

Original Article

Domination of Filamentous Anoxygenic Phototrophic Bacteria and Prediction of Metabolic Pathways in Microbial Mats from the Hot Springs of Al Aridhah

(microbial mats / hot springs / 16S amplicon / *Chloroflexus* / Saudi Arabia)

M. YASIR^{1,5}, A. K. QURESHI¹, S. SRINIVASAN², R. ULLAH¹, F. BIBI^{1,5},
M. REHAN^{3,5}, S. B. KHAN⁴, E. I. AZHAR^{1,5}

¹Special Infectious Agents Unit, King Fahd Medical Research Center, King Abdulaziz University, Jeddah, Saudi Arabia

²Institute of Bioinformatics and Applied Biotechnology, Bangalore, Karnataka-560100, India

³King Fahd Medical Research Center, King Abdulaziz University, Jeddah, Saudi Arabia

⁴Department of Chemistry, King Abdulaziz University, Jeddah, Saudi Arabia

⁵Medical Laboratory Technology Department, Faculty of Applied Medical Sciences, King Abdulaziz University, Jeddah, Saudi Arabia

Abstract. Microbial mats in hot springs form a dynamic ecosystem and support the growth of diverse communities with broad-ranging metabolic capacity. In this study, we used 16S rRNA gene amplicon sequencing to analyse microbial communities in mat samples from two hot springs in Al Aridhah, Saudi Arabia. Putative metabolic pathways of the microbial communities were identified using phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt). Filamentous anoxygenic phototrophic bacteria associated with phylum

Chloroflexi were abundant (> 50 %) in both hot springs at 48 °C. *Chloroflexi* were mainly represented by taxa *Chloroflexus* followed by *Roseiflexus*. Cyanobacteria of genus *Arthrospira* constituted 3.4 % of microbial mats. Heterotrophic microorganisms were mainly represented by Proteobacteria, Actinobacteria, Bacteroidetes, and Firmicutes. Archaea were detected at a lower relative abundance (< 1 %). Metabolic pathways associated with membrane transport, carbon fixation, methane metabolism, amino acid biosynthesis, and degradation of aromatic compounds were commonly found in microbial mats of both hot springs. In addition, pathways for production of secondary metabolites and antimicrobial compounds were predicted to be present in microbial mats. In conclusion, microbial communities in the hot springs of Al Aridhah were composed of diverse bacteria, with taxa of *Chloroflexus* being dominant.

Received June 17, 2019. Accepted November 8, 2019.

This project work was funded by General Directorate of Research Grants (GDRG), King Abdulaziz City for Science and Technology (KACST), Saudi Arabia under grant number AT-12-33.

Corresponding author: Muhammad Yasir, Special Infectious Agents Unit, King Fahd Medical Research Center, King Abdulaziz University, Jeddah-21589, Saudi Arabia. E-mail: yasirkhattak.mrl@gmail.com

Abbreviations: AAR4 – Al Aridhah4, AAR5 – Al Aridhah5, AMDR – antimicrobial drug resistance, BID – bacterial infectious disease, BOSM – biosynthesis of other secondary metabolites, FAP – filamentous anoxygenic phototrophic, FSD – folding, sorting and degradation, GBM – glycan biosynthesis and metabolism, KEGG – Kyoto encyclopaedia of genes and genomes, MCV – metabolism of cofactors and vitamins, MTP – metabolism of terpenoids and polyketides, OM – organic matter, OTUs – operational taxonomic units, PICRUSt – phylogenetic investigation of communities by reconstruction of unobserved states, RR – replication and repair, TK – total potassium, TN – total nitrogen, TP – total phosphorus.

Introduction

Hot springs represent an extremophilic environment and a suitable habitat for studying the thermophilic microorganisms that flourish at the high temperatures found in extreme geochemical conditions (Wilkins et al., 2019). Microbial mats from geothermal ecosystems provide a model for the ecological analysis of microbial communities that thrive in such conditions (Rozanov et al., 2017). Phototrophic microbial mats form as a result of a gradient in sunlight and the physicochemical conditions supported by the activity of microorganisms (Rozanov et al., 2017; Ward et al., 2019). Microbial mats are commonly observed in hot springs across geographical regions (Alcaman-Arias et al., 2018; Ward et al., 2019).

These mats are mainly composed of Cyanobacteria and filamentous anoxygenic phototrophs (FAPs), including Chloroflexi, in neutral and alkaline hot springs (Rozanov et al., 2017; Uribe-Lorio et al., 2019). The relative abundance of these taxa mainly depends on the temperature, with Cyanobacteria primarily being found in lower-temperature hot springs and Chloroflexi having a higher relative abundance in hot springs that are $> 60\text{ }^{\circ}\text{C}$ (Alcama-Arias et al., 2018; Uribe-Lorio et al., 2019). *Chloroflexus* spp. and *Roseiflexus* spp. have been well studied from microbial mats in Mushroom Spring in Yellowstone National Park, United States (Klatt et al., 2013; Thiel et al., 2017). The majority of Cyanobacteria are mesophilic in nature with a few exceptions, including moderate thermophiles, such as *Fischerella*, and the thermophilic *Synechococcus*, which grows optimally above $55\text{ }^{\circ}\text{C}$ (Allewalt et al., 2006).

Thermophilic microorganisms from hot water springs were initially studied with the use of optical microscopy (Castenholz, 1973), which was later followed by separation and classification of thermophilic strains, using culture-dependent approaches (Pierson and Castenholz, 1974). Studies in the late 1990s revealed that 80–99 % of bacteria present in the environment had not yet been explored by laboratory-based culture-dependent protocols and hence remained overlooked in terms of their ecological functions and potential biotechnological applications (Amann et al., 1995). Recent developments in molecular biology and associated technological advances,

such as 16S amplicon sequencing, shotgun sequencing, single-cell genomics, and transcriptomics, have provided a tremendous opportunity for the identification and characterization of microorganisms (Tripathy et al., 2016; Thiel et al., 2017; Wilkins et al., 2019). Such advances have revolutionized research on microbial diversity, evolution, and adaptation in specific environments (Uribe-Lorio et al., 2019; Wilkins et al., 2019). For instance, distinct bacterial taxa and diverse metabolic capacities were revealed through a metagenomic study of microbial mats from Mushroom Spring (Thiel et al., 2017).

Geological surveys have identified several hot springs in the volcanic region of Jazan and four in the Al-Lith region of Saudi Arabia. These hot springs have been partially studied by culture-dependent approaches (Khiyami et al., 2012; Yasir et al., 2019). In this study, we were the first to use 16S metagenomic analysis to investigate the taxonomic diversity in the microbial mats from the hot springs located at Al Aridhah in the Jazan region.

