



Microbial diversity and ecology of geothermal springs in the high-grade metamorphic terrain of Sri Lanka

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ARTICLE INFO

Keywords:

Bacterial abundance
Ecological diversity
Geothermal ecosystems
Extreme environments
16S rRNA amplicon sequencing

ABSTRACT

The current study evaluated the bacterial diversity of six hot water spring clusters in Sri Lanka by Illumina MiSeq sequencing of the V3–V4 region of the 16S rRNA gene. Bacterial abundance measures and diversity statistics were assessed using QIIME2 metagenomics workflow, and the results were compared according to the region, the water temperature at the surface (36–59 °C), and pH (6.25–8.35). The predominant phyla observed were *Proteobacteria*, *Actinobacteria*, *Firmicutes*, [*Thermi*], and *Cyanobacteria*. A low abundance of *Bacteroidetes*, *Chloroflexi*, *Acidobacteria*, TM7, and *Spirochaetes* was detected in most of the springs. Several important bacterial species such as *Deinococcus geothermalis* that can tolerate Martian-like conditions, genera such as *Legionella* and *Campylobacter* that contain pathogenic species, sulfur metabolizing *Desulfovibrio*, *Desulfatirhabdium*, *Desulforhabdus*, *Desulfacium*, *Thermodesulfovibrio*, *Desulfovirga*, and *Thiobacter* species, and several other species with the potential practical industrial application were detected. Several opportunistic human pathogens were detected in the water samples and raised a public health concern about the management of post-bathing water. Based on the Bray Curtis beta diversity metric, the microbial distribution correlated with temperature rather than the geographic distance. This study provides valuable new insights into the bacterial diversity of the hot springs in Sri Lanka. Future research needs to be conducted on industrially important thermophiles identified in this study.

1. Introduction

Natural geothermal springs are widely distributed in various locations in the world and most of them are associated with volcanism or active tectonic settings such as recent uplifts (Diamond and Harris, 2000; McCall, 2013). Yellowstone National Park in the United States, the Blue Lagoon Geothermal springs and Strokkur geyser in Iceland, Pamukkale travertine thermal pools in Turkey, Rotorua hot springs in New Zealand, and Beppu springs in Japan are among some of the well-known geothermal springs (McCall, 2013). Magma in the crust is considered as the primary heat source for hot water springs, while active volcanic belts and fault zones act as possible geological formations that bring the magma to the shallower levels of the earth's crust. In addition to the magmatic heat, chemical reactions and decay of radioactive

elements in minerals act as heat sources for the hot springs (Mohammadzadeh and Kazemi, 2017). Hot springs closer to volcanoes have shown acidic pH, while neutral or alkaline pH can be observed in hot springs associated with limestone (Urbietta et al., 2015). Sri Lanka has ten hot water springs, and none of them are associated with active or dormant volcanic activities (Chandrajith et al., 2013; Dissanayake and Jayasena, 1988). These hot water springs are located in the Proterozoic crust with high-grade metamorphic rocks, which are divided into three main lithological units, namely Highland Complex (HC), Vijayan Complex (VC), and Wannu Complex (WC) (Chandrajith et al., 2013). All geothermal springs in Sri Lanka are closely associated with the boundary between Highland and Vijayan Complexes (Fig. 1), where an expectedly higher geothermal gradient was found (Chandrajith et al., 2013). These geothermal waters are of meteoric origin that infiltrated deep geological

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<https://doi.org/10.1016/j.envadv.2022.100166>

Received 30 September 2021; Received in revised form 4 January 2022; Accepted 5 January 2022

Available online 7 January 2022

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units and returned to the surface after acquiring heat under a higher geothermal gradient. Fractures or fault zones may act as the hydraulically convective zones that transmit heat through ascending groundwater to the surface (Chandrajith et al., 2013). Premasiri et al. (2006) classified hot water springs in Sri Lanka into three groups by considering the geochemical parameters, geological and geographical settings. All these hot water springs are located in the dry and intermediates climatic zones, where semi-arid conditions prevail, and also in lower altitude regions (< 100 m), except Marangala spring (about 100 m above sea level) (Chandrajith et al., 2013) (Fig. 1).

Geothermal environments are extreme biological niches that support the life of a variety of microorganisms (Roy et al., 2020). Many studies are conducted on the hydro-geochemical properties of the hot springs and their microbial diversity to understand the interaction of the groundwater bodies and the surrounding environment. Geothermal springs harbor diverse groups of microorganisms (Satyanarayana et al., 2005). The versatility among the microbiome depends on the physical and chemical properties of the geothermal water, the source of origin of the hot spring, and geothermal gradient and pressure within the spring (Merino et al., 2019; Rampelotto, 2013). A variety of prokaryotes,

archaea, and eukaryotes can grow at high temperatures forming bioherms and stromatolites (McCall, 2013). Organisms that use 35–70 °C as the optimal growth temperature are referred to as thermophiles, and those that can grow in 55–85 °C are referred to as extreme thermophiles. Organisms that grow optimally above 80 °C are designated as hyper-thermophiles (Baker et al., 2001; Mehta et al., 2016; Satyanarayana et al., 2005). Identification and classification of the extremophiles are important since most of these organisms are metabolically diverse and possess enzymes that are stable in extreme environmental conditions (Mehta et al., 2016). Populations of unicellular cyanobacteria and algae dated to as far back as 3500 million years are identified in bioherms and stromatolites found near hot springs (McCall, 2013). (Damer and Deamer, 2020) Damer and Deamer (2020) suggested that the study of hot springs would provide a better understanding of the origin of life on Earth. Identification of microbes that can thrive under extreme conditions such as high acidity and salinity by mechanisms of sulfur oxidation and reduction (Counts et al., 2017; Willis et al., 2019), carbon dioxide fixation, metal oxidation, and hydrogen production are important, and they can be valuable resources for industrial processes such as 'white biotechnology' in which organisms and enzymes are used for

