Environmental Microbiology

Analysis of bacterial communities and characterization of antimicrobial strains from cave microbiota

Muhammad Yasir

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

ARTICLE INFO

ABSTRACT

In this study for the first-time microbial communities in the caves located in the mountain range of Hindu Kush were evaluated. The samples were analyzed using culture-independent (16S rRNA gene amplicon sequencing) and culture-dependent methods. The amplicon sequencing results revealed a broad taxonomic diversity, including 21 phyla and 20 candidate phyla. *Proteobacteria* were dominant in both caves, followed by *Bacteroidetes*, *Actinobacteria*, *Firmicutes*, *Verrucomicrobia*, *Planctomycetes*, and the archaeal phylum *Euryarchaeota*. Representative operational taxonomic units from Koat Maqbari Ghaar and Smasse-Rawo Ghaar were grouped into 235 and 445 different genera, respectively. Comparative analysis of the cultured bacterial isolates revealed distinct bacterial taxonomic profiles in the studied caves dominated by *Proteobacteria* in Koat Maqbari Ghaar and *Firmicutes* in Smasse-Rawo Ghaar. Majority of those isolates were associated with the genera *Pseudomonas* and *Bacillus*. Thirty strains among the identified isolates from both caves showed antimicrobial activity. Overall, the present study gave insight into the great bacterial taxonomic diversity and antimicrobial potential of the isolates from the previously uncharacterized caves located in the world's highest mountains range in the Indian sub-continent.

Introduction

Caves with surface entrances represent one of the unique and poorly studied ecosystems on Earth. They include hydrological systems that are relatively isolated from the surface, and share basic physicochemical conditions, including complete darkness, constant humidity, and thermal stability.[1,2] Caves comprised of unique underground communities of organisms, and cave microclimates often support dense populations of extremophiles.[3] Multidisciplinary studies on cave microbiology have implicated microorganisms in the geological processes of caves, and have opened several avenues of research, including cave geochemistry, cave environmental microbiology and identification of new microbial species and exploration of novel biotechnological molecules from cave sources.[4,5] Recently, several new members of the genera *Catellatospora* and *Nonomuraea* were discovered in caves of Mexico

and Northern Thailand.[6,7] In 2005, *Agromyces subbeticus* sp. nov., was isolated from a cave in the Cordoba area of southern Spain.[8] Jurado et al. reported *Aurantimonas altamirensis* sp. nov., a Gram-negative member of the order *Rhizobiales* from Altamira Cave, located in the Cantabria, Spain.[9] These microorganisms newly discovered in caves demonstrate the potential for identifying secondary metabolites that have yet to be fully evaluated and exploited.[4,7]

Caves are found worldwide, and biospeleological research has been much increased in the last twenty years.[2,4] Available literature indicates the existence of short caves in the world's highest mountains range called Karakoram, Hindu Kush, and Pamir, which incorporate some of the world's highest peaks, including the K2 (8610 m) and Nanga Parbat (8125 m) located in the sub-continent.[10] In the Chitral area of Pakistan, the Hindu Kush mountain is surrounded by a high limestone plateau, and there are various unverified reports of caves in this region.[11] The Nanga Parbat has Rakhiot Cave, which is 73.2 m long and the highest known cave at an altitude of 6644 m.[11] So far, no systematic studies have been conducted to explore the caves system in those mountains. The caves identified by local people have never been surveyed for microorganisms. In this study for the first time, microbial communities were investigated in the two caves, Koat Maqbari Ghaar (KMG) and Smasse-Rawo Ghaar (SRG), located in the Hindu Kush Mountain and situated in the northern Khyber Pakhtunkhwa province of Pakistan. Advances in sequence-based metagenomic approaches have made studies on microbial diversity and community composition in diverse environments more possible and informative. However, using high-throughput sequencing techniques alone to study the microbial community in an environment has the possibilities to miss the low abundant taxa as previously observed.[12] In this study, an integrated approach of culture-based and culture-independent pyrosequencing methods were used to gain a more accurate representation of the microbial communities in the study sites. Bacterial strains isolated in this study were screened for antibacterial and antifungal activities.