Material and Methods

Sample collection and physicochemical analysis

In this study, microbial mat samples were collected during January 2015 from two hot springs in close proximity to one another at Al Aridhah (AAR4 and AAR5) in the Jazan region of Saudi Arabia (Fig. 1). The pH and

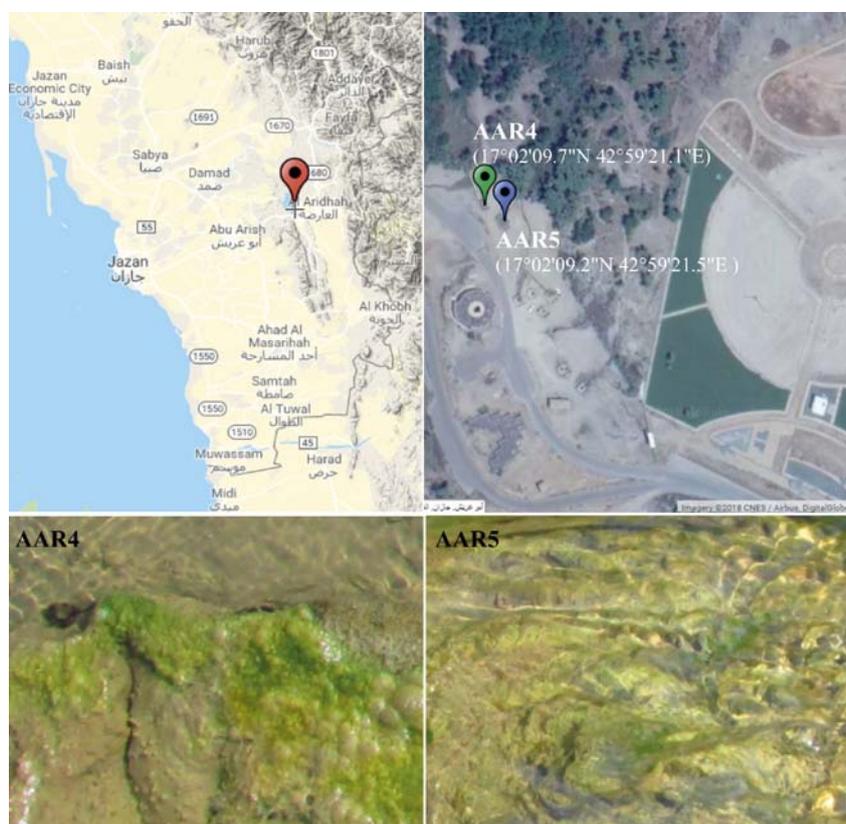


Fig. 1. Map of sampling sites and close up of microbial mats of Al Aridhah hot springs located in the Jazan region of Saudi Arabia. The map was obtained from DigitalGlobe.

temperature of the springs were measured using a Martini portable meter (Martini, Cragieburn, Australia). The microbial mats were 2–3 mm thick, and samples were collected with sterilized spatulas and placed in sterilized containers. Temperature and pH at the sampling spots were 48 °C and 7.3, respectively. Samples were stored at 4 °C during transportation and were kept at –20 °C in the laboratory before further processing. Organic matter was determined through the loss of ignition according to the protocol of Dean (1974), and total nitrogen was determined using the Kjeldahl method. To determine other elements, 0.5 g of each sample was digested with a nitric acid-perchloric acid (HNO₃-HClO₄) mixture (Alzubaidy et al., 2016; Ullah et al., 2017). Following digestion, the solution was diluted with 50 ml of distilled water, and the level of total phosphorus was determined calorimetrically and that of potassium by atomic absorption spectroscopy. The other metals in the samples were determined by inductively coupled plasma-optical emission spectroscopy, using the instrument according to the manufacturer's instructions (Ullah et al., 2017).

DNA extraction and 16S amplicon sequencing

Samples were vigorously homogenized and metagenomic DNA was extracted from the microbial mats with a beads beating protocol using a power soil DNA extraction kit (MO BIO Laboratories, Inc., Carlsbad, CA). Samples were processed for 16S rRNA gene amplicon paired-end sequencing in a MiSeq instrument (Illumina, San Diego, CA) as previously described (Yasir et al., 2015a). Briefly, a PCR-amplified template was generated from genomic DNA using the universal primer set of 515F and 806R targeting the V4 variable region of bacteria and the archaeal 16S rRNA gene. PCR products were purified on AMPure beads, and concentrations were then measured with a dsDNA BR Assay Kit using a Qubit fluorometer (Invitrogen, Carlsbad, CA). Subsequently, limited-cycle PCR with 1 ng of each PCR product was performed to add adapters and dual-index barcodes to each amplicon. After purification, the libraries were normalized according to the Nextera XT protocol (Illumina). The multiplexed samples were pooled into a single flow cell for sequencing using the MiSeq platform (Illumina) following the manufacturer's protocol.

Data analysis and prediction of metabolic functions

Raw FASTQ files were collected from the MiSeq system (Illumina), and paired-end reads were joined using PANDAseq (Masella et al., 2012). Sequences were filtered; they were cleaned of primers and barcodes, all reads with 'N' and those with sequences < 200 bp were deleted, and high-quality sequences were de-replicated (Yasir et al., 2015b). The filtered sequence reads were clustered at $k = 10$ (97 % similarity), and chimeras and singleton reads were deleted. Sequence reads of high quality were grouped into operational taxonomic units

(OTUs) using a sequence similarity threshold of 97 %. The sequences were taxonomically classified using BLAST against the Greengenes database and by an RDP classifier and an RDP training set as described previously (Dowd et al., 2008; Yasir, 2018) using QIIME 1.9.1 (Caporaso et al., 2010).

Putative metabolic functions of microbial communities were identified using the PICRUSt (1.1.3) software, which compares the identified 16S rRNA gene sequences to related known genome sequenced species (Langille et al., 2013). OTUs were closed-referenced picked against the Greengenes (v13.5) database at 97 % similarity from each sample sequence file using QIIME (1.9.1) and saved in biome format following the protocol of PICRUSt (1.1.3). Resultant OTUs were used to predict metabolic functions by referencing the Kyoto Encyclopaedia of Genes and Genomes (KEGG) on the web-based Galaxy application. Sequencing data were submitted to the European Nucleotide Archive under accession Nos. SAMEA5203386–SAMEA5203387.

Results

Physicochemical analysis

The temperature of both AAR4 and AAR5 was 48 °C, and their pH was neutral (7.3). Both hot springs were nutritionally poor, and the total organic matter in microbial mats was 1.50 ± 0.06 % and 1.70 ± 0.09 %, respectively (Table 1). The total nitrogen level in both hot springs was 0.02 %, and the phosphorus level was 0.02 % and 0.01 % in AAR4 and AAR5, respectively. The iron concentration was 139.1 ± 0.5 mg/kg in AAR4 and 133.6 ± 0.8 mg/kg in AAR5. No substantial differences were found in the concentration (mg/kg) of magnesium (AAR4 = 25.90 ± 0.07 , AAR5 = 26.1 ± 0.1) and sodium (AAR4 12.13 ± 0.02 , AAR5 = 12.05 ± 0.05) between the two hot springs. The total potassium concentration was 0.27 ± 0.01 % in AAR4 and 0.28 ± 0.01 % in AAR5. Both hot springs had a Zn concentration of < 0.5 mg/kg (Table 1).

Table 1. Physicochemical analysis of the hot springs of Al Aridhah

Variable	AAR4	AAR5
pH	7.3	7.3
Temperature (°C)	48	48
OM (%)	1.50 ± 0.06	1.70 ± 0.09
TK (%)	0.27 ± 0.01	0.28 ± 0.01
TN (%)	0.02	0.02
TP (%)	0.02	0.01
Iron (mg/Kg)	139.1 ± 0.5	133.6 ± 0.8
Magnesium (mg/Kg)	25.90 ± 0.07	26.10 ± 0.10
Sodium (mg/Kg)	12.13 ± 0.02	12.05 ± 0.05
Zinc (mg/Kg)	0.44 ± 0.03	0.49 ± 0.04

Microbial diversity analysis

In total, 342,489 trimmed and high-quality (> 200 bp) sequences were obtained from 343,206 raw reads of Al Aridhah hot springs. Sequence reads were classified into 1,074 OTUs at 97 % identity, excluding singletons, and assigned to microbial domains. A total of 36 phyla were detected in the microbial mat samples, including four phyla from archaea. Thirty phyla were commonly present in microbial mats from both hot springs. The dominant six bacterial phyla constituted > 90 % of all the sequence reads. The most dominant phylum was Chloroflexi, with a mean relative abundance value of 55.7 ± 5.6 %, and it was present at a relatively higher abundance in AAR4 (Fig. 2A). Other dominant phyla were Actinobacteria (12.3 ± 2.3 %) and Proteobacteria (10.9 ± 5.1 %), whereas Bacteroidetes, Cyanobacteria, and Firmicutes represented bacterial sequences in the range of 3.3–6.3 % (Fig. 2A). Proteobacteria were predominantly comprised of δ -Proteobacteria (Fig. 2B). Among archaea, Euryarchaeota, Crenarchaeota, Thaumarchaeota, and Korarchaeota were detected in the microbial mat from both hot springs, but were relatively less abundant (≤ 1 %) compared to bacteria.