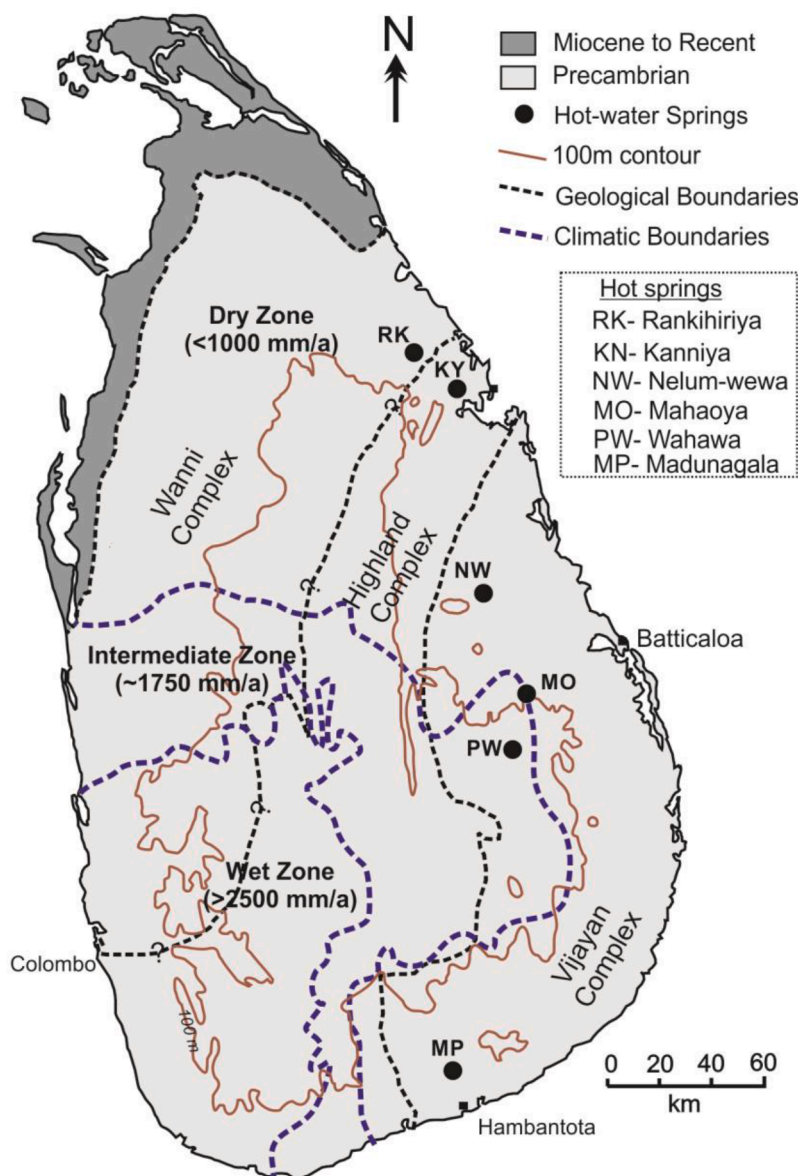


Fig. 1. Locations of the investigated hot water springs in Sri Lanka and the geological and climatic boundaries

industrial processing and production of materials, chemicals, and energy (Urbietta et al., 2015).

Several studies have been conducted on the bacterial diversity of geothermal springs in Sri Lanka by culture-dependent methods (Medhavi et al., 2018; Samarasinghe et al., 2021). *Bacillus thermoamylovorans*, *Meiothermus* sp, *Bacillus schlegelii thermophilic*, and cyanobacteria were cultured and isolated from Mahaoya thermal springs (Medhavi et al., 2018). Magana-arachchi and Wanigatunge (2008) characterized the cyanobacteria and archaea present in Mahapelessa geothermal springs. Isolation of bacteria from the Madunagala, Mahaoya, Wahawa and Nelumwewa hot springs and study on the amylase and cellulase activity of some of the isolates have shown a potential of using these thermophiles for industrial applications (Nandaneel et al., 2015).

New-generation sequencing technologies emerged as a powerful tool in characterizing microbes in specific environments. Microbial ecology of several hot springs around the world has been studied using 16S rRNA amplicon sequencing (Baker et al., 2001; Chan et al., 2017; Kambura et al., 2016; Kanokratana et al., 2004; Saxena et al., 2017; Verma et al., 2014; Wemheuer et al., 2013). Samarasinghe et al. (2021) conducted a 16S rRNA metagenome analysis on a water sample collected from the Mahapelessa thermal spring. That study provided an insight into the bacterial diversity of the thermal spring and showed the importance of culture-independent new generation sequencing technologies for characterizing the extremophiles and other important bacterial species living in this extreme biological niche. Therefore, this study aims to characterize the bacterial species in geothermal springs in Sri Lanka by 16S rRNA amplicon sequencing and compare them with habitats of other thermal springs around the world. We hypothesized that geothermal springs in Sri Lanka have a diverse population of extremophiles and some of them will have the potential to be utilized for industrial and biotechnological applications since the springs are found in a high-grade metamorphic terrain where volcanic-related activities are absent.

2. Materials and methods

2.1. Sample collection

Water samples from geothermal springs were collected between December 2016 – January 2017 from six clusters of geothermal springs located in the intermediate and dry climatic zones (Fig. 1). Each cluster was made of several springs, and each spring was enclosed by a stone-wall to form individual wells, except at the Wahawa cluster in which the mainspring is an artesian type that releases water about 1.5 m above the land surface. Water samples were collected using an aluminum bucket sterilized with 70% ethanol and flame heating. Surface and deep-water samples were collected from wall-enclosed wells, whereas water samples of the artesian type well were collected directly to the sterile tubes from the water flow. Deep samples were collected by submerging the sterilized depth sampler up to 2.5–3.5 m below the water surface, while surface samples were collected by submerging the bucket 0.3–0.6 m below the water surface. The samples were drawn out immediately, and 150 mL of water was collected into 50 mL conical tubes. Temperature and pH of spring water were measured *in situ* using a portable meter (Hach SensIon, Loveland, CO, USA). Samples were immediately transferred to a cool box with ice and transported to the molecular biology laboratory at the Faculty of Veterinary Medicine and Animal Science, University of Peradeniya, and stored at -20 °C until further processing.

2.2. DNA extraction and sequencing

100 mL of water collected from each well was filtered through a 0.22 µm filter membrane using a sterilized reusable filter funnel attached to a vacuum source. Microbial DNA was extracted from the filter membrane using the Power Water DNA isolation kit (Germantown, MD, USA) according to the manufacturer's instructions. Extracted DNA was quantified using the NanoDrop™ 2000 Spectrophotometer (Thermo

Scientific™) and shipped to Massey Genome Service for paired-end sequencing of the 16S rRNA gene (Massey University, Palmerston North, New Zealand). Quality checking of samples was conducted at Massey University by Qubit fluorometric quantization. For the PCR amplification, barcoded forward and reverse V3-V4 amplicon primers were used (barcoded 16Sf V3: (5'-AATGATACGGCGACCACCGA-GATCTACAC-barcode-TATGGTAATTGGCCTACGGGAGGCAGCAG-3') and barcoded 16Sr V4:

(5'-CAAGCAGAAGACGGCATACGAGAT-barcode-AGTCAGT-CAGCCGGACTACHVGGGTWCTAAT-3')) as described by Kozich et al. (2013) and Paturi et al. (2021).

For the PCR reaction, 1 µL of genomic DNA (5 ng/µL) was added to 17 µL of AccuPrime™ Pfx SuperMix (Invitrogen™ Number: 12344040), and 1 µL each of the barcoded forward (V3) and reverse (V4) amplicon primers were added, and the PCR was run at 95 °C for 2 min, then 30 cycles of 95 °C for 20 s, 55 °C for 15 s, 72 °C for 5 min, followed by a final extension of 72 °C for 10 min. PCR products were cleaned up using SequalPrep Normalization Plate Kit (Thermo Fisher Scientific), and the library concentration was quantified by Qubit™ HS Assay (Invitrogen™ number: Q32854), and the full library size of ~630 bp was verified on a LabChip® GX Touch HT (Perkin Elmer) instrument using the DNA High Sensitivity LabChip® Assay. The libraries were pooled by equal molarity. The pooled library was diluted to 8 pM, and PhiX was diluted to 12.5 pM with ice-cold HT-1. Finally, 800 µL of the pooled library and 200 µL of PhiX were combined to give a calculated spike of 20% v/v PhiX. Samples were mixed, and 600 µL was loaded into a thawed Illumina MiSeq V2 cartridge for sequencing on the Illumina MiSeq platform. The amplicon libraries were paired-end sequenced (2 × 250 bp).