Materials and methods

Samples collection and chemical analysis

This study represents a polyphasic analysis of microbial communities from two caves in the Hindu Kush mountain range situated in the North-West region of Pakistan. The local names of those caves are Koat Maqbari Ghaar (KMG, 34°49′13.06″ N, 72°30′41.81″ E) and Smasse-Rawo Ghaar (SG, 34°33′38.1″ N, 71°51′03.4″ E), and they are located in the Swat and Malakand division, respectively. The KMG cave is about 2.5 m wide and 15 m deep, and is located at about 1260 m above sea level. The SRG cave has an almost horizontal orientation and around 2 m wide, 10 m long, and is located at 1062 m above sea level. Both caves are of dried nature and very limitedly influenced by anthropogenic activities. Sediment samples in three replicates were collected from each cave using autoclaved and sterilized bottles. The samples were collected in February 2013. All of the samples were stored at 4 °C for around 20 h during transportation, and were immediately processed for culturing after

arriving in the laboratory. A part of each sample was stored at −80 °C for metagenomic DNA extraction. No specific permission was required for sampling the studied caves. These lands were not privately owned or protected in any way and the caves are not part of a national park or reserve. Our sampling did not involve endangered or protected species.

The pH of each sample was measured using Sartorius pH meters (Denver, Germany) in a 1/10 (w/v) saturated colloid solution of sediment in deionized water. Temperature was measured using ASTM thermometers (Gilson, USA) on each site. Total soil organic matter and total nitrogen were determined using the partial oxidation method and micro Kjeldahl method.[13] Total phosphorus was measured colorimetrically.[13] Physicochemical analysis were performed in triplicate for each sample.

DNA extraction and pyrosequencing

Total DNA was extracted from each homogenized cave sediment replicates using the protocol for the PowerSoil® DNA extraction kit (Mo Bio Laboratories, Carlsbad). Amplification of the 16S rRNA gene hypervariable region V4 was performed using bar-coded 515F and 806R universal primers containing A and B sequencing adaptors, following the procedure previously described.[14] PCR products were quantified using high-sensitivity Qubit technology (Invitrogen, USA), and were purified using Agencourt Ampure beads (Agencourt, USA). The 454 FLX–titanium pyrosequencing platform (Roche, Basel Switzerland) was used to perform high-throughput sequencing following the manufacturer's protocol. Raw pyrosequencing data was processed using the analysis pipeline of MR DNA (Texas, USA).[14] Sequence reads <150 bp were removed, and the remaining reads were screened for homopolymer runs exceeding 6 bp, chimeric sequences, and sequences containing Ns; all of these were also excluded. Barcodes and primers were depleted from sequences. High quality sequence reads were clustered into OTUs using a threshold of 97% sequence similarity. For singleton reads the default value of 2 reads in QIIME v1.9 software was used to exclude from further analysis.[15] OTUs were taxonomically classified using BLASTn against the curated databases GreenGenes, RDP (http://rdp.cme.msu.edu), and NCBI (www.ncbi.nlm.nih.gov).[16] The alpha diversity analysis was performed with Chao1 and the non-parametric Shannon formula using QIIME v1.9 software.[15]

Culture-dependent samples processing

Sediment samples were serially diluted for the isolation of bacterial colonies with improved culture methods, based on an increased number of inoculation plates for each dilution, longer incubation times, selection of micro-colonies, and use of modified culture media as previously described.[17] Briefly, two different concentrations of R2A medium (full strength 18 g/L and half strength 9 g/L) and diluted nutrient broth (1/5 strength 3 g/L and 1/10 strength 1.3 g/L) supplemented with 1.5% agar, 20% aqueous extract of the collected cave sediment, and two incubation conditions, 17 °C and 37 °C, were used to culture bacteria from the caves samples. The plates were incubated in aerobic condition for one week in seal

plastic bags containing wet tissue papers to avoid drying of culture media during this long incubation. Colony forming units (CFU) were counted, and the CFU of the three replicates were used to estimate the size of the bacterial population. Colonies were purified by sub-culturing and then stored using a 15% glycerol stock in cryogenic vials at −80 °C. Eighty-four isolates were selected for 16S rRNA gene sequencing based on the colony morphology and growth characteristics. Genomic DNA was extracted from the isolated strains using 1% Triton X-100 (Sigma) and boiling at 95 °C for 20 min. The 16S rRNA gene was amplified using the universal bacterial primers, 27F and 1492R, as described previously.[17] Purified PCR products were sequenced with ABI Prism Sequencer 3730 (Applied Biosystems, USA) according to manufacturer's protocol. An analysis of chimeras in the 16S rRNA gene sequences was performed using Bellerophon software.[18] Sequences were blasted at the EzTaxon server (http://www.ezbiocloud.net/) to identify the related type strains.[19] A multiple sequence alignment was performed, and a phylogenetic tree was constructed by the neighbor-joining distance method using Jukes-Cantor model to compute evolutionary distance in MEGA6 software.[20] Bootstrap values were calculated based on 1000 replications. The 16S rDNA sequences were deposited in GenBank (NCBI) under accession numbers KF747002–KF747085.