The sequence reads were classified into 243 bacterial and 24 archaeal families. A relatively high diversity was observed in AAR5 and 232 bacterial families were identified, compared with 193 families in AAR4 (Fig. 3A).

One hundred eighty-two families were commonly detected in both hot springs, whereas 49 families were unique to AAR5 and 12 were specifically detected in AAR4 (Fig. 3A). Chloroflexaceae was the dominant family, followed by Roseiflexaceae, Micrococcaceae, and Oscillatoriales in both hot springs (Fig. 3B). Among archaea, 16 families were commonly found in both hot springs, and eight families were unique to AAR5 (Fig. 3C). Methanobacteriaceae, Cenarchaeaceae, Methanosaetaceae, and Thermococcaceae were present at relatively higher abundance in both hot springs, whereas Desulfurococcaceae and Thaumarchaeota had higher abundances among archaeal families in the AAR5 microbial mat.

The sequences read were classified into 697 OTUs at the genus level, comprising 657 bacteria and 40 archaea. The highest number of bacterial genera (619) was detected in AAR5, whereas 424 genera were found in AAR4 (Fig. 4A). Among the genera detected, 386 were present in both hot springs, 233 were unique to AAR5, and 38 genera were specifically found in AAR4 (Fig. 4A). Moreover, eight genera in AAR4 and 15 genera in AAR5 were present at ≥ 1 % abundance, accounting for 84 % and 79 % of the total sequence reads in the respective samples (Fig. 4B). The genera *Chloroflexus* (34.6 ± 5.6 %), *Roseiflexus* (14.2 ± 6.8 %), and *Arthrobacter* (12.2 ± 2.3 %) were present at a relatively higher abundance in both hot springs' mats (Fig. 4B), whereas

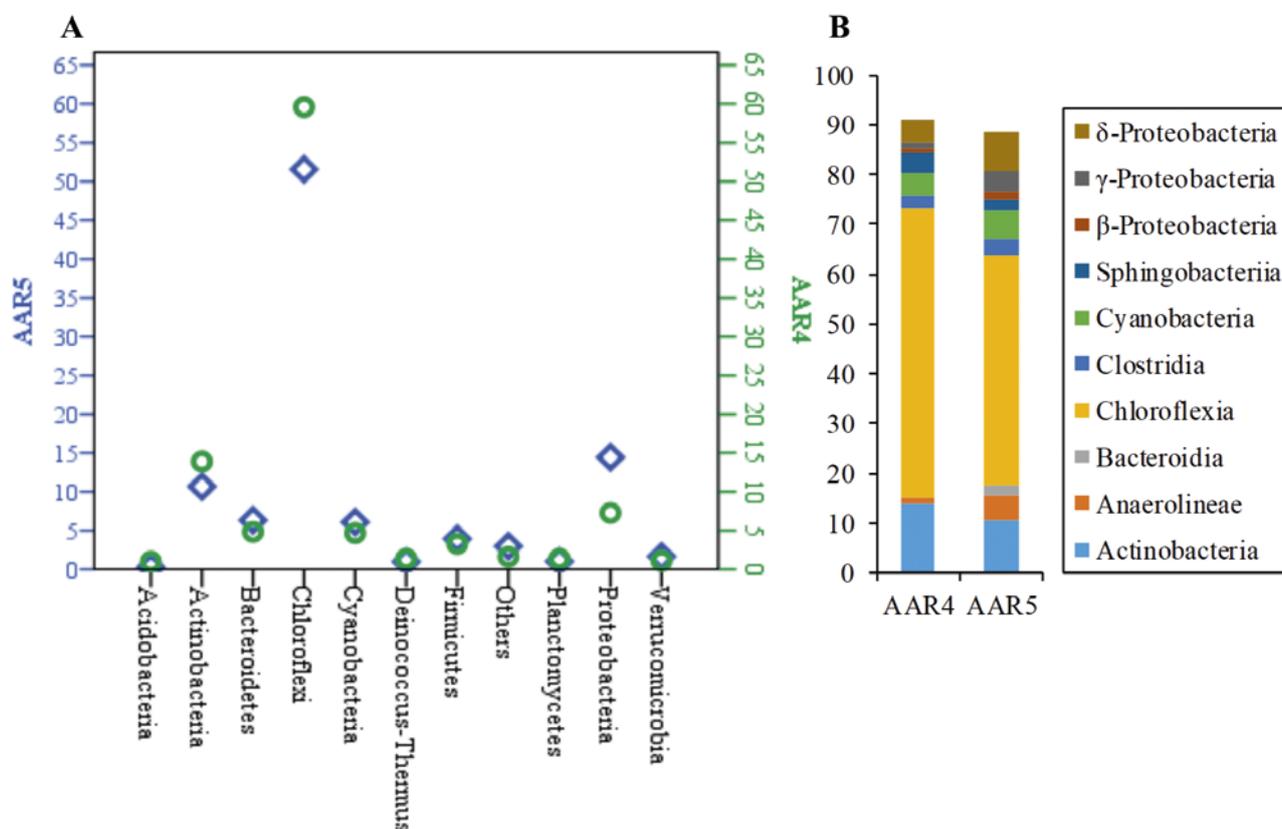


Fig. 2. Percentage of relative abundance of the dominant (A) phyla and (B) classes identified from microbial mats of the hot springs of Al Aridhah