2.3. Sequence quality checking and analysis

Before QC trimming, reads were joined with FLASH 1.2.11 (Magoč and Salzberg, 2011). The joined reads were then processed with SolexaQA (Cox et al., 2010) with a quality probability threshold of 0.05. The majority of the reads were 429 bp in length following the trimming. Metadata associated with the samples were arranged into tables, and sequence reads associated with the metadata were analyzed using the QIIME2 metagenomics workflow (version 2019.1). In brief dada2 plugin was used to generate a feature table and representative sequence variants, which were then aligned using the mafft plugin (Katoh et al., 2002). Rooted and unrooted phylogenies were generated using a fast tree plugin (Price et al., 2010). The ASVs generated by the dada2 plugin were classified to operational taxonomic units (OTU) using a naïve Bayes taxonomy classifier (Bokulich et al., 2018) against the Greengenes database (version 13.8; 99% OTU reference sequences) (McDonald et al., 2012). This was performed to reduce the overrepresentation of the variation compared to actual biological variation when ASVs are directly used. The OTU analysis was performed using the diversity plugin (<https://github.com/qiime2/q2-diversity>), and the resulting OTU table was converted to a BIOM file for further analysis (McDonald et al., 2012).

QIIME2 output provided the taxonomic summaries of microbes living in different water sources and diversity statistics at a sampling depth of 997. Nonparametric statistical tests were performed in QIIME2 to identify pairwise differences between each sample and group comparisons based on hot water spring clusters.

2.4. Measurements of the diversity and statistics

Within-sample diversity was studied using the alpha diversity measures Pielou's evenness and Faith's phylogenetic diversity (Faith's PD) indexes. Data obtained from the current study cannot be assumed normal because samples were not collected randomly, and there can be a spatial aggregation of microbial populations in water. Therefore, non-parametric tests were used for further analysis. Differences in alpha diversity metrics across the hot water spring clusters and samples with

all pairwise comparisons were determined using Kruskal-Wallis tests. The between-sample and cluster diversity were assessed using the beta diversity measures phylogenetic UniFrac distance (weighted and unweighted) and non-phylogenetic distance (Bray Curtis and Jaccard) metrics. Principal Coordinates Analysis (PCoA) plots were generated to visualize individual and group differences. Due to the high sensitivity in unweighted UniFrac beta diversity metrics for low abundances of rare organisms present in the data and the denoising method used in the analysis, downstream analysis was performed based on the weighted UniFrac distance metrics in addition to the non-phylogenetic distance methods. All the statistical analyses were executed with a p -value ≤ 0.05 as the significant cut-off. Differential abundance between geothermal springs was analyzed by balance taxonomy in gneiss to overcome the issues generated due to the differential compositional nature of microbiota data. Balance taxonomy assesses the logarithmic ratio of abundances between the numerator and denominator taxa at each node in the dendrogram created using correlation clustering. Differences in the logarithmic ratio of a balance were compared between geothermal spring clusters to identify differences in microbiota composition.

3. Results

3.1. Geothermal spring characteristics

A total of 23 water samples were collected from six (6) hot spring clusters in Sri Lanka (Table 1). Samples were collected from four wells in the Kanniya hot water cluster, and two of them showed gas bubbling at the time of the sampling. A total of nine (9) samples were collected from five (5) active wells in the Nelumwewa cluster. The Wahawa cluster is comprised of 18–20 springs scattered in the village and the paddy fields (Senaratne and Chandima, 2011), however, only the artesian well was accessible for sampling. Rankihiriya spring has one well with about 1.5 m of depth, and gas bubbling was observed at the sampling time. Four wells from the Mahaoya spring cluster were sampled. The Madunagala cluster also comprises four (4) wells, and surface and deep-water samples were collected from the mainspring.

In the current study, Nelumwewa and Mahaoya spring water showed the highest surface temperature (59 °C), while Kanniya and Rankihiriya

Table 1
Details of hot spring water samples from in Sri Lanka (nd- not measured).

Name of the cluster	Number of accessible wells	Number of samples	Sample Identity	Temp. (°C)	pH (25 °C)
Nelumwewa	5	5 surface and 4 deep	NW1_S	50	nd
			NW1_D	51	7.85
		NW2_S	59	nd	
		NW2_D	54	8.27	
		NW3_S	41	nd	
		NW3_D	42	8.35	
		NW4_S	nd	nd	
		NW5_S	42	nd	
		NW5_D	nd	nd	
		Mahaoya	7	4 deep	MO1
MO2	59				7.74
MO3	56				7.71
MO4	56				7.71
Wahawa	1	1 flow water basin	PW_S	47	7.47
			PW_F	46	nd
Kanniya	7	4 surface	KN1	38	6.39
			KN2	36.8	6.25
			KN3	32.8	6.44
			KN4	38.2	6.46
Rankihiriya	1	1 surface deep	RK1_S	36.3	7.13
			RK1_D	nd	nd
Madunagala (main spring)	4	1 surface deep	MP1_S	45.5*	7.11*
			MP1_D	nd	nd

hot springs showed the lowest temperature during the sampling (36 °C). Nelumwewa, Mahaoya, and Rankihiriya hot springs were observed with alkaline pH, while Kanniya was the only hot spring that showed an acidic pH (Table 1).

3.2. Taxonomic composition of the prokaryotic communities

The total number of demultiplexed sequences obtained from the 23 samples was 3,179,994 and ranged from 5152 to 273,080, with a mean of 138,261 reads (Supplementary Table S1). The lowest number of sequence reads were found in the water samples collected from the Wahawa artisan well. Low numbers of sequence reads were found in two surface water samples collected from the wells in the Nelumwewa cluster. Samples contained a total of 2,101,234 operational taxonomic units (OTUs) after denoising, and they were distributed from 997 to 191,801 among samples with the mean of 91,358 OTUs. However, there were only 2398 unique sequences (OTUs), and they had 262 to 454 nucleotides with the mean and standard deviation of 417.81 ± 11.87 nucleotides. Ninety-eight percent (98%) of total OTUs had 430 nucleotides. The mean frequency of OTUs was 876 times across all samples. Obtained sequences belonged to the kingdom bacteria and included 37 affiliated phyla. The predominant phyla were *Proteobacteria* (29.72–98.86%), *Actinobacteria* (0.0–61.41%), *Firmicutes* (0.0–20.11%), *[Thermi]* (0.0–14.36%), *Cyanobacteria* (0.0–10.72%), *Bacteroidetes* (0.0–4.30%), *Chloroflexi* (0.0–3.22%), *Acidobacteria* (0.0–1.41%), *TM7* (0.0–1.25%), and *Spirochaetes* (0.0–1.76%) (Fig. 2 and Supplementary Table S1).

Based on the 16S rRNA amplicon sequencing, the phylum *Proteobacteria* was the signature phylum that dominated the bacterial population in all the six hot water springs, with the highest proportions in the Mahaoya spring and the lowest proportions in the Wahawa spring. The phylum *Proteobacteria* was represented by five classes (*Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Deltaproteobacteria*, and *Epsilonproteobacteria*) (Fig. 3) and 56 families.

The phylum *Actinobacteria* was observed with low relative abundance (0.0–7.95%) in all the geothermal water springs, except for Wahawa (34.70 and 61.41%) and Madunagala (12.32 and 14.30%). Three species were observed in the Wahawa hot spring cluster (*Arthrobacter* sp, *Rothia terrae*, and *Nocardiopsis* sp.), while five species (*Helcobacillus massiliensis*, *Micrococcus terreus*, *Nocardioides*, *Rothia amarae*, and *Arthrobacter* sp.) were observed in the Madunagala hot spring. The phylum *Firmicutes* was detected from all the hot springs, although the relative abundance was low. Only one well of Madunagala hot spring showed a higher relative abundance (20.11%) with 19 families affiliated to three classes (Classes *Bacilli*, *Clostridia*, and *Erysipelotrichi*).

