform using 2 × 250 bp paired-end reads with v2/v4 chemistry. The sequences are available on the NCBI SRA portal under BioProject ID PRJNA1048161.

Bioinformatics analysis

Initial quality assessment of Illumina-generated raw paired-end reads was conducted using the FastQC tool. Sequence analysis was carried out using the DADA2 package (version 1.8), involving filtering, trimming, denoising, dereplication, and chimera removal, which resulted in amplicon sequence variants (ASVs) (Callahan et al., 2016). Taxonomic classification of ASVs was done using the SILVA (v1.38) (Quast et al., 2013) and UNITE (Abarenkov et al., 2023) reference databases for bacterial and fungal communities, respectively. The resulting ASVs and taxonomic tables were used for diversity and compositional analyses. Statistical analyses were conducted in R, leveraging relevant bioinformatics packages such as phyloseq (McMurdie and Holmes, 2013), ggplot2 (Wickham, 2016), corrplot, and vegan (Oksanen et al., 2013). A Venn diagram was constructed using Venny (v.2.1a), a web-based tool, to represent common and unique ASVs within the samples (Oliveros, 2007-2015).

Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt)

To discern metabolic pathways, metagenomic functional genes, and enzymatic reactions potentially associated with the spring samples, PICRUSt2 analysis was conducted on the 16S rRNA gene dataset. The ASV dataset underwent pre-processing to eliminate rare ASVs and singletons that could introduce noise into the analysis, along with the removal of low-depth samples. The PICRUSt2 pipeline (version 2.3.0_b) was subsequently executed on the resulting 16S rRNA sequence data table following the methodology outlined in Douglas et al. (2020). The data obtained was analysed at the KEGG (Kyoto Encyclopedia of Genes and Genomes), with primary focus on Carbon, Nitrogen, Sulphur and Methane utilisation pathways.

Results and discussion

Water is crucial for life, yet accessing it in the Himalayan regions is challenging due to uneven landscapes, limited infrastructure, and remote communities. The increasing population and pilgrimage not only lead to water scarcity but also raise concerns about the safety and hygiene of these water bodies, necessitating continuous monitoring. Therefore, it is important to perform microbiological examinations to identify bacterial communities that pose potential safety threats. Significant volumes of human-associated waste can lead to the emergence of pathogens, making such examinations critical. In the current study, targeted metagenomics sequencing yielded a total of 359,473 and 107,408 raw reads for bacterial and fungal sequences, respectively. Following quality checks, trimming, and chimera removal, 266,443 (74.12%) and 82,356 (76.67%) reads were retained for bacterial and fungal sequences, respectively (Sup Table 1).

These high-quality sequences were further assigned to 3,232 bacterial and 1,828 fungal ASVs. Alpha and beta diversity analyses were conducted to assess the microbial diversity and richness of spring water samples collected from different altitudes. Bacterial communities exhibited the highest richness in Sp3 with a Chao1 index of 1298, whereas Sp4 had the lowest at 469 (Fig. 1a). The Shannon and Simpson indices ranged from 4.57 to 6.66 and 0.97 to 1.00, respectively. Sp3 exhibited the highest Shannon and Simpson index values (6.66 and 1.00), followed by Sp2, Sp1, and Sp4, which had the lowest (4.67 and 0.98). Conversely, the mycobiome diversity was highest in Sp1, followed by Sp2, Sp4, and Sp3. The Chao1 estimator was highest in Sp1 (1207) and lowest in Sp3 (87) (Sup Table 2). Shannon and Simpson index values for fungi were: Sp1 (5.34, 0.98), Sp2 (4.2, 0.94), Sp4 (1.99, 0.69), and Sp3 (1.29, 0.42) (Fig. 1b).

Notably, among spring water samples from different altitudes, Sp3 exhibited the highest bacterial diversity, while fungal diversity was lowest in Sp3. The alpha diversity indices highlighted these differences across the samples. Furthermore, the NMDS plot effectively portrays beta diversity (Sup Fig. 1), illustrating the dissimilarities in microbial community composition. The dispersion and proximity of the samples depict the greater dissimilarity between the bacterial and fungal communities (Sup Fig. 1(a) and (b)). Alpha and beta diversity analyses unveiled the heterogeneity within spring water samples, providing valuable insights into the unique ecological characteristics of microbial communities at different altitudes. Detailed sequence analysis revealed the dominance of bacterial phyla *Proteobacteria* (35.38%), *Firmicutes* (22.47%), *Acidobacteriota* (14.73%), *Actinobacteriota* (5.35%), *Chloroflexi* (4.75%), *Bacteroidota* (3.80%), and Planctomycetota (2.24%) (Fig. 2a). Proteobacteria were dominant across all springs, comprising 44.92% in Sp1, 40.73% in Sp2, 19.12% in Sp3, and 36.78% in Sp4. Firmicutes showed notable presence, particularly in Sp1 (31.82%) and Sp4 (46.03%). *Acidobacteriota*, *Actinobacteriota*, and *Chloroflexi* displayed diverse abundance patterns, highlighting the intricate microbial composition within these high-altitude Himalayan springs.