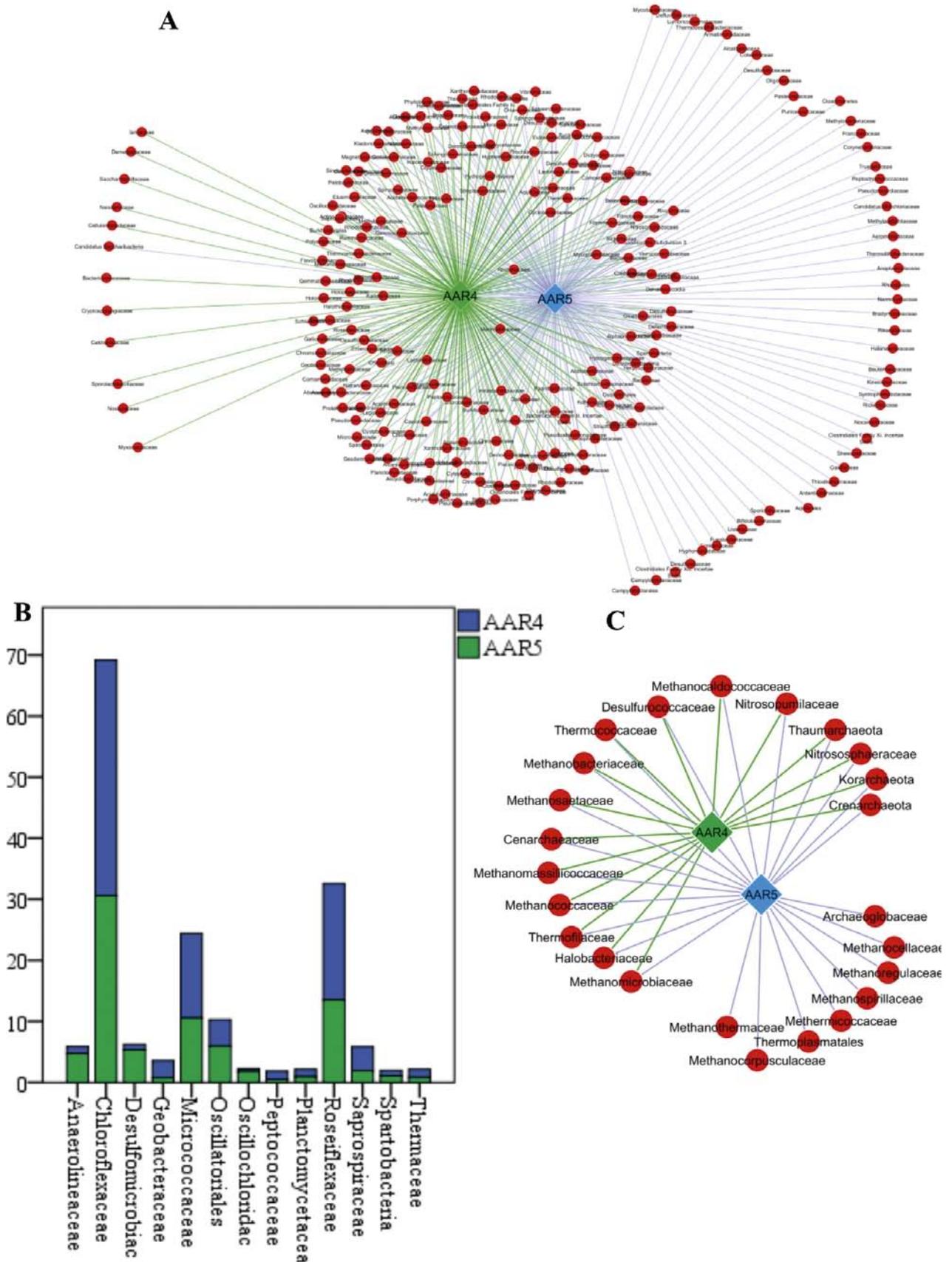


Fig. 3. (A) Network analysis of the OTUs identified at the family taxonomic level of bacteria in microbial mats from the hot springs of Al Aridhah. (B) Percentage of relative abundance of the top 13 families found in the studied samples, and (C) network presentation of identified archaeal families. Nodes connected with two lines are commonly detected in both hot springs, and nodes connected with a single line are unique to the respective hot spring.

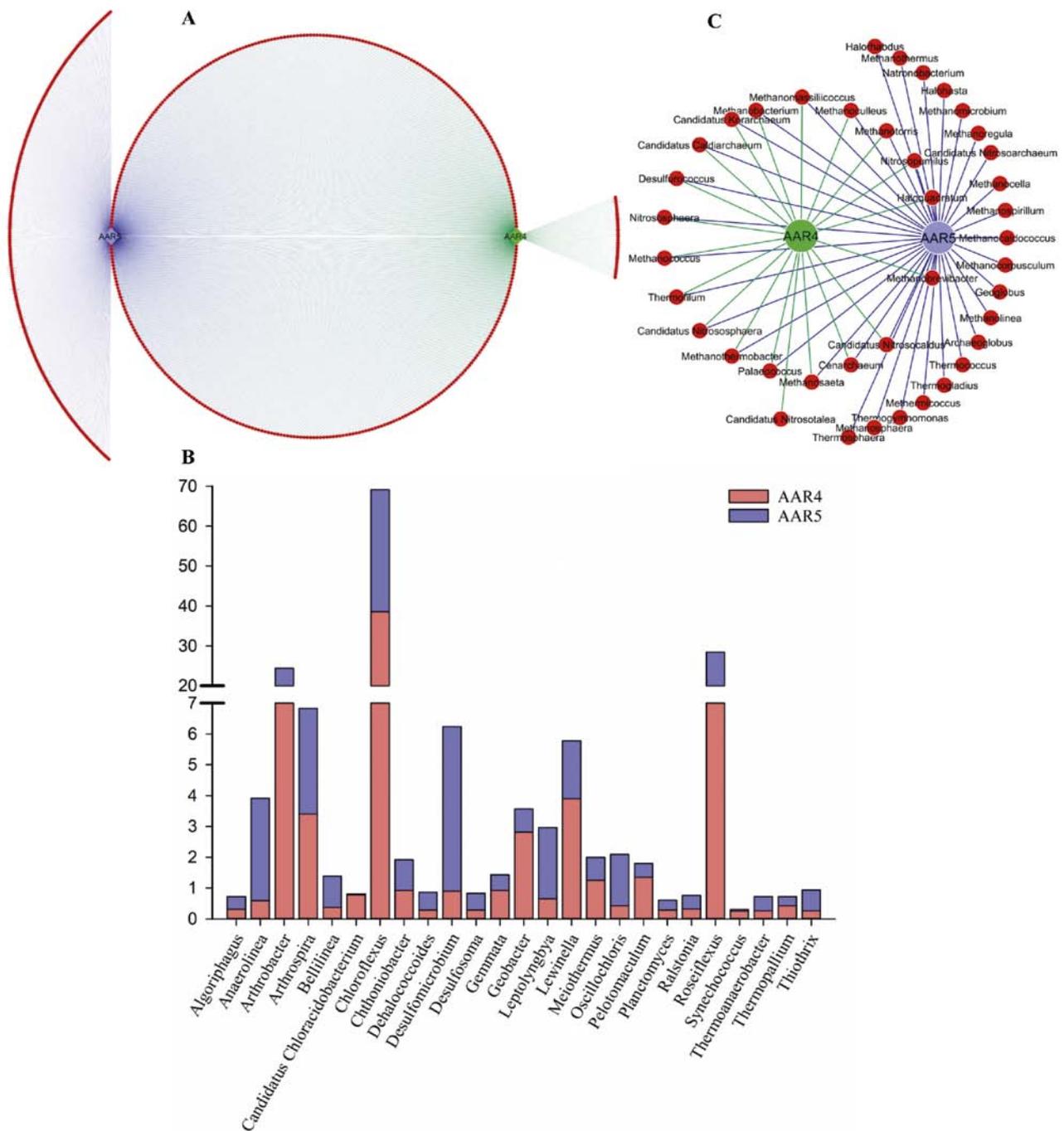


Fig. 4. (A) Network analysis of OTUs identified at the genus level of bacteria in microbial mats from the hot springs of Al Aridhah. (B) Percentage of relative abundance of the top 25 genera found in the studied samples, and (C) network presentation of identified archaeal genera. Nodes connected with two lines are commonly detected in both hot springs, and nodes connected with a single line are unique to the respective hot spring.

Lewinella, *Geobacter*, *Pelotomaculum*, and *Meiothermus* were present at relatively higher abundance in AAR4 compared with AAR5. Genera *Desulfomicrobium*, *Heliothrix*, *Anaerolinea*, and *Leptolyngbya* were detected at a higher abundance in AAR5 compared with AAR4. Among archaea, 19 genera were commonly present in both hot springs and 20 genera were unique to AAR5 (Fig. 4C). *Candidatus Nitrosocaldus*, *Methanosaeta*,

Methanobrevibacter, *Cenarchaeum*, and *Palaeococcus* were present at relatively higher abundance in both hot springs.

Chloroflexi and Cyanobacteria

From sequence reads, 1,018 bacterial OTUs were identified at the species level. Five hundred thirty-one OTUs were commonly present in both hot springs from

among the 618 OTUs of AAR4 and the 934 OTUs of AAR5. Taxa associated with Chloroflexi were predominantly identified in AAR4 and AAR5 and mainly belonged to class Chloroflexia followed by Anaerolineae. Forty-two OTUs linked to Chloroflexi at the species level were identified and were predominantly composed of *Chloroflexus* spp. (36.2 % and 26.5 %) followed by *Roseiflexus castenholzii* (14.4 % and 7.1 % in AAR4 and AAR5, respectively; Fig. 5A). OTUs of *Roseiflexus* spp. and *Chloroflexus aurantiacus* were detected at a relatively higher abundance in both hot springs. *Anaerolinea* spp. and *Oscillochloris trichoides* were present at a relatively higher abundance of 3.4 % and 1.7 % in AAR5, respectively, and were detected at < 1 % in AAR4 (Fig. 5A). Other OTUs were detected at < 1 % in the microbial mat from both hot springs. Moreover, only two taxa of *Chlorobium* spp. and *Pelodictyon phaeoclathratiforme* from phylum Chlorobi were detected and were present at < 1 % abundance.