The phylum *Thermi* was observed in all the studied wells. Both the deep (1.89–12.09%) and surface (0.18–14.36%) water samples collected from the Nelumwewa hot spring cluster showed a high relative abundance of the phylum *Thermi*. In all the other springs, *Thermi* showed less than 5% relative abundance. *Cyanobacteria*, a phylum that is important in the evolution of oxygenic photosynthesis (Klatt et al., 2011), was detected at a fairly low relative abundance (0.0–10.72%). Kanniya and Rankihiriya hot springs had the highest relative abundance (6.41–10.72%) of *Cyanobacteria*, whilst Mahaoya and Madunagala hot springs were free from *Cyanobacteria*. A low relative abundance of *Cyanobacteria* was observed in surface (2.92%) and deep (0.88 and 1.90%) water samples from almost all the wells in the Nelumwewa hot spring cluster. The filamentous anoxygenic photosynthetic phylum *Chloroflexi* was present in the Kanniya, Rankihiriya, and Nelumwewa hot spring clusters. The phylum *Chloroflexi* was dominated by the class *Anaerolineae* with families *S47*, *Caldilineaceae*, and *Anaerolineaceae* present. The family *S47* was observed in Kanniya and Rankihiriya.

A relative abundance of less than 5% was observed in phylum *Bacteroidetes* in the six hot water spring clusters. Six classes were observed in this phylum: *Bacteroidia*, *Cytophagia*, *Flavobacteriia*, *Sphingobacteriia*, *Rhodothermi*, and *Saprospirae*. The abundance of each class varied among

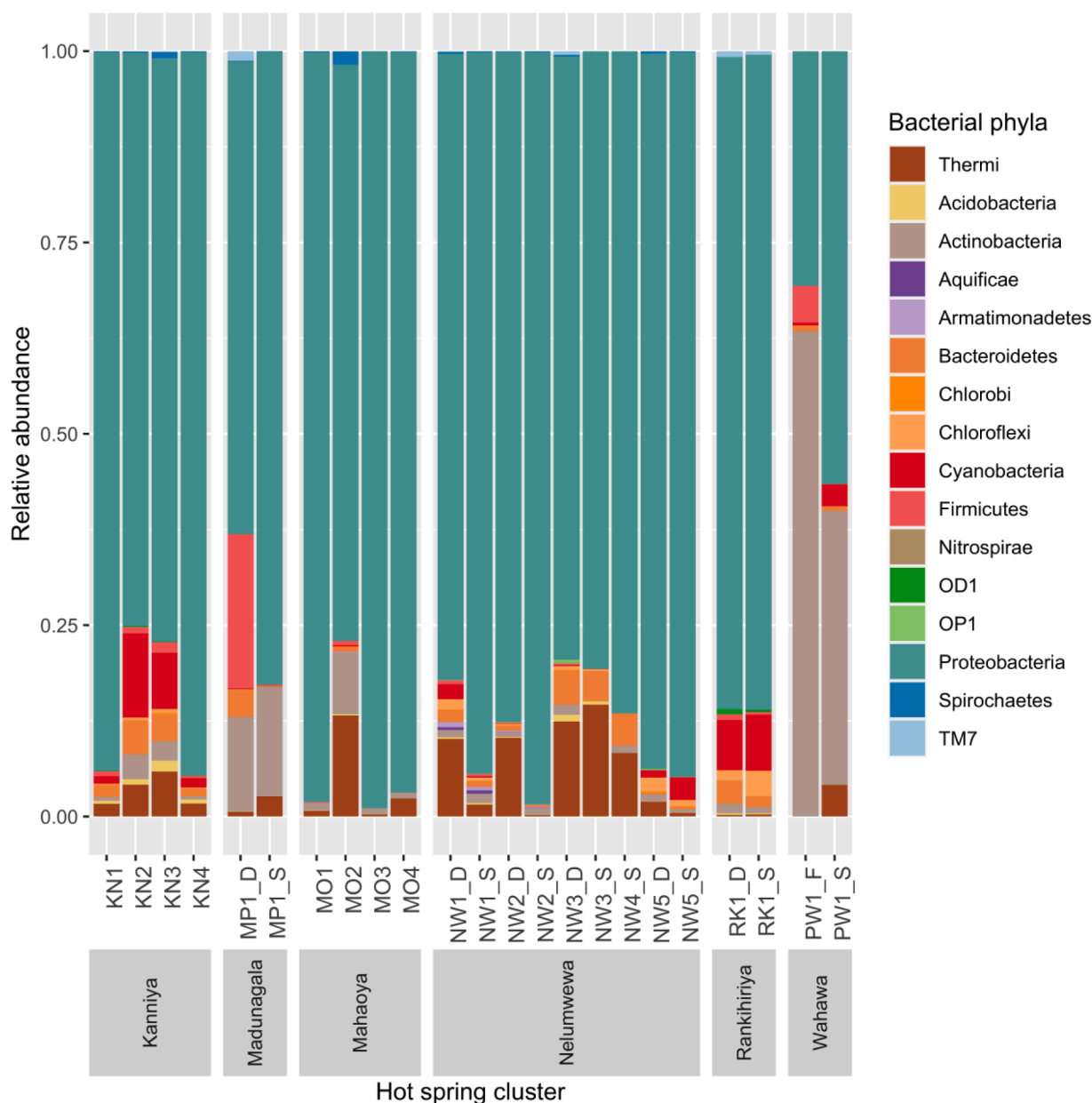


Fig. 2. Relative frequency of major phyla observed in each sample. Short labels in the x-axis represent the samples collected from each hot spring cluster as mentioned in Table 1.

the hot springs. *Cytophagia* was the most prevalent *Bacteroidetes* class detected. All six *Bacteroidetes* classes were detected in two wells of the Nelumwewa hot spring cluster (both surface and deep samples). Very low relative abundances were observed in the phyla *Acidobacteria* (0.0–1.41%), *TM7* (0.0–1.25%), *Spirochaetes* (0.0–1.76%), *OD1* (0.0–0.68%), *Armatimonadetes* (0.0–0.63%), *OP1* (0.0–0.40%), *Chlorobi* (0.0–0.25%), *Nitrospirae* (0.0–0.14%), *OP8* (0.0–0.25%), *Aquificae* (0.0–0.42%), *GN02* (0.0–0.12%), and *TM6* (0.0–0.15%) in all geothermal springs.

3.3. Important bacterial species detected in the study

Several important bacterial species with potential applications in biotechnology, bioremediation, pharmaceuticals, cosmetics, and food and agrochemical industries were detected in some of the studied hot water springs (Fig. 4). Sulfate-reducing species (*Desulfatirhabdium sp*, *Desulfobivrio sp*, *Desulfacinum sp*, *Desulforhabdus sp*, *Desulfovirga sp*, and *Thermodesulfobivrio sp*) were observed in the Nelumwewa, Rankihiriya,

and Kanniya hot springs clusters. Sulfate oxidizing *Thiobacter sp* was present in the Nelumwewa hot springs, while sulfur metabolizing *Gulbenkiania mobilis* was observed in the Kanniya hot spring cluster (Table 2). Extremely radiation-resistant *Deinobacterium sp*; *Deinococcus murrayi*, *Deinococcus geothermalis*, and *Truepera sp* were mainly detected from the Kanniya hot springs. *Deinococcus murrayi* were present in all the analyzed geothermal water samples, except for the Wahawa spring cluster (Table 2). *Deinococcus geothermalis*, an extremophile studied in astronomy due to its potential interplanetary survivability and ability to survive in space and Mars-like conditions tolerating high extra-terrestrial ultraviolet radiation (Panitz et al., 2019) was observed in water samples obtained from the Kanniya hot spring cluster (Table 2).