Antimicrobial activity

Antimicrobial activities of the isolated strains on growth of the pathogenic bacteria *Salmonella typhi* and *Staphylococcus aureus*, and the yeast *Candida albicans* were determined by confrontation bioassay using paper discs. A concentration of 0.5 McFarland of the target strains were spread on media-agar plates. Twenty microliters of each bacterial suspension containing approximately 10^8 cfu/mL was added to a sterile paper disk (Advantec, Japan), and then discs were placed on the target strain plates. The plates were incubated for 72 h at 25 °C, and zones of inhibition around the paper discs were measured.[21] Chloramphenicol (30 μg/disk) and amphotericin-B (20 μg/disk) were used as reference to estimate the antimicrobial activities of the tested isolates against bacteria and yeast respectively.

Statistical analysis

One-way ANOVA and Tukey's HSD (Honestly Significant Difference) tests were used to statistically compare physicochemical parameters, and CFU obtained at different media compositions and incubation temperature. The Statistical Package for the Social Sciences (SPSS) version 20 was used for the statistical analysis.

Results

Physicochemical analysis

The pH values of KMG (7.6 ± 0.21) and SRG (8.0 ± 0.25) were not significantly ($p = 0.29$) different from one another, and the sediment of SRG was slightly basic. Temperature of KMG was 14 °C and SRG 17 °C. Chemical analysis indicated that both caves were nutritionally poor. Total organic matter and nitrogen content in KMG (1.7 ± 0.08% and 0.09 ± 0.002%) and SRG (1.05 ± 0.02% and 0.05 ± 0.004%) were significantly ($p = 0.01$ and $p = 0.001$) different between the two caves. Phosphorus level in the KGM and SRG were 191.4 ± 0.4 ppm and 82.6 ± 0.6 ppm, respectively.

Microbial taxonomic diversity

Taxonomic assignment of about 44,015 high-quality pyrosequencing reads from KMG (23,838) and SRG (20,177) were obtained using the V4 region of the 16S rRNA gene. The taxonomic composition of the bacterial communities was highly diverse in both caves sediments. The majority of the identified operational taxonomic units (OTUs), defined by 97% sequence similarity, were affiliated with 21 phyla that included 19 bacterial phyla, 2 archaea, and 20 candidate phyla. Among the identified phyla, 10 phyla and 5 candidates divisions were detected in both caves. The percentage sequence reads from KMG and SRG were affiliated with 6 bacterial phyla detected at relatively higher abundance including; *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, *Firmicutes*, *Verrucomicrobia* and *Planctomycetes* (Fig. 1). Among the *Proteobacteria*, α-*Proteobacteria* (45.6% KMG and 34.1% SRG) and γ-*Proteobacteria* (35.2% KMG and 32.1% SRG) were dominant in both caves, followed by δ-*Proteobacteria* (12.6% KMG and 16.9% SRG) and β-*Proteobacteria* (9.2% KMG and 16.7% SRG). The phylum *Deinococcus-Thermus* was detected only in KMG (6.8%), and WS3 (candidate division, 4.3%) was detected in SRG. The 15 candidate divisions and 10 bacterial phyla, including 2.9% *Acidobacteria*, 2.8% KSB1, and 1.2% OP3 (candidate division), were detected specifically in SRG. The archaeal phylum *Euryarchaeota* was detected at 3.4% and 0.2% abundance in KMG and SRG, respectively. *Cyanobacteria* were detected at <1% abundance in both caves.

A total of 498 different genera were detected. The highest number of genera (445) was detected in SRG, while 235 genera were present in KMG. Among these, 185 genera were present in both caves, while 53 genera were specifically detected in KMG and 263 in SRG. Moreover, 17 genera in KMG and 21 genera in SRG were present at an abundance ≥1%, accounting for 74% and 58% of the total sequence reads in the respective samples (Fig. 2). In KMG, the following bacterial genera were detected at relatively high abundances: *Cellvibrio* (17.1%), *Saccharophagus* (15.5%), *Bacillus* (7.6%), and *Deinococcus* (6.6%). In SRG, the dominant genera were *Chthoniobacter* (6.9%), *Opitutus* (6.6%), *Chondromyces* (3.4%), *Rubrobacter* (3.4%), *Rhodoplanes* (3.1%), and *Prosthecobacter* (3%). Only the genus *Cytophaga* was present in both caves at ≥1% abundance.