These phyla play crucial roles in nutrient cycling, soil health, and overall ecosystem functioning, contributing significantly to various ecological functions and essential environmental processes (Wang et al., 2024). The dominance of *Acidobacteriota, Bacteroidota, Chloroflexota, Gemmatimonadota*, and *Planctomycetota* in high-altitude regions was consistent with previous reports (Rambia et al., 2023). *Gemmatimonadota*, reported by Chee-Sanford et al. (2019) and Park et al. (2017) for its potential role in N2O reduction and greenhouse gas emissions, suggests similar ecological relevance in the current study. Furthermore, the presence of heavy metal resistance and arsenic metabolizing genes in the uncultured candidate phylum MBNT15, distantly related to *Desulfobacterota*, and the presence of mercury (Hg) pollution bioindicators in *Desulfobacterota* in specific spring samples (Sp1, Sp2, and Sp3) underscore the environmental challenges posed by heavy metals. This raises concerns about the potential toxicity of the springs for living organisms and highlights the importance of monitoring and addressing heavy metal pollution in Himalayan spring ecosystems (Begmatov et al., 2022; Rincón-Tomás et al., 2023).

Our mycobiome analysis depicted the presence of *Ascomycota, Basidiomycota*, and *Mortierellomycota* phyla across all samples. *Ascomycota* was the most dominant phylum, with a relative abundance of 72.11%, followed by *Basidiomycota* (16.04%), *Mortierellomycota* (9.21%), and *Chytridiomycota* (1.2%) (Fig. 2 (b)). When examining specific springs, Sp1 showed a higher abundance of *Ascomycota* (58.72%), followed by *Basidiomycota* (25.05%), *Mortierellomycota* (11.64%), and Chytridiomycota (2.5%). Sp2 exhibited a higher abundance of Ascomycota (40.05%), with notable contributions of *Basidiomycota* (32.14%) and *Mortierellomycota* (23.85%). The phylum *Ascomycota* is dominant in Sp3 and Sp4, constituting 99.73% and 89.9%, respectively. *Ascomycota* abundance showed variations with higher altitude, attributed to lower temperatures and pH conditions in the mountainous regions of the Himalayas. *Ascomycota* is well-known to thrive in diverse environments owing to its stress-tolerant capacity (Egidi et al., 2019). Its prevalence is particularly favored in higher altitude mountainous regions with acidic soils and cold temperatures. Basidiomycota is the second most abundant phylum observed in the study. It is known as wood-decaying fungi and is involved in breaking down complex organic materials, contributing to nutrient cycling (Yadav et al., 2023). Ascomycota, *Basidiomycota*, and *Mortierellomycota* are the key phyla in the high altitude regions of the Himalayas (Rambia et al., 2023). *Ascomycota* and *Basidiomycota* are referred to as the "dynamic duo" of the fungal world, playing a crucial role in organic matter decomposition and the carbon cycle (Wang et al., 2017). *Chytridiomycota*, known as chytrids, are predominantly water-dwelling organisms. These aerobic zoosporic fungi exhibit dual roles as saprotrophs and pathogens, thriving in freshwater, brackish, and marine environments, and displaying abundance in soil ecosystems (Volk TJ, 2013).

At a lower taxonomic rank, the analysis unveiled the dominance of the bacterial genera *Tumebacillus* (13.85%), *Massilia* (12.97%), *Brevibacillus* (7.02%), *HSB OF53-F07* (3.67%), *Rhodanobacter* (3.08%), *Acidibacter* (2.8%), and *Alicyclobacillus* (2.78%) (Fig. 2(c)). Distinct variations in genera abundance were observed among the springs, with *Brevibacillus* exhibiting a higher prevalence in Sp1 (26.14%) compared to Sp2 (1.94%), however, it was not found in other higher altitude springs, i.e., Sp3 and Sp4. This is particularly noteworthy due to its significant role in the bioremediation of heavy metals, attributed to its metal-resistant nature and participation in the biodegradation of hydrocarbons (Wani et al., 2021). *Acidibacter* demonstrated significantly higher representation in Sp2 (3.77%) and Sp3 (6.65%), suggesting the acidic nature of these specific springs. Sp4 displayed different genera abundance in comparison to other springs, with *Rhodanobacter* (11.93%), *Alicyclobacillus* (6.92%), *Dokdonella* (5.69%), and *Pseudolabrys* (5.52%) emerging as dominant over other genera. *Massilia* is found across all spring samples; however, their prevalence is significantly higher in lower altitude springs, i.e Sp1 and Sp2 and is also reported in the Himalayan geothermal springs (Mahato et al., 2019). The presence of genus *Massilia* is of concern as it causes nosocomial infections in immunocompromised patients and co-infections in humans and animals (Gupta et al., 2023).