Protease-producing *Sporosarcina aquimarina* (Kanniya) and amylase-producing *Caldimonas taiwanensis* (Nelumwewa, Kanniya, Rankihiriya, Mahaoya, and Madunagala) belonging to thermozyme producing microbes were observed. Nitrogen-fixing *Azoarcus indigenus* (Nelumwewa), *Azospira restricta* (Nelumwewa, Kanniya, and Rankihiriya), *Pseudacidovorax intermedius* (Kanniya and Madunagala), *Novosphingobium*

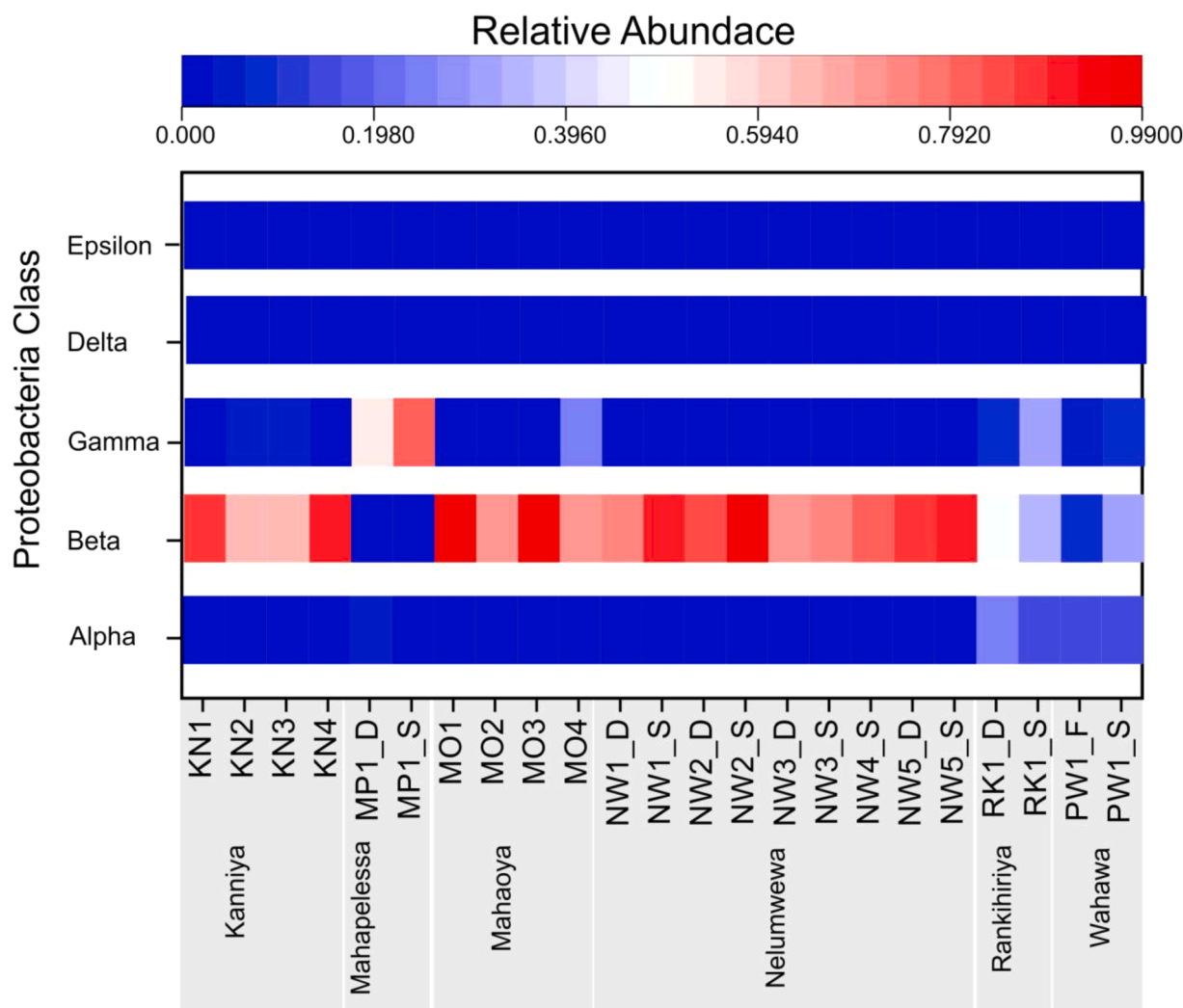


Fig. 3. Heat map of 5 major classes of Proteobacteria categorized by hot water spring sample type and the region. Short labels in the x-axis represent the samples collected from each hot spring cluster as mentioned in Table 1.

nitrogenifigens (Kanniya and Rankihiriya), *Gluconacetobacter* (Madunagala), and *Xanthobacter flavus* (Nelumwewa, Wahawa, and Rankihiriya) were also detected. The only nitrifying organism, *Nitrobacteria hamadaniensis* was detected in all the hot springs, except Kanniya. All the investigated hot springs had organisms that have previously shown capabilities of degrading various chemical compounds, including aromatic hydrocarbons (Supplementary Table S2).

Waterborne opportunistic human pathogens were mainly identified from the Rankihiriya, Madunagala, Kanniya, and Mahaoya hot springs (Supplementary Table S3) with lower relative abundance ($< 5\%$) (Fig. 4). *Brevundimonas sp* was found in all the hot springs. *Haemato-bacter massiliensis*, *Weeksella virosa*, *Orientia chuto*, *Roseomonas sp*, *Helicobacter sp*, and *Legionella sp* were detected in the Rankihiriya hot spring cluster. Six possible human pathogens, including *Ochrobactrum pseudogrignonense*, *Herbaspirillum sp*, *Massilia sp*, *Helicobacillus massiliensis*, *Elizabethkingia sp*, and *Staphylococcus saprophyticus* were detected in the Madunagala hot spring cluster. Common human pathogens *Helicobacter sp* and *Legionella sp* were detected in the Kanniya hot spring cluster. *Ochrobactrum Pseudintermedium*, *Bordetella holmesii*, *Neisseria sp*, *Campylobacter rectus*, *Massilia sp*, *Helicobacillus massiliensis*, and *Bergeyella zoohelcum* were detected in the Mahaoya hot spring cluster (Supplementary Table S3).

3.4. Statistics of bacterial diversity

According to Pielou's evenness and Faith's PD indices, Kanniya and Rankihiriya had the highest alpha diversity compared to the other clusters (Supplementary Figs. S1 and S2). The Wahawa samples had the highest average Pielou's evenness index (0.73), although microbiomes were phylogenetically less diverse. The Mahaoya samples had the lowest diversity in terms of evenness and richness with a range of 0.05–0.42 (mean = 0.21) and phylogenetic relationship (Supplementary Figs. S1 and S2). There were no significant differences in the diversity between surface and deep-water samples ($p > 0.05$). Multiple springs were sampled from Kanniya, Mahaoya, and Nelumwewa clusters, and no significant differences were identified between different springs in each cluster at the significance level of $p \leq 0.05$. Overall, evenness ($p = 0.51$) and Faith's PD ($p = 0.36$) alpha diversities were not significant between all the hot water samples. Nevertheless, an overall significant difference was observed in evenness ($p = 0.01$) and Faith's PD ($p = 0.02$) between hot water spring clusters.