Fifty-three OTUs from Cyanobacteria were detected from the microbial mats of Al Aridhah hot springs. Twenty-nine OTUs were common in both hot springs. Twenty OTUs were unique to AAR5, and four OTUs were specifically detected in AAR4. Taxa of *Arthrospira* spp. from Cyanobacteria were predominant, at a relative abundance of 3.4 %, in both hot springs (Fig. 5A). *Leptolyngbya* spp. were detected at a relatively high abundance of 1.1 % in AAR5 compared with AAR4 (0.5 %). Other OTUs were present at < 1 %, including *Synechococcus* spp. (Fig. 5A).

Heterotrophic microbial community analysis

Heterotrophic bacteria represented < 55 % of the community of the microbial mats and mainly comprised Actinobacteria, Proteobacteria, Bacteroidetes, and Firmicutes. Proteobacteria were present at relatively higher abundance in AAR5 compared with AAR4 and were mainly composed of δ -Proteobacteria followed by γ -Proteobacteria and β -Proteobacteria (Fig. 2A, B). A total of 436 OTUs were classified to Proteobacteria. *Desulfomicrobium terraneus* from δ -Proteobacteria and *Pseudoalteromonas* spp. from γ -Proteobacteria were identified in AAR5 at a relatively higher abundance of 5.3 % and 1.7 %, respectively (Fig. 5B). *Geobacter* spp. were found at a relatively higher abundance of 2.8 % in AAR4, and other OTUs were present at < 1 % abundance (Fig. 5B).

Fifty-five OTUs were identified from Actinobacteria, and OTUs linked to *Arthrobacter* spp. were detected at >10 % abundance in both hot springs (Fig. 5B). Of Firmicutes, 188 OTUs were detected and were predominantly composed of taxa from the strictly anaerobic class of Clostridia (Fig. 2B). In total, 97 OTUs were detected from Bacteroidetes, and *Lewinella* spp. were commonly found in both hot springs at > 1 % abundance (Fig. 5B). Other OTUs were detected at < 1 % abundance, except for *Schleiferia* spp. and *Anaerophaga* spp. (Fig. 5B).

Predicted metabolic functions

The putative metabolic functions of the hot spring mat microbiota were predicted from 16S rDNA amplicon sequence data using the PICRUSt tool. The estimated KEGG orthologies were found to be involved in the main pathways (level 1) of metabolism, genetic information processing, environmental information processing, cellular processes, and human diseases. Expanding the main pathways at level 2 resulted in 24 sub-pathways, which were further classified into 203 sub-sub-pathways at level 3. In both hot springs, most of the predicted pathways were associated with the metabolism of amino acids (10.4 ± 0.4 %), carbohydrates (10.3 ± 0.5 %), energy (7.3 ± 0.3 %), lipids (3.7 ± 0.1 %), and glycan biosynthesis and metabolism (2.2 ± 0.1 %). Apart from common amino acid synthesis pathways, different pathways were predicted for carbohydrate metabolism including pyruvate, propanoate, glycolysis/gluconeogenesis, butanoate, and pentose phosphate pathway (Fig. 6).

Notably, xenobiotic biodegradation and metabolism pathways were found at a considerable percentage (3.4 ± 0.3 %) and were mainly linked to the degradation of naphthalene, benzoate, toluene, aminobenzoate, caprolactam, styrene, xylene, dioxin, and polycyclic aromatic hydrocarbons and the metabolism of xenobiotics by cytochrome P450. The predicted energy metabolic pathways in the bacterial communities involved oxidative phosphorylation and the metabolism of methane and nitrogen. Carbon fixation pathways in prokaryotes were found at > 1 % in both hot springs (Fig. 6), whereas sulphur metabolism was not a major pathway in microbial communities of the mat samples. Secondary metabolite, terpenoid, and polyketide pathways for the following antibiotics were predicted: streptomycin, tetracycline, novobiocin, ansamycins, penicillin, and cephalosporin (Fig. 6). Pathways involved in infectious diseases, including the *Vibrio cholerae* pathogenic cycle and *Staphylococcus aureus* infection and bacterial toxins, were identified. Moreover, resistance to beta-lactam antibiotics was predicted at a relatively lower abundance of < 0.5 %.

Consistent with the harsh environment of hot springs, sequences linked with DNA repair and recombination proteins (2.4 ± 0.1 %) were predominantly identified at level 3 from the sub-pathway of replication and repair within the genetic information processing pathway. In addition, protein folding and associated processing function were predicted at a relative abundance of 0.9 % in both hot springs. Different types of membrane transport, mainly the ABC transporter (2.9 ± 0.2 %) and a two-component system (2.1 ± 0.3 %) from the environmental information processing pathway, were predicted. Bacterial motility proteins (1.7 ± 0.4 %) were prominently found in the cellular processes pathway. Overall, the predicted metabolic pathways and relative abundance did not vary markedly in mats from either of the hot springs.