Previous studies have revealed that genera *Dokdonella* and *Rhodanobacter* are key players in bioremediation and biogeochemical cycling, which provides a significant importance of these groups of microorganisms in ecological functioning (Figueroa-González et al., 2016; Green et al., 2012).

The mycobiome analysis showed the predominance of fungal genera, including *Aspergillus, Fusarium, Hygrocybe, Mortierella, Solicoccozyma, Trichosporiella, Linnemannia, Podila, Lachnellula, Penicillium, Archaeorhizomyces, Phialea,* and *Scutellinia,* collectively having an average relative abundance >1% across spring samples. Interestingly, distinct genera with substantial abundance were observed across samples (Fig. 2(d)). Water sample (Sp1) from Baniyakund spring showed the presence of *Aspergillus* (12%), *Trichosporiella* (8%), and other taxa affiliated with *Linnemannia* (6.81%), *Lachnellula* (6.13%), and *Mortierella* (6.12%). In contrast, Sp2 displayed the presence of *Hygrocybe* (25%), *Mortierella* (18%), *Podila* (5.65%), and *Solicoccozyma* (5.61%). Sp3 showed a significantly higher abundance of *Aspergillus* (91.24%), while Sp4 was dominated by *Fusarium* (82.04%). These findings highlight the intriguing fungal diversity in extreme environments, where certain genera seem to thrive and dominate while some are getting eliminated across higher elevations. In this study, *Aspergillus* emerged as the most dominant group, contrasting with a previous study in the alpine forests of the Himalayan region, where its abundance was minimal (Rambia et al., 2023). The presence of *Aspergillus is* attributed to its adaptability across various habitats. It is one of the most resilient groups of fungi and thrives in diverse environmental conditions. *Aspergillus* and *Penicillium* spp. are found in diverse habitats, including underground water, surface water, and tap water, and have been reported as opportunistic pathogens (Babič et al., 2017). Additionally, the presence of *Fusarium* species at the altitude of 3480m in the Himalayas is noteworthy. These filamentous fungi are known to cause various fungal plant diseases, which also pose risks to animal and human health by producing harmful mycotoxins (Perincherry et al., 2019). *Hygrocybe* belongs to the agaric family *Hygrophoraceae* and is commonly found in grassland habitats, avoiding areas dominated by *Ectomycorrhizal* fungi. Our observations, aligning with previous studies, reveal the exclusive presence of the *Hygrocybe* genus in Sp2, where *Ectomycorrhiza* dominance is notably low compared to Sp1, Sp3, and Sp4. Despite its unique habitat preferences, the ecological role of *Hygrocybe* remains unexplored, necessitating further studies for a comprehensive understanding of its contribution to ecosystems (Halbwachs et al., 2013).

For comparative analysis of the ASVs, a Venn diagram (Fig. 3 (a) and (b)) showing the shared bacterial and fungal genera among the spring samples was performed to understand the variations in the diversity across different altitudinal gradient. The analysis revealed that 49 bacterial and 278 fungal genera were unique to the Sp1 spring, 23 bacterial and 103 fungal genera were unique to the Sp2, 43 bacterial and 10 fungal genera were unique and 50 bacterial and 24 fungal genera were found to be unique in the Sp4. This highlights the unique microbial composition at different altitudes within each spring.

An in-depth analysis of the KEGG pathways revealed a diverse range of bacterial abundance across various metabolic pathways. The priority pathways to study the presence and relative abundance of bacteria involved in the metabolic processes were Carbon metabolism, Nitrogen metabolism, Sulphur metabolism and Methane metabolism. All the targeted metabolism pathways revealed a multimodal curve where the most abundance was observed in Carbon metabolism followed by Nitrogen, Sulphur and Methane utilisation (Sup Fig 2). Members of phyla *Proteobacteria*, *Methylomirabilota*, and *Gemmatimonadota* play crucial roles in methane metabolism, contributing significantly to the global carbon cycle (Zhu et al., 2022). At the genus level, some nitrifying and denitrifying bacteria were present, such as *Rhodanobacter, Bryobacter, Nitrospira,* and *Dokdonella*. Notably, *Bryobacter* was consistently detected across all sites and is a representative denitrifying bacterium (Pang et al., 2021). However, *Nitrospira* and *Rhodanobacter* exhibited variations across sites, i.e., absence in Sp1 and Sp4, respectively. *Rhodanobacter* and *Nitrospira*, known as aerobic nitrite-oxidizing bacteria, can reduce nitrate to nitrite and thus play a crucial role in the nitrogen cycle (Green et al., 2012; Mehrani et al., 2020). Within the *Proteobacteria* phylum, *Dokdonella* predominant in Sp4, has been reported for its significant role in nitrogen-containing organic pollutant removal. Additionally, sulphur bacteria contained *Tumebacillus* and *Alicyclobacillus* which have been previously reported to oxidize sulphur (Duan et al., 2020; Jiang et al., 2009). Sulphur metabolism was evident through the presence of *Tumebacillus* and *Alicyclobacillus* in Sp4, where sulphur-related metabolism appeared higher as compared to other sites. Furthermore, there is intricate interconnection between microorganisms involved in nitrate reduction and sulphur oxidation. Nitrate-reducing sulfide-oxidizing bacteria, crucial in these processes, were found in diverse ecological niches, including soil, rhizosphere, and freshwater habitats (Duan et al., 2020). This underscores the ecological versatility and significance of these microorganisms in biogeochemical cycles.