Group-wise comparisons showed that Kanniya was significantly different from Mahaoya ($p = 0.02$) and Nelumwewa ($p = 0.01$) according to Pielou's evenness index. Similar results were obtained for Faith's PD measures between Kanniya and Mahaoya clusters ($p = 0.02$), and Kanniya and Nelumwewa clusters ($p = 0.05$). Based on both methods, Nelumwewa was significantly different from Rankihiriya ($p =$

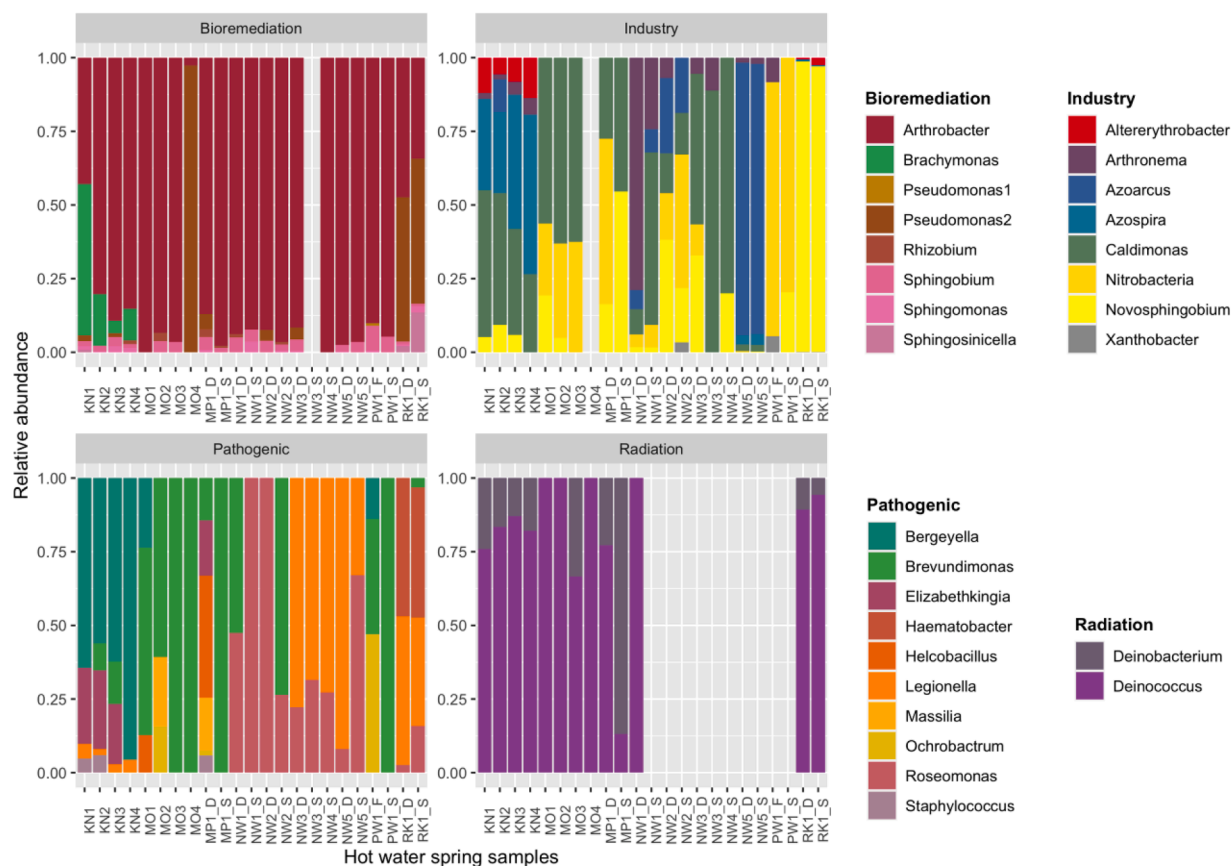


Fig. 4. Major genera identified in the six hot water springs. They are categorized based on the importance of bioremediation, industry, human health (pathogenic), and radiation. Short labels in the x-axis represent the samples collected from each hot spring cluster as mentioned in Table 1.

Table 2

Sulfur metabolizing and extremely radiation-resistant bacterial genera/species detected in the six hot spring clusters.

Genus	Species	Hot Spring	Source
<i>Thiobacter</i>	-	Nelumwewa	Hirayama et al. (2005)
<i>Desulfatirhabdium</i>	<i>Butyrativorans stermophilum</i>	Nelumwewa	Galushko and Kuever (2019)
<i>Desulfovibrio</i>	<i>cuneatus</i>	Rankihiriya	Sass et al. (1998)
	<i>litoralis</i>	Rankihiriya	Sass et al. (1998)
	<i>mexicanus</i>	Kanniya	Hernández-Eugenio et al. (2000)
	<i>putealis</i>	Kanniya	Basso et al. (2005)
<i>Desulfacinum</i>	-	Nelumwewa	Rees and Patel (2015)
<i>Desulforhabdus</i>	<i>amnigena</i>	Nelumwewa	Elferink et al. (1995)
<i>Desulfovirga</i>	<i>adipica</i>	Rankihiriya,	Tanaka et al. (2000)
		Nelum wewa	
<i>Thermodesulfovibrio</i>	-	Nelumwewa	Maki (2015)
<i>Gulbenkiania</i>	<i>mobilis</i>	Kanniya	Saxena et al. (2017)

0.03). Additionally, Nelumwewa was significantly different from Wahawa ($p = 0.03$) only according to the Pielou’s evenness index, while Nelumwewa was significantly different from Mahaoya ($p = 0.05$) based on Faith’s PD index. However, q-values (adjusted p-values with Benjamini and Hochberg corrections) were not significant for any pairwise or group-wise comparisons in both methods.

Beta Diversity utilizes an inter-sample distance matrix and thereby accounts for the whole distribution of the species in the entire community and showed samples are most similar to one another. In this study, similarities of the microbiome between hot spring clusters were examined using the weighted UniFrac (phylogenetic-based) matrix and Bray Curtis (non-phylogenetic) method, and visualized by principal coordinate analysis (PCoA) (Fig. 5). Based on the phylogenetic UniFrac

distances (weighted) analysis, a clear separation was observed in the Madunagala, Wahawa, and Rankihiriya hot water springs. However, the Mahaoya and Nelumwewa springs were clustered together with two other Kanniya wells (Fig. 5a). Based on Bray Curtis, all the spring samples from Kanniya were clustered together and separately from other regions. However, Wahawa and Madunagala were closely associated, while only four (4) samples of Nelumwewa and Mahaoya were clustered together by dividing the Nelumwewa samples into two clusters. The balance taxonomy analysis was performed to identify taxa that were differentially abundant between samples and hot spring clusters. The denominator of balance (y_0) had sparse OTUs (134 taxa) compared to the numerator of y_0 (2264 taxa) (Supplementary Fig. S3). All the hot water spring clusters except Kanniya appeared to be at (or near) a logarithmic ratio of 0. The balance (y_0) was considerably lower at the Kanniya cluster compared to the other hot water spring clusters (Supplementary Fig. S4).