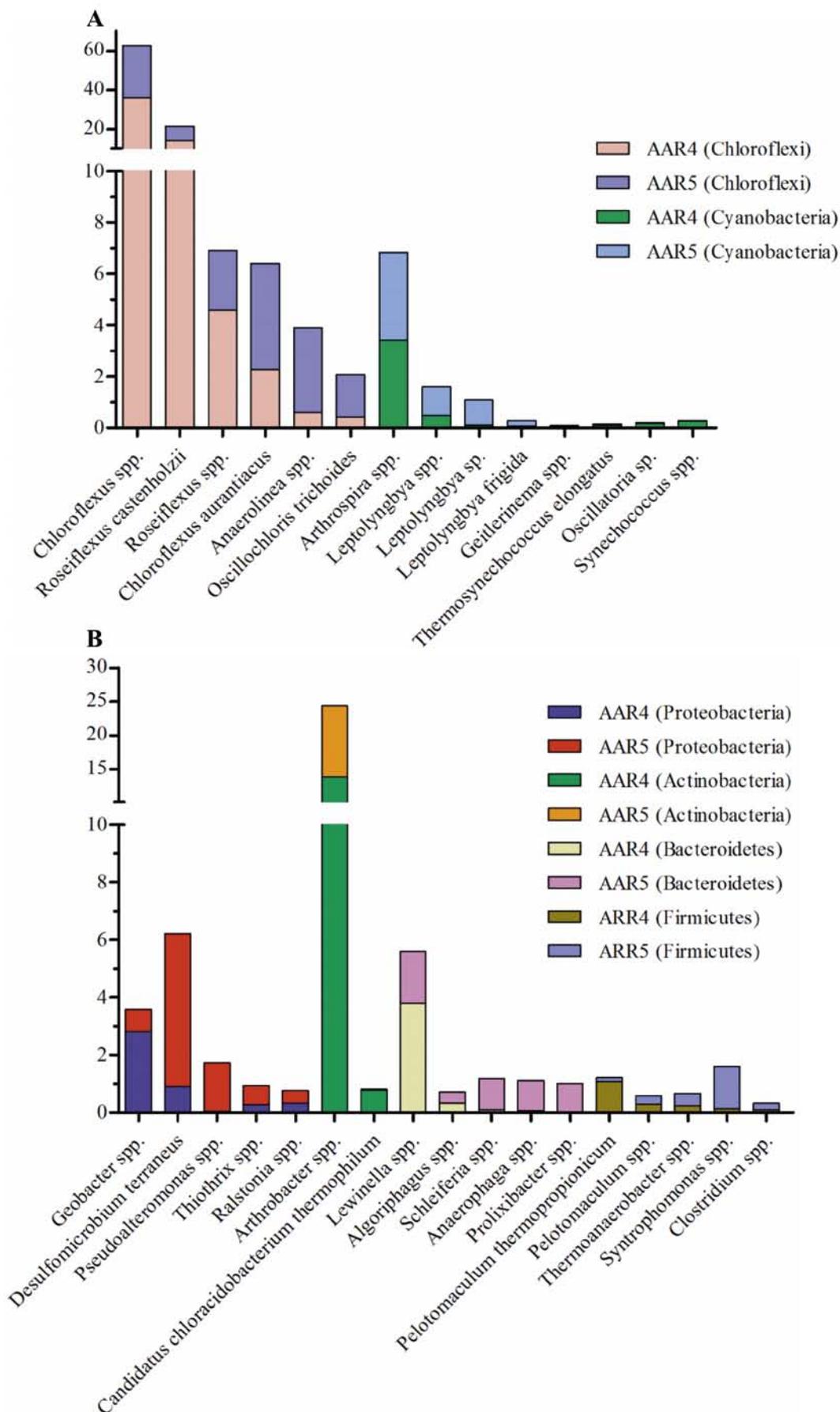


Fig. 5. (A) Percentage of relative abundance of the dominant taxa associated with Chloroflexi and Cyanobacteria, and (B) heterotrophic bacteria

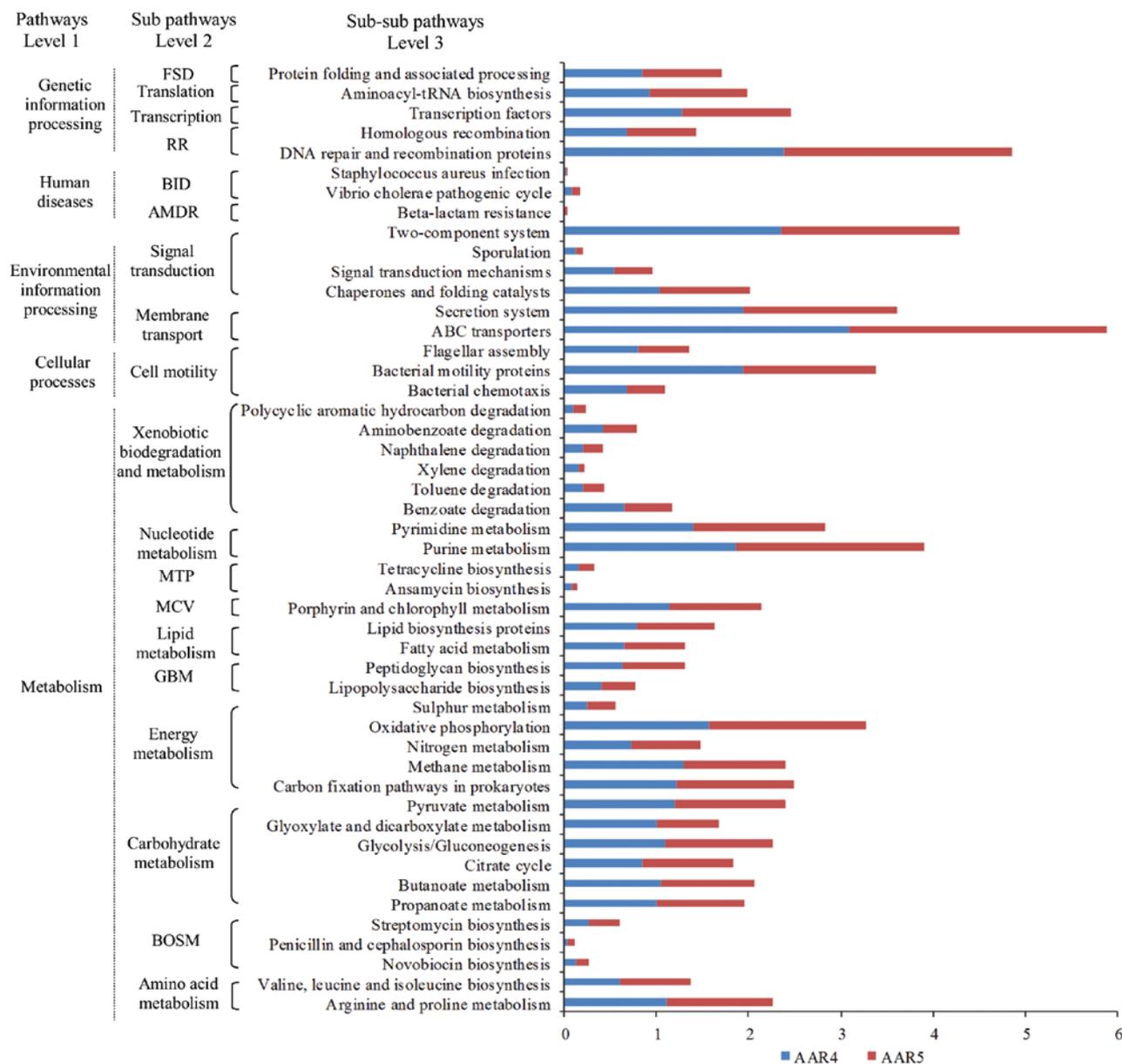


Fig. 6. Relative abundance of putative pathways of microbial communities in microbial mats from the hot springs of Al Aridhah using PICRUSt. The pathways are presented according to KEGG.

Discussion

Physicochemical parameters influence the microbial diversity and community composition in hot springs. The studied hot springs and other geothermal springs in the Jazan region in Saudi Arabia have a neutral pH (Khiyami et al., 2012). The temperature of these hot springs is lower than that of the hot spring at Ain Khulab (71 °C) but in the range of the hot spring of Bani Malik (45 °C) of this region. These hot springs are oligotrophic, with a low nutrient level and relatively abundant iron. The poor nutrient level in the Al Aridhah hot springs is in agreement with previous studies from Saudi Arabia, although variation was observed in the chemical analysis, which may affect the microbial diversity and composition of the microbial mats of the AAR4 and

AAR5 hot springs (Al-Dayel, 1988; Khiyami et al., 2012).

Microbial mats in hot springs exhibit spatial and temporal heterogeneity and are composed of diverse species capable of a wide range of metabolic processes (Kim et al., 2015; Thiel et al., 2017). Our results demonstrated that the dominant phyla in both of the hot springs were Chloroflexi, Actinobacteria, Proteobacteria, Bacteroidetes, Cyanobacteria, and Firmicutes, all of which are commonly reported in hot springs around the world (Uribe-Lorio et al., 2019; Wilkins et al., 2019). However, their abundance differed, with members of the Chloroflexi phylum being highly abundant and Cyanobacteria having low abundance in the Al Aridhah hot springs. Both types grow at 48 °C, but in general, the taxa of Cyanobacteria grow optimally in low-temperature hot

springs and members of Chloroflexi phylum grow better at higher temperatures (Nubel et al., 2002; Sahoo et al., 2017). Alcaman-Arias et al. (2018) observed Cyanobacteria and Chloroflexi at a similar relative abundance in the Porcelana hot spring at a temperature of 58 °C. However, several published reports have identified a negative or exclusion relationship between Cyanobacteria and Chloroflexi, as found in the AAR4 and AAR5 hot springs (Sahoo et al., 2017; Ward et al., 2019). These findings suggest that variation in microbial mats with regard to the taxa abundance across different geographical regions might be influenced by other physicochemical and environmental factors.