In the next step, we analyzed the OTUs at species level in the KMG and SRG caves. A total of 873 different species were identified, including 384 species in KMG and 628 species in SRG. Only 139 species were present in both caves. In each cave, more than 60% species were affiliated with four bacterial phyla: *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, and *Actinobacteria*. In total, 170 (44.3%) identified species from KMG and 309 (35.4%) from SRG were affiliated with *Proteobacteria*. Among them, 89 species were commonly present in both caves including *Saccharophagus degradans* and *Lysobacter spongicola* that were present at relatively higher abundance in KMG, and were
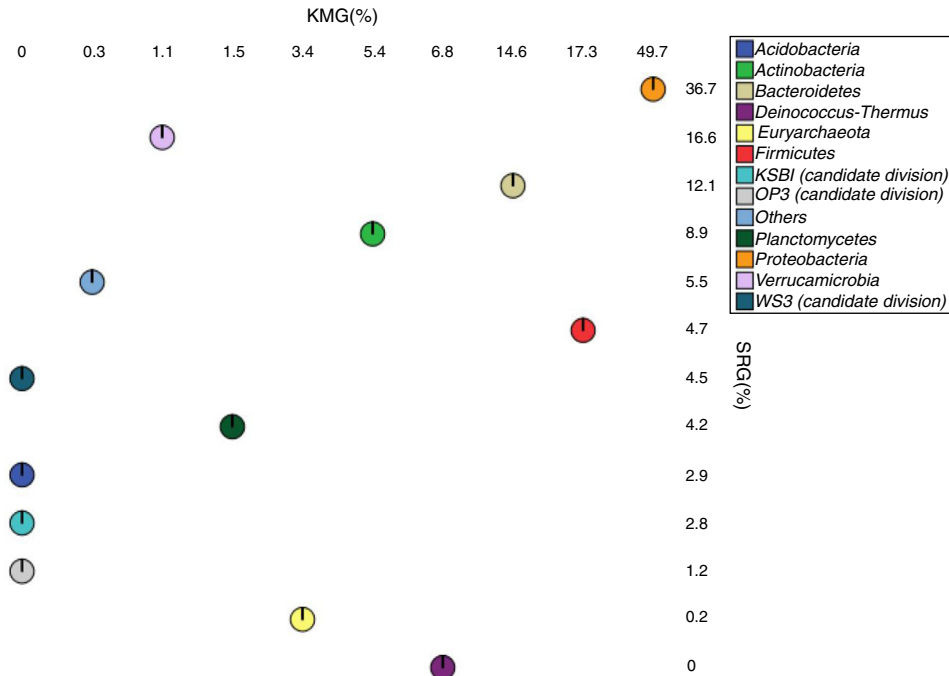
Fig. 1 – Distribution of microbial phyla and candidate divisions detected at relatively higher abundance in the KMG and SRG caves. Percentage abundance from KMG are shown on the x-axis and from SRG on the y-axis. The category "others" represents microbial phyla and candidate divisions that were present at <1% abundance in each cave. KMG, Koat Maqbari Ghaar; SRG, Smasse-Rawo Ghaar.
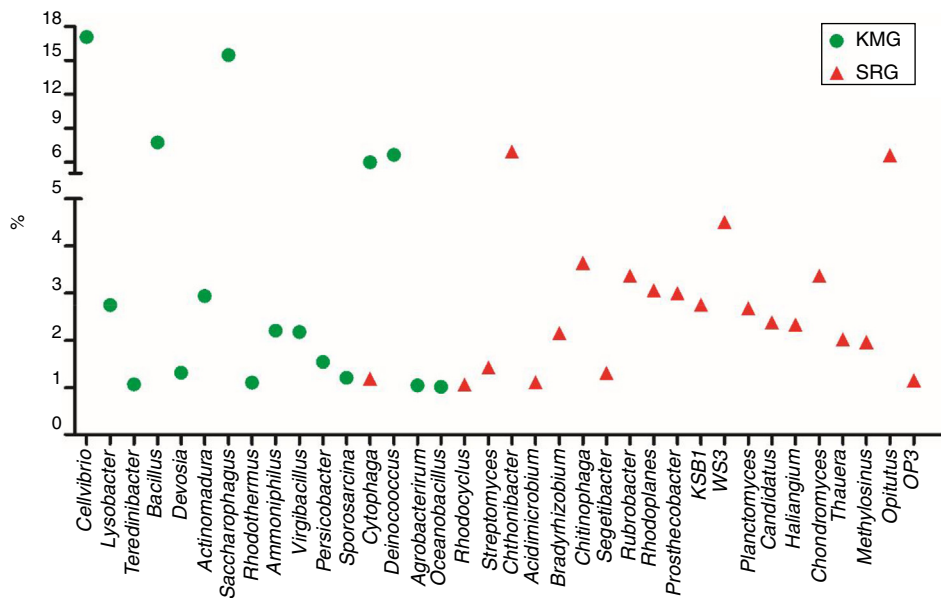