4. Discussion

Many studies have been conducted on microbial communities in hot springs, particularly in the United States (Inskip et al., 2013), Japan (Everroad et al., 2012), China (Liu et al., 2016), Iceland (Tobler and Benning, 2011), and Malaysia (Chan et al., 2015). Sri Lanka has ten hot springs, and a few culture-based studies were carried out to characterize the microbes in some of these springs (e.g., Medhavi et al. 2018; Samarasinghe et al. 2021). In the current study, we performed a detailed characterization of the thermal spring microbiome by culture-independent 16S rRNA sequencing of 23 water samples collected from six thermal spring clusters in Sri Lanka.

The hot springs in the current study showed an overall dominance by the bacterial phylum *Proteobacteria*. Similarly, hot springs in Western

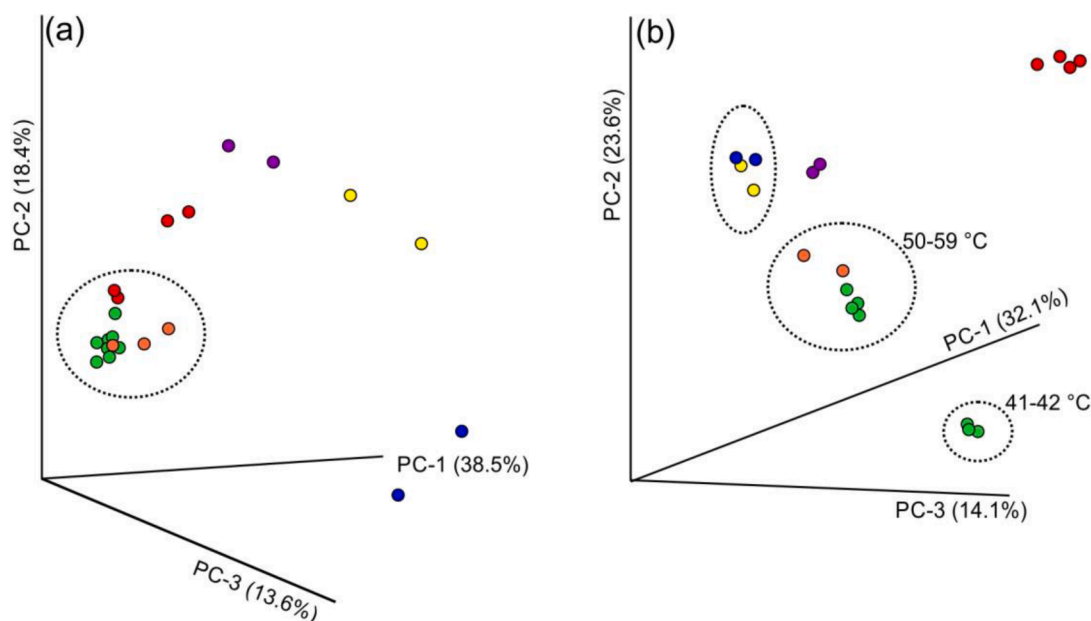


Fig. 5. Principle coordinate analysis (PCoA) of (a) phylogenetic unifracs distances (weighted); and (b) non-phylogenetic distance (Bray Curtis). The wells are colored as: Red – Kanniya; Purple – Rankihiriya; Green – Nelumwewa; Orange – Mahaoya; Blue – Madunagala; and Yellow – Wahawa.

Sichuan, China (Tang et al., 2018), Eritrea in Eastern Africa (Ghilamical et al., 2017), Pakistan (Amin et al., 2018; Baker et al., 2001), and Bullicame travertine, Italy (Valeriani et al., 2018) are dominated by the phylum *Proteobacteria*. In addition, hot springs with a wide range of temperatures (44–110 °C) at various geographical locations have been reported with abundant *Proteobacteria* (Ghilamical et al., 2017). Several hot springs in this study harbored various *Proteobacteria* genera that have important industrial applications. Even though an individual organism does not possess all the enzymes involved in the degradation pathway, together as a community, they have shown the ability to digest hydrocarbons (Saxena et al., 2017). Microbes involved in the degradation of hydrocarbons such as *o*-xylene, naphthalene, cyclohexane, toluene, tetrachloroethane, methane, and hexane were observed in the Kanniya, Madunagala, and Rankihiriya springs (Supplementary Table S4). Chemoorganotrophic organisms, such as *Pseudomonas stutzeri*, *Novosphingobium naphthalenivorans*, and *Novosphingobium resinovorum* that utilize hydrocarbon as a source of energy were observed in the Madunagala hot spring cluster. Methanotrophic species, such as *Methylococcus capsulatus*, were also observed in the Madunagala hot spring cluster (Supplementary Table S4). Previous studies conducted in India have also shown that thermophiles (i.e., *Pseudomonas stutzeri*, *Acidovorax sp.*, and *Methylococcus capsulatus*) identified from some hot water springs have the ability to degrade a variety of hydrocarbon compounds (Saxena et al., 2017).

Alphaproteobacteria was observed with high relative abundance in the Rankihiriya and Wahawa hot springs. The genus *Methylobacterium* from the class *Alphaproteobacteria* is a facultative methylotrophic bacterium with the ability to utilize C1 compounds such as methanol or formaldehyde and other multi-carbon compounds. *Methylobacterium* spp is reported in high-temperature petroleum reservoirs (50–120 °C) (Saxena et al., 2017). They have been shown to degrade a range of toxic compounds, such as phenol, monomethyl isophthalate, dimethyl isophthalate, and methyl tert-butyl ether (Salam et al., 2015). Therefore, this organism has an industrial potential in polymer production and degradation. In this study, the highest relative abundance of *Methylobacterium* was detected in the Wahawa springs (Supplementary Table S4).

Balneotherapy, therapeutic bathing in thermal springs, is still practiced in some European countries and Japan and is thought to provide relief from certain dermatologic conditions, arthritis, and fibromyalgia

(Aburto-Medina et al., 2020). Elements such as sulfur, calcium, sodium, magnesium, chloride, potassium, and bicarbonate found in hot springs, are believed to be responsible for these healing properties (Bojadgieva et al., 2002). Bacteria involved in the sulfur cycle were identified in hot springs in the Indo-Burma region (Panda et al., 2015). In the current study, sulfate-reducing obligatory anaerobes, affiliated with the class *Deltaproteobacteria*, were found in the Nelumwewa, Rankihiriya, and Kanniya hot springs. Previous studies have shown that Nelumwewa, Madunagala, and Wahawa have a high sulfur concentration in the water (Chandrajith et al., 2013). There is a belief that water in Kanniya, Rankihiriya, Wahawa, and Madunagala hot springs has medicinal and healing properties, and people come in from all over the country to bathe in these waters. Therefore, the presence of sulfur metabolizing bacteria together with sulfur, chloride, and calcium-rich water might be playing an important role in the believed healing and medicinal property of these hot water springs. Ecosystems in the hot springs can be disturbed and become polluted due to human activity. Waterborne opportunistic human pathogens were present in the samples collected from Rankihiriya, Madunagala, Kanniya, and Mahaoya hot springs (Supplementary Table S3) and these springs are popular for therapeutic bathing. *Legionella* species were also detected with a very low relative abundance in the Nelumwewa and Rankihiriya hot springs. Pathogenic *Legionella pneumonia* is a public health concern, and they were previously found in higher abundance in hot springs in Eritrea (Ghilamical et al., 2018). These results raise a concern and highlight the importance of a good drainage management system of post-bathing water.