In agreement with previous studies, *Chloroflexus* spp. and *Roseiflexus* spp. were abundantly found from among FAP bacteria, as found in the extensively studied Mushroom Spring (Allewalt et al., 2006; Bhaya et al., 2007). However, the taxa of *Candidatus Thermochlorobacter aerophilum* was not detected in the microbial mats of Al Aridhah hot springs, although they were dominantly identified along with other newly identified dominant taxa of *Candidatus Chloracidobacterium thermophilum* and class *Anaerolineae* from phylum Chloroflexi in the metagenomic study from microbial mats of Octopus Spring and Mushroom Spring, both in Yellowstone National Park (Klatt et al., 2011; Garcia Costas et al., 2012).

The relative abundance of heterotrophic bacteria varied between the AAR4 and AAR5 hot springs. Actinobacteria were present at a relatively higher abundance in AAR4 and Proteobacteria in AAR5, followed by Bacteroidetes and Firmicutes. Actinobacteria, which are commonly found in soil and aquatic environments, are generally involved in geochemical cycles and the turnover of organic matter. Proteobacteria were dominated by sulphate-reducing δ -Proteobacteria, and the relative abundance of *Desulfomicrobium* was markedly different between AAR4 and AAR5. Similarly, bacteria from the phyla Firmicutes, Actinobacteria, Proteobacteria, and Bacteroidetes were found in most of the studied hot springs such as those in Tengchong in China (Pagaling et al., 2012) and Nevada in the United States (Song et al., 2009). In contrast to previous studies, Planctomycetes and Verrucomicrobia were detected at lower abundance in the microbial mats of the hot springs studied (Coman et al., 2013; Chan et al., 2015).

Metabolic pathways in the hot springs of Ain Al Aridhah are probably based on interactions between autotrophic and heterotrophic microbial communities. An increased abundance of Chloroflexi indicates low photosynthetic activity and the presence of a redox gradient. The presence of anaerobic Clostridia from phylum Firmicutes suggests an anaerobic environment at the lower layer, where organic material is decomposed into low-molecular-weight molecules. Moreover, predicted sulphur pathways in the microbial mat probably exist and may be associated with Proteobacteria, Firmicutes (Urbieta et al., 2015), Chloroflexi, and Chlorobi (Bryant et al., 2012). In line with the methane metabolism pre-

dicted from the taxonomic data, methanotrophs belonging to genera *Hyphomicrobium*, *Methyloligella*, and *Methylobacterium* were identified in the Al Aridhah hot springs. In addition, methane-oxidizing *Methyloversatilis* and *Methyloversatilis* from β -Proteobacteria were found in both hot springs (Tsubota et al., 2005). Consistent with our KEGG functional analysis from 16S amplicon sequencing data, xenobiotic degradation, aromatic compound metabolism, and environmental information processing pathways have previously been reported in Amazonian and Odisha hot spring microbial communities (Panda et al., 2016; Sahoo et al., 2017). However, variation exists in the percentages of the predicted functional categories among this and previous studies, as observed in the taxonomic data (Panda et al., 2016; Sahoo et al., 2017).

In conclusion, 16S metagenomic analysis provided deep insight into the microbial community structure of the microbial mats of the Al Aridhah hot springs in Saudi Arabia. Specific lineages of FAP bacteria, mainly *Chloroflexus* and *Roseiflexus*, were dominantly associated with microbial mats of both hot springs. This work and other studies that have observed variation in microbial community composition suggest a biogeographic pattern in hot spring microbial mat microbiota. In addition, transcriptomic studies and metagenomic studies based on shotgun sequencing are required to further analyse the metabolic potential of the hot spring microbial mats estimated from the taxonomic data in this study.

References

- Al-Dayel, M. (1988) Geothermal resources in Saudi Arabia. *Geothermics* **17**, 465-476.
- Alcaman-Arias, M. E., Pedros-Alio, C., Tamames, J., Fernandez, C., Perez-Pantoja, D., Vasquez, M., Diez, B. (2018) Diurnal changes in active carbon and nitrogen pathways along the temperature gradient in Porcelana hot spring microbial mat. *Front. Microbiol.* **9**, 2353.
- Allewalt, J. P., Bateson, M. M., Revsbech, N. P., Slack, K., Ward, D. M. (2006) Effect of temperature and light on growth of and photosynthesis by *Synechococcus* isolates typical of those predominating in the octopus spring microbial mat community of Yellowstone National Park. *Appl. Environ. Microbiol.* **72**, 544-550.
- Alzubaidy, H., Essack, M., Malas, T. B., Bokhari, A., Motwalli, O., Kamanu, F. K., Jamhor S. A., Mokhtar, N. A., Antunes, A., Simões, M. F., Alam, I., Bougouffa, S., Lafi, F. F., Bajic, V. B., Archer, J. A. (2016) Rhizosphere microbiome metagenomics of gray mangroves (*Avicennia marina*) in the Red Sea. *Gene* **576**(2 Pt 1), 626-636.
- Amann, R. I., Ludwig, W., Schleifer, K. H. (1995) Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol. Rev.* **59**, 143-169.
- Bhaya, D., Grossman, A. R., Steunou, A. S., Khuri, N., Cohan, F. M., Hamamura, N., Melendrez, M. C., Bateson, M. M., Ward, D. M., Heidelberg, J. F. (2007) Population level functional diversity in a microbial community revealed by