Fig. 2 – Percentage distribution of the relatively dominant genera detected in the KMG and SRG caves. KMG, Koat Maqbari Ghaar; SRG, Smasse-Rawo Ghaar.

detected at <0.01% abundance in SRG (Fig. 3). The *Haliangium* spp. and *Bradyrhizobium lupini* were present at higher abundances in SRG than in KMG. The species *Cellvibrio ostraviensis*, *Cellvibrio japonicus*, and *Teredinibacter turnerae* were dominant and specific to KMG. While, *Rhodoplanes* spp., *Methylosinus trichosporium*, *Chondromyces crocatus*, *Rhodocyclus tenuis*, and *Chondromyces apiculatus* were specifically detected in SRG at ≥1% abundance. From the phylum *Firmicutes*, 250 different

species were identified including 27 species that were commonly present in both caves. While, 79 *Firmicutes* associated species were specifically detected in KMG and 71 species OTUs were specific to SRG. Only the species; *Ammoniphilus oxalaticus*, *Bacillus asahii*, *Sporosarcina ginsengisoli*, and *Virgibacillus halodenitrificans* were detected at >1% abundance in KMG. Other *Firmicutes* species were detected at <1% abundance in KMG and SRG. From the phylum *Actinobacteria*, *Rubrobacter*
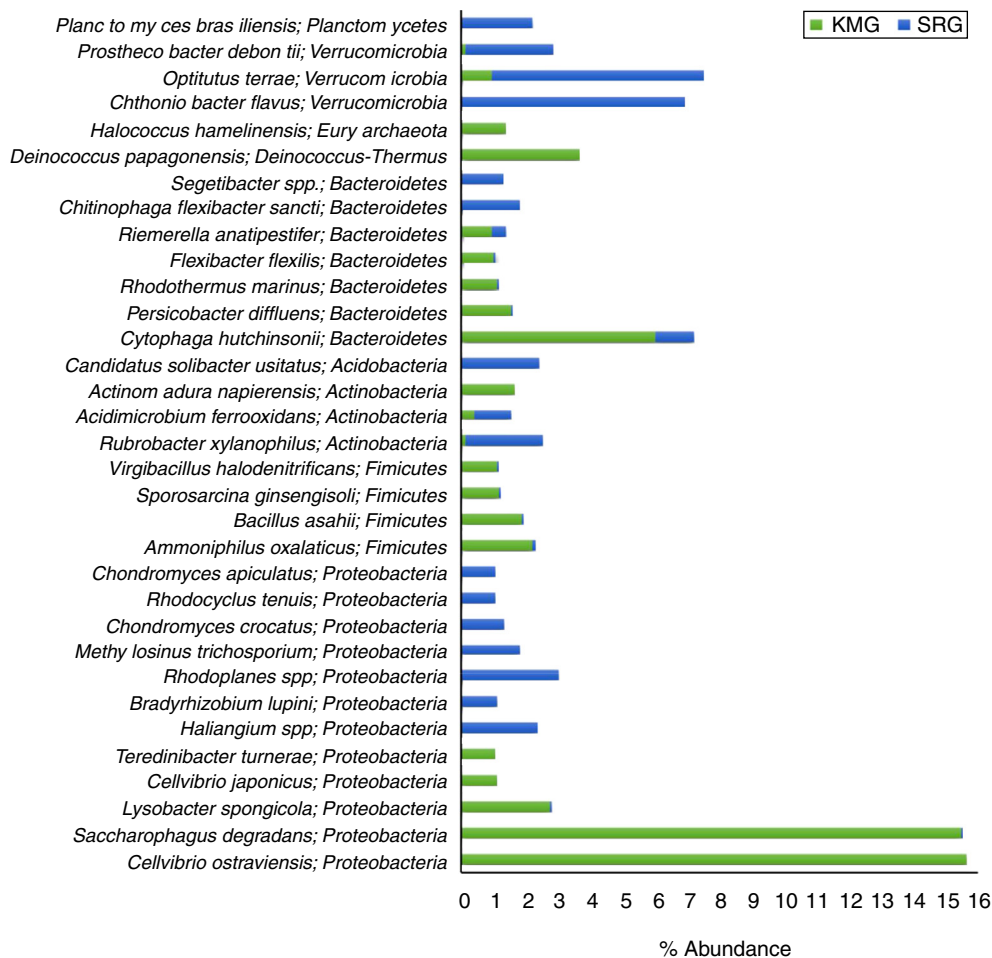
Fig. 3 – Percentage distribution of relatively dominant OTUs at species level detected in ≥1% at least in one cave. KMG, Koat Maqbari Ghaar; SRG, Smasse-Rawo Ghaar.