It is essential to understand that climate change, road construction, urbanization and other anthropogenic activities have a substantial impact on the quality of natural water. Also, it is crucial to emphasize the importance of studying the microbial diversity of springs as a continuous surveillance and identifying threats in the era where there is an increasing number of infections and developing AMR (Sharma et al., 2023). The assessment of the water quality in two natural springs, Raj Naula and Badi Naula, located in the Kosi River catchment area of Almora city in the Kumaon Himalaya region of Uttarakhand, unveiled a notable decline in both water quantity and quality. Notably, both springs exhibited contamination by coliform bacteria, signalling the presence of organic pollutants (Sati and Paliwal, 2008, Panwar, 2020). Other studies conducted in the Uttarakhand Himalayan region in the springs of the Sumari village of the Pauri Garhwal district and Raikholi village at Bageshwar reported faecal matter contamination (Chauhan, Badwal, and Badola 2020, Chhimwal et al., 2022). The observed bacteriological contamination, attributed to local drains, runoff, and unhygienic open defecation practices, poses a significant health risk to the village population, leading to ailments such as dysentery, diarrhoea, and typhoid. Additionally, tourism also contributes to the water quality in the springs, polluting the pristine water. Reports by Rashid & Romshoo showed a deterioration

of water quality in the Kashmir Lidder valley due to an influx of pilgrim tourists visiting Amarnath Cave (Rashid & Romshoo, 2013).

Fewer studies have explored the bacterial communities in spring samples in IWH; however, their ecological functions and metabolic potential remain largely unexplored. The present study provides valuable insights into the diverse bacterial and fungal communities across the altitudinal gradient. Nevertheless, there were certain limitations to our study, which included a lack of temporal data on physicochemical parameters, organic matter content and sulphur concentrations. Future investigations should delve into the impact of environmental variations on the microbial composition of spring samples. Special emphasis should be placed on unravelling the ecological and metabolic potential of microbial communities, considering their role as reservoirs of biodegrading, bioremediation, and metal-resistant microbes.

In conclusion, our study underscores the importance of understanding microbial diversity and its functional roles within natural water springs of the Himalayan regions. Through targeted metagenomics, we identified the detailed microbial dynamics associated with the springs. Our findings, supported by high-throughput sequencing data, emphasize the prevalence of Proteobacteria and highlight key genera like *Tumebacillus* and *Massilia*. Additionally, functional analysis revealed insights into carbon metabolism, which is crucial for assessing ecosystem health. This study provides valuable insights for effective water resource management and underscores the need for sustainable conservation efforts in the fragile Himalayan ecosystem. Furthermore, continuous monitoring of Himalayan springs is vital to protect human health and preserve indigenous microflora in microbial repositories amidst unique environmental challenges.

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Data Availability

The sequences obtained from the high throughput sequencing effort were submitted to the NCBI database using the sequence read archive (SRA) submission with the project number PRJNA1048161

Author contribution

YO: MS writing, 16S rRNA gene sequencing data analysis, MK: MS writing, Fungal data analysis; RG: community DNA extraction, purification and PCR amplification; JMR, SP: Sample collection, MS writing; AZ: MS writing, data interpretation; BSR: conceptualization and

MS writing; AS: conceptualization, Project administration, Funding acquisition, Supervision, MS writing.

Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors approved the manuscript.

Competing interests

The authors declare no competing interests.

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Figure legend

Figure 1 Alpha diversity parameters of spring samples (a) Bacterial (b) Fungal

Figure 2 Relative abundance of bacterial and fungal communities of spring samples: (a) Top 20 bacterial phyla (b) fungal phyla (c) bacterial genera (d) fungal genera

Figure 3 Venn Diagram representing the common (a) Bacterial and (b) Fungal genera among spring samples