- comparative genomic and metagenomic analyses. *ISME J.* **1**, 703-713.
- Bryant, D. A., Liu, Z., Li, T., Zhao, F., Costas, A. M. G., Klatt, C. G., Ward, D. M., Frigaard N-U, Overmann J. (2012) Comparative and functional genomics of anoxygenic green bacteria from the taxa Chlorobi, Chloroflexi, and Acidobacteria. In: *Advances in Photosynthesis and Respiration: Functional Genomics and Evolution of Photosynthetic Systems*. eds. Burnap, R., Vermaas, W., vol 33, pp. 47-102. Springer, Dordrecht, The Netherlands.
- Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., Fierer, N., Peña, A. G., Goodrich, J. K., Gordon, J. I., Huttley, G. A., Kelley, S. T., Knights, D., Koenig, J. E., Ley, R. E., Lozupone, C. A., McDonald, D., Muegge, B. D., Pirrung, M., Reeder, J., Sevinsky, J. R., Turnbaugh, P. J., Walters, W. A., Widmann, J., Yatsunenko, T., Zaneveld, J., Knight, R. (2010) QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* **7**, 335-336.
- Castenholz, R. W. (1973) The possible photosynthetic use of sulfide by the filamentous phototrophic bacteria of hot springs. *Limnol. Oceanogr.* **18**, 863-876.
- Chan, C. S., Chan, K. G., Tay, Y. L., Chua, Y. H., Goh, K. M. (2015) Diversity of thermophiles in a Malaysian hot spring determined using 16S rRNA and shotgun metagenome sequencing. *Front. Microbiol.* **6**, 177.
- Coman, C., Druga, B., Hegedus, A., Sicora, C., Dragos, N. (2013) Archaeal and bacterial diversity in two hot spring microbial mats from a geothermal region in Romania. *Extremophiles* **17**, 523-534.
- Dean, W. E. (1974). Determination of carbonate and organic matter in calcareous sediments and sedimentary rocks by loss on ignition: comparison with other methods. *J. Sediment. Res.* **44**, 242-248.
- Dowd, S. E., Sun, Y., Wolcott, R. D., Domingo, A., Carroll, J. A. (2008) Bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) for microbiome studies: bacterial diversity in the ileum of newly weaned Salmonella-infected pigs. *Foodborne Pathog. Dis.* **5**, 459-472.
- Garcia Costas, A. M., Liu, Z., Tomsho, L. P., Schuster, S. C., Ward, D. M., Bryant, D. A. (2012) Complete genome of *Candidatus Chloracidobacterium thermophilum*, a chlorophyll-based photoheterotroph belonging to the phylum Acidobacteria. *Environ. Microbiol.* **14**, 177-190.
- Khiyami, M. A., Serour, E. A., Shehata, M. M., Bahklia, A. H. (2012) Thermo-aerobic bacteria from geothermal springs in Saudi Arabia. *Afr. J. Biotechnol.* **11**, 4053-4062.
- Kim, Y. M., Nowack, S., Olsen, M. T., Becraft, E. D., Wood, J. M., Thiel, V., Klapper, I., Kuhl, M., Fredrickson, J. K., Bryant, D. A., Ward, D. M., Metz, T. O. (2015) Diel metabolomics analysis of a hot spring chlorophototrophic microbial mat leads to new hypotheses of community member metabolisms. *Front. Microbiol.* **6**, 209.
- Klatt, C. G., Wood, J. M., Rusch, D. B., Bateson, M. M., Hamamura, N., Heidelberg, J. F., Grossman, A. R., Bhaya, D., Cohan, F. M., Kuhl, M., Bryant, D. A., Ward, D. M. (2011) Community ecology of hot spring cyanobacterial mats: predominant populations and their functional potential. *ISME J.* **5**, 1262-1278.
- Klatt, C. G., Liu, Z., Ludwig, M., Kuhl, M., Jensen, S. I., Bryant, D. A., Ward, D. M. (2013) Temporal metatranscriptomic patterning in phototrophic Chloroflexi inhabiting a microbial mat in a geothermal spring. *ISME J.* **7**, 1775-1789.
- Langille, M. G., Zaneveld, J., Caporaso, J. G., McDonald, D., Knights, D., Reyes, J. A., Clemente, J. C., Burkepille, D. E., Vega Thurber, R. L., Knight, R., Beiko, R. G., Huttenhower, C. (2013) Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. *Nat. Biotechnol.* **31**, 814-821.
- Masella, A. P., Bartram, A. K., Truszkowski, J. M., Brown, D. G., Neufeld, J. D. (2012) PANDAseq: paired-end assembler for illumina sequences. *BMC Bioinformatics* **13**, 31.
- Nubel, U., Bateson, M. M., Vandieken, V., Wieland, A., Kuhl, M., Ward, D. M. (2002) Microscopic examination of distribution and phenotypic properties of phylogenetically diverse Chloroflexaceae-related bacteria in hot spring microbial mats. *Appl. Environ. Microbiol.* **68**, 4593-4603.
- Pagaling, E., Grant, W. D., Cowan, D. A., Jones, B. E., Ma, Y., Ventosa, A., Heaphy, S. (2012) Bacterial and archaeal diversity in two hot spring microbial mats from the geothermal region of Tengchong, China. *Extremophiles* **16**, 607-618.
- Panda, A. K., Bisht, S. S., De Mandal, S., Kumar, N. S. (2016) Bacterial and archeal community composition in hot springs from Indo-Burma region, North-east India. *AMB Express* **6**, 111.
- Pierson, B. K., Castenholz, R. W. (1974) A phototrophic gliding filamentous bacterium of hot springs, *Chloroflexus aurantiacus*, gen. and sp. nov. *Arch. Microbiol.* **100**, 5-24.
- Rozaanov, A. S., Bryanskaya, A. V., Ivanisenko, T. V., Malup, T. K., Peltek, S. E. (2017) Biodiversity of the microbial mat of the Garga hot spring. *BMC Evol. Biol.* **17(Suppl 2)**, 254.
- Sahoo, R. K., Gaur, M., Das, A., Singh, A., Kumar, M., Subudhi, E. (2017) Comparative analysis of 16S rRNA gene Illumina sequence for microbial community structure in diverse unexplored Hot Springs of Odisha, India. *Geomicrobiol. J.* **34**, 567-576.
- Song, Z., Zhi, X., Li, W., Jiang, H., Zhang, C., Dong, H. (2009) Actinobacterial diversity in hot springs in Tengchong (China), Kamchatka (Russia), and Nevada (USA). *Geomicrobiol. J.* **26**, 256-263.
- Thiel, V., Hugler, M., Ward, D. M., Bryant, D. A. (2017) The dark side of the Mushroom spring microbial mat: life in the shadow of chlorophototrophs. II. metabolic functions of abundant community members predicted from metagenomic analyses. *Front. Microbiol.* **8**, 943.
- Tripathy, S., Padhi, S. K., Mohanty, S., Samanta, M., Maiti, N. K. (2016) Analysis of the metatranscriptome of microbial communities of an alkaline hot sulfur spring revealed different gene encoding pathway enzymes associated with energy metabolism. *Extremophiles* **20**, 525-536.
- Tsubota, J., Eshinimaev, B., Khmelenina, V. N., Trotsenko, Y. A. (2005) *Methylothermus thermalis* gen. nov., sp. nov., a novel moderately thermophilic obligate methanotroph from a hot spring in Japan. *Int. J. Syst. Evol. Microbiol.* **55(Pt 5)**, 1877-1884.
- Ullah, R., Yasir, M., Khan, I., Bibi, F., Sohrab, S. S., Al-Ansari, A., Al-Abbasi, F., Al-Sofyani, A. A., Daur, I., Lee, S. W., Azhar, E. I. (2017) Comparative bacterial community analysis in relatively pristine and anthropogenically influenced

- mangrove ecosystems on the Red Sea. *Can. J. Microbiol.* **63**, 649-660.
- Urbieta, M. S., Gonzalez-Toril, E., Bazan, A. A., Giaveno, M. A., Donati, E. (2015) Comparison of the microbial communities of hot springs waters and the microbial biofilms in the acidic geothermal area of Copahue (Neuquen, Argentina). *Extremophiles* **19**, 437-450.
- Uribe-Lorio, L., Brenes-Guillen, L., Hernandez-Ascencio, W., Mora-Amador, R., Gonzalez, G., Ramirez-Umana, C. J., Díez, B., Pedrós-Alió, C. (2019) The influence of temperature and pH on bacterial community composition of microbial mats in hot springs from Costa Rica. *Microbiologyopen*, **8**, e893.
- Ward, L. M., Idei, A., Nakagawa, M., Ueno, Y., Fischer, W. W., McGlynn, S. E. (2019) Geochemical and metagenomic characterization of Jinata Onsen, a proterozoic-analog hot spring, reveals novel microbial diversity including iron-tolerant phototrophs and thermophilic lithotrophs. *Microbes Environ.* **34**, 278-292.
- Wilkins, L. G. E., Ettinger, C. L., Jospin, G., Eisen, J. A. (2019) Metagenome-assembled genomes provide new insight into the microbial diversity of two thermal pools in Kamchatka, Russia. *Sci. Rep.* **9**, 3059.
- Yasir, M., Angelakis, E., Bibi, F., Azhar, E. I., Bachar, D., Lagier, J. C., (2015a) Comparison of the gut microbiota of people in France and Saudi Arabia. *Nutr. Diabetes* **5**, e153.
- Yasir, M., Azhar, E. I., Khan, I., Bibi, F., Baabdullah, R., Al-Zahrani, I. A., Al-Ghamdi, A. K. (2015b) Composition of soil microbiome along elevation gradients in southwestern highlands of Saudi Arabia. *BMC Microbiol.* **15**, 65.
- Yasir, M. (2018) Analysis of bacterial communities and characterization of antimicrobial strains from cave microbiota. *Braz. J. Microbiol.* **49**, 248-257.
- Yasir, M., Qureshi, K. A., Khan, I., Bibi, F., Rehan, M., Khan, S. B., Azhar E. I. (2019) Culturomics-based taxonomic diversity of bacterial communities in the hot springs of Saudi Arabia. *OMICS* **23**, 17-27.