xylanophilus and *Acidimicrobium ferrooxidans* were detected in both cave but present at relative higher abundance in SRG. *Actinomadura napierensis* and *Candidatus solibacter* were the two dominant spices detected at >1% abundance specifically in KMG and SRG, respectively. From the phylum *Bacteroidetes*, *Cytophaga hutchinsonii* was detected at a relatively high abundance in both caves. The species *Chitinophaga flexibacter* and *Segetibacter* spp. were more abundant in the SRG, and *Persicobacter diffluens* and *Rhodothermus marinus* were detected at >1% abundance in KMG. Apart from the OTUs at species level from dominant phyla, the *C. solibacter usitatus*, *Opitutus terrae* and *Planctomyces brasiliensis* were detected at relatively higher abundance in SRG sample. The Gram-negative bacterium *Deinococcus papagonensis* and *Halococcus hamelinensis* from archaea phylum *Euryarchaeota* were specifically detected in KMG (Fig. 3).

Chao 1 and Shannon index

Chao1 and Shannon indices were calculated to estimate alpha diversity. Rarefaction curves were established at a 97% sequence similarity level to define OTUs. In rarefaction curves, both samples were tended to approach saturation plateau, and the samples SRG was plotted in the upper part of the graph (Fig. 4A). The Chao1 analysis demonstrated a decreased trend of richness in the sample KMG (3622) compared to SRG (4701, Fig. 4B). The Shannon diversity index for KMG was 9.0 and for SRG was 10.0, indicating high diversity in both caves (Fig. 4C). However, species diversity in SRG was higher than that of KMG.

Cultured bacterial diversity and antimicrobial activity of isolates

Incubation at 37 °C and in full-strength R2A medium yielded higher numbers of bacteria from both caves KMG (8.5) and SRG (8.4) (counts expressed as log^{10} cfu/g) that were significantly ($p < 0.05$) higher than the CFU obtained from all other tested culture media and temperature used in this study. From incubation at 17 °C, significantly higher numbers of bacteria were noticed on half-strength R2A from KMG (7.5), and on full-strength R2A from SRG (7.7) compared to other tested media with respective samples. Overall, the recovery of viable bacteria colonies was higher at 37 °C than at 17 °C. Initially, 350 strains were purified on the basis of colony morphologies and were screened for antimicrobial activity. The 16S rRNA genes of 84 isolates from the two caves were sequenced,
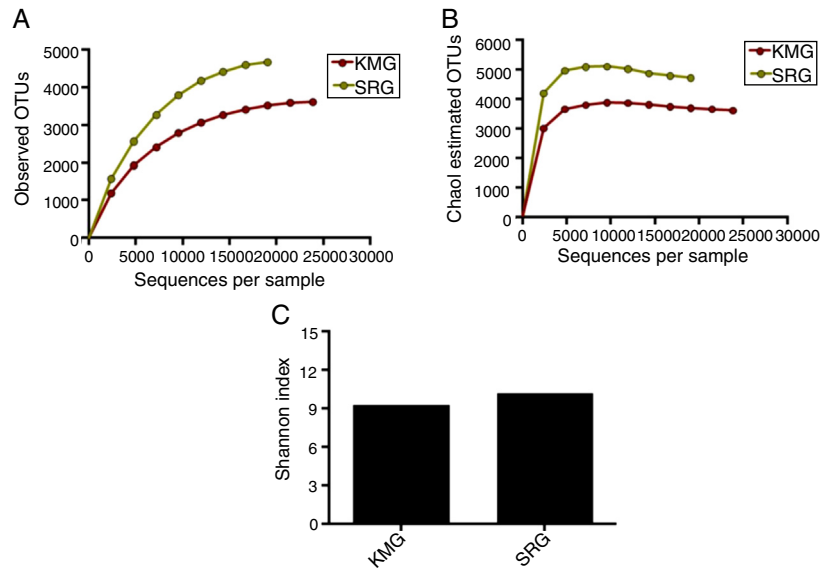
Fig. 4 – Alpha diversity of the sequence reads. (A) Rarefaction curves of the experimentally observed OTUs versus diversity (B) estimated by Chao1. (C) Boxplots of the Shannon index. KMG, Koat Maqbari Ghaar; SRG, Smasse-Rawo Ghaar.

and they displayed 96.2–100% identity to the sequences available in GenBank, and were assigned to major bacterial groups using phylogenetic analysis (Fig. 5). Three strains showed <97% sequence similarity with the closest related type strain, suggesting as candidate novel species. *Proteobacteria* was the dominant phylum among KMG isolates (52.6%), and representing 25.9% of isolates from SRG. The γ-*Proteobacteria* sequences represented majority of isolates from KMG (43.5%) and SRG (11.1%). α-*Proteobacteria* and β-*Proteobacteria* sequences were evident in 8.8% of KMG isolates, and 14.8% of isolates from SRG were affiliated with α-*Proteobacteria*. *Firmicutes* and *Actinobacteria* accounted for 40.4% and 7%, respectively, of isolates from KMG. *Firmicutes* was the dominant phylum in SRG (71.4%), and no *Actinobacteria* isolates were detected from SRG. Cultured isolates from both caves were classified into 14 distinct genera. Majority of the isolates were associated with the genera *Pseudomonas* (40.3% KMG and 11.1% SRG) and *Bacillus* (22.8% KMG and 44.4% SRG). In total 39 distinct species were identified among the cultured isolates from both caves. Four species were common between the two caves. Twenty-six species were unique to KMG, and 9 species were specifically detected in SRG. The following four species, *Exiguobacterium undae*, *Pseudomonas koreensis*, *Sphingomonas melonis*, and *Pseudomonas mosselii* were isolated from the sediment of both caves. In KMG, the dominant cultured species was *P. koreensis*, followed by *Bacillus humi*, *Pseudomonas peli*, *Pseudomonas baetica*, and *Pseudomonas cremoricolorata*. In SRG, the dominant cultured species were *Paenibacillus lautus*, *Bacillus timonensis*, *Bacillus simplex*, and *Caulobacter vibrioides*.

Thirty strains showed antimicrobial activity among the total isolates from both caves (Table 1). Fifteen strains showed antibacterial activity against *S. typhi*, and 20 strains were active against *S. aureus*. Six strains showed antibacterial activity against both bacterial pathogens tested. Isolated strains belonging to the genera *Pseudomonas* and *Bacillus* were more antagonistic, as demonstrated strong antimicrobial activity against tested pathogenic bacteria. Only two strains, which showed around 100% sequence similarity with *Brevibacillus borstelensis* and *P. mosselii*, inhibited the growth of the human fungal pathogen *C. albicans*.

Discussion

Organic material in the studied caves was close to starvation level; this is common in caves with limited external influence.[22] Microorganisms residing in such cave systems are mostly oligotrophic or chemolithotrophic in nature, and require specific nutrients for their growth.[5] Therefore, many bacteria are difficult to cultivate from these sources. Despite the starved condition, phylogenetically diverse bacterial taxa colonized both caves, and mainly comprised of *Proteobacteria* followed by *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Verrucomicrobia*, and *Planctomycetes* as observed in previous caves studies.[23,24] We found an important difference in the relative abundance of different *Proteobacteria* subdivisions. The α-*Proteobacteria* and γ-*Proteobacteria* were dominant in the studied caves, followed by δ-*Proteobacteria* and β-*Proteobacteria*. In the microbiologically well-studied Spanish Altamira cave, cosmopolitan *Proteobacteria* groups were dominant in dripping waters and cave walls.[5,25] Similarly, *Proteobacteria* represented half of an entire wall's bacterial community in the Tito Bustillo cave in Spain, and probably due to their versatile metabolic capabilities survive on available ions in the rock contents and used them for chemolithotrophic energy production.[26] The Gram-negative *Proteobacteria*, *S. degradans* that degrades a number of complex polysaccharides as energy source, was commonly identified in the studied KMG and SRG caves.[27] While, *C. crocatus* from myxobacteria that predominantly lives in the soil and feed on insoluble organic substances was specifically present at higher abundance in the SRG.[28]
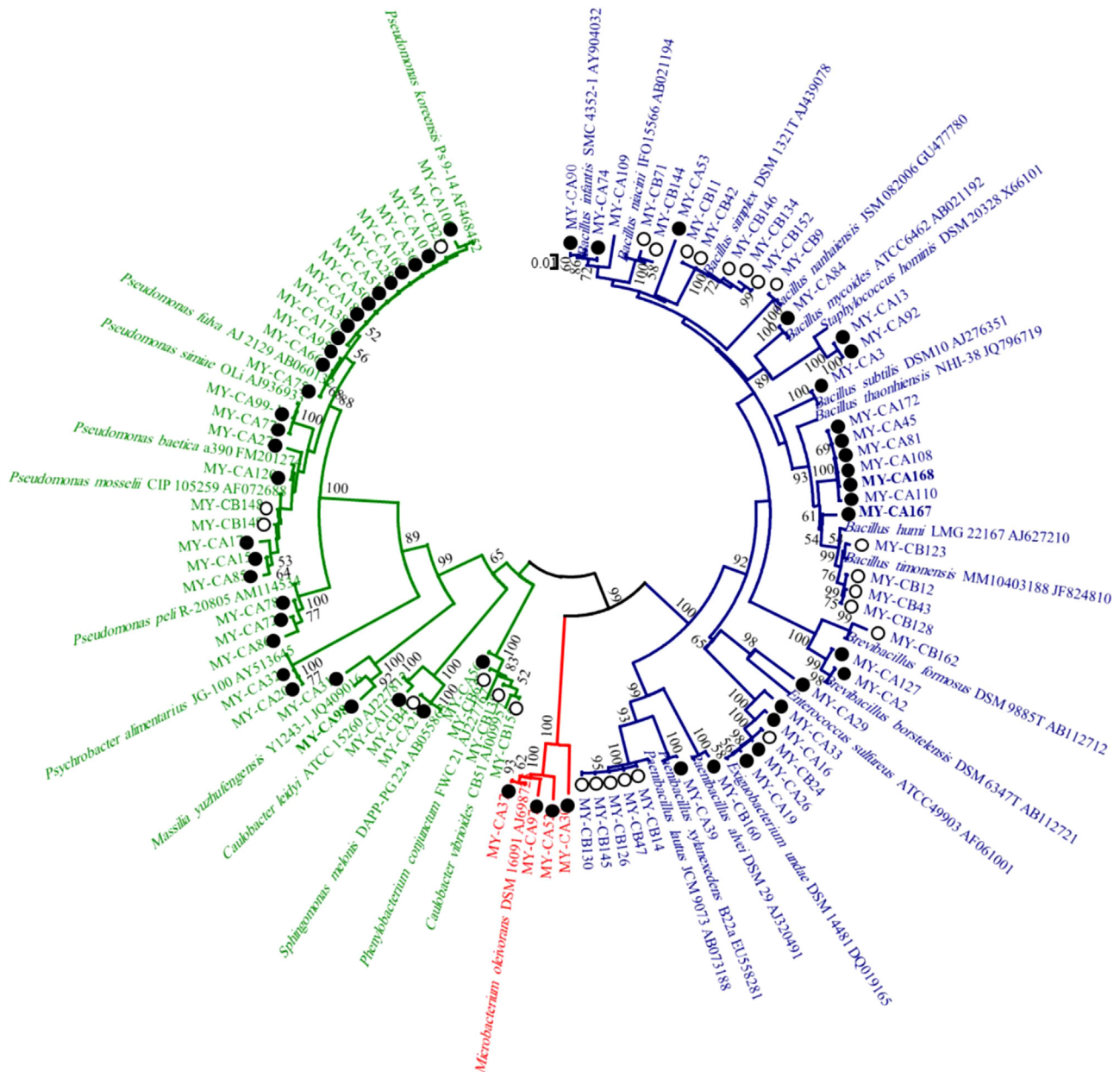
Fig. 5 – Phylogenetic analysis based on 16S rRNA gene sequences of the bacterial isolates. The neighbor-joining clustering method was used to construct the tree. Bootstrap values were calculated based on 1000 replications and are shown at branch points. The bar represents 0.01 sequence divergence. Sequences derived from the NCBI GenBank database are shown with their accession numbers. Filled circles following the experimental sequence names correspond to KMG, and non-filled circles correspond to SRG. KMG, Koat Maqbari Ghaar; SRG, Smasse-Rawo Ghaar.

Over the past decade, an abundance of *Actinobacteria* has been found in caves and other subterranean environments suggesting that caves are favored habitats for this group of bacteria, and actively involved in the formation of crystals in cave walls and biomineralization process.[29,30] In our study, several *Actinobacteria* were commonly identified in both caves including the *R. xylanophilus* and *A. ferrooxidans*. Member from *Rubrobacter* is