A study conducted on the hot spring-microbialite system in the Trans-Himalayan Plateau showed that different springs with different water geochemistry, such as higher concentrations of borates, sodium, thiosulfate, sulfide, and bicarbonate, can be inhabited by diverse groups of microbes, including *Aquificae*, *Deinococcus-Thermus*, *Chlorobi*, *Chloroflexi*, *Cyanobacteria*, *Bacteroidetes*, *Proteobacteria* and *Firmicutes* (Roy et al., 2020). Predominance among the communities has been shown to vary according to the physicochemical characteristics of hot spring water, such as pH, redox potential, temperature, the concentration of trace elements (Mehetre et al., 2018), and geothermal gradient (Chandrajith et al., 2013; Roy et al., 2020). Studies have proved variations in physicochemical properties of Sri Lankan hot springs (Chandrajith et al., 2013; Dissanayake and Jayasena, 1988). In the current study, we measured the water temperature and the pH during sample collection. In

general, the Nelumwewa, Wahawa, and Mahaoya were hot springs with higher temperatures (50–60 °C), and water temperatures of Rankihiriya, Madunagala, and Kanniya springs were lower (30–50 °C) (Supplementary Table S7). Based on alpha diversity metrics, the microbiomes of the Rankihiriya and Kanniya spring clusters were more diverse compared to the other hot water springs. Higher species richness, evenness, and phylogenetic diversity were shown in the Rankihiriya and Kanniya clusters. It has been shown that in thermophilic microbial communities, the alpha diversity indices (Shannon, Chao1, PD-whole tree, and the total number of observed OTUs) are negatively correlated with the increase in water temperature (Chiriac et al., 2017). Several other studies conducted in hot spring samples from Yellowstone National Park, USA (Miller et al., 2009), Japan (Everroad et al., 2012), and Iceland (Tobler and Benning, 2011) are also in agreement with this correlation. The lowest diversity was encountered in the Mahaoya spring, which has the highest reported temperature (59 °C).

The influence of temperature on the microbial community composition was also demonstrated in the beta diversity Bray Curtis metric. Based on the current study, the Nelumwewa samples were divided into two groups in the Bray Curtis PCoA, where one group had two wells (4 samples) with higher temperature (50–59 °C) and clustered with the Mahaoya samples (55–59 °C), while the rest (3 samples) were at a moderate temperature (41–42 °C) and clustered separately. It indicates that microbial abundances are different between these two groups of wells in Nelumwewa, most likely due to the temperature variation, although they are in the same region. Therefore, we suggest that the microbial abundances of the studied hot water springs were consistent with temperature rather than the geographic location. Wang et al. (2013) also identified the significance of temperature over biogeography in shaping microbial community composition in hot springs of the Tibetan Plateau.

Actinobacteria is a phylum that can inhabit wide ranges of temperatures and is shown to survive well under neutral or alkaline pH conditions (Song et al., 2009). We observed *Actinobacteria* in the Wahawa hot spring that had a high reservoir temperature, alkaline pH (7.47), and Madunagala hot spring which had low reservoir temperature and alkaline pH (7.11). There were no *Actinobacteria* in the Rankihiriya hot spring that had the lowest pH (6.6). The Rankihiriya hot spring is characterized as high bicarbonate water (Chandrajith et al., 2013). The weak acidic environment may be the reason for the absence of alkaliphilic. The existence of *Firmicutes* together with *Actinobacteria* is identified as an adaptation to low nutrient conditions of the hot springs (Kambura et al., 2016). We have observed the presence of both phyla in significant levels in the Madunagala and Wahawa hot springs. The microbial composition of these two hot spring clusters was comparable according to the Bray Curtis alpha diversity metric.

Hot springs with rich sulfur content and high temperature provide a favorable environment for the phylum *Thermi* (Panda et al., 2016). In the current study, the phylum *Thermi* was found abundantly in Nelumwewa and Mahaoya hot springs. These two springs showed higher sulfur content and high temperature. Thermophiles acclimatized to lower temperature include *Bacteroidetes*, *Proteobacteria*, and photosynthetic bacteria, such as *Cyanobacteria* and *Chloroflexi* (Amin et al., 2018; Chan et al., 2017). In the current study, Rankihiriya and Mahapelessa, which had relatively lower temperatures, showed a higher relative abundance of *Bacteroidetes*, while Wahawa and Mahaoya with higher temperatures showed a very low relative abundance of this phylum. Kanniya and Rankihiriya, which had the lowest temperature, showed a higher abundance of *Cyanobacteria* and *Chloroflexi* sp. The Kanniya and Nelumwewa springs showed the presence of *Arthronema* sp in a low relative abundance. *Arthronema* sp is known to have a commercial value as a natural colorant in nutraceuticals, cosmetics, and pharmaceutical industries (Panda et al., 2015).

Due to field constraints and limited availability of previous data, it was not possible to conduct random sampling or collect a large number of samples per well in the current study. Except for the two surface water

samples of Nelumwewa that had a low number of sequence reads, there were no significant differences between the sequence reads obtained from deep and surface samples. The lowest number of sequence reads were found in the two samples collected from the Wahawa artesian well. Because of the spatial aggregation of microbial populations in water and a limited number of samples collected per well, the whole diversity of the ecosystem in each well might not have been revealed in the current study. However, we were able to get valuable insights into the microbial diversity of thermal springs in Sri Lanka, and the results of this study would be valuable in designing future studies on target species found in these springs.

5. Conclusions

We conducted 16S rRNA amplicon sequencing and identified the bacterial diversity, community composition, and abundances in six hot water spring clusters in Sri Lanka. There was a diverse community of extremophile bacterial species inhabiting the geothermal water springs in Sri Lanka. Temperature was identified as the main factor in differentiating microbial diversity in investigated springs. Several important bacterial species, such as *Deinococcus geothermalis* that can tolerate Martian-like conditions, genera such as *Legionella* and *Campylobacter* that contain pathogenic species, sulfur metabolizing *Desulfovibrio*, *Desulfatirhabdium*, *Desulforhabdus*, *Desulfacinum*, *Thermodesulfovibrio*, *Desulfvirga*, and *Thiobacter* species, were detected. Industrially significant organisms, such as heat-stable protease and amylase producers, nitrogen-fixing organisms, and potential bioremediation bacterial species, were detected in all the wells. This study provides valuable insights into the ecological diversity of bacteria populating the hot springs in Sri Lanka. Results of this study provide valuable information on industrially important thermophiles, and future studies need to be conducted on thermostable enzymes and other by-products of these bacteria.

Funding

This work was supported by the National Research Council (NRC) of Sri Lanka grant: NRC 15-057.

Data availability statement

Data supporting this paper is available as a supplementary file and additional data can be obtained from corresponding authors.

CRedit authorship contribution statement

Ruwini Rupasinghe: Methodology, Investigation, Writing – original draft, Visualization. **Sathya Amarasena:** Data curation, Writing – original draft, Visualization. **Sudeera Wickramarathna:** Investigation, Writing – review & editing. **Patrick J. Biggs:** Validation, Formal analysis, Data curation, Writing – review & editing. **Rohana Chandrajith:** Resources, Visualization, Writing – review & editing. **Saumya Wickramasinghe:** Conceptualization, Methodology, Investigation, Resources, Writing – review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial or commercial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The authors would like to acknowledge Dr. Kaushalya Wijenayake and Dr. Saranga Diyabalanage for their assistance in water sample collection and processing. Mr. Xiaoxiao Lin of the Massey Genome Service is acknowledged for the assistance provided during the

sequencing. The authors appreciate the support provided by Dr. David Wheeler during the preliminary data analysis.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.envadv.2022.100166](https://doi.org/10.1016/j.envadv.2022.100166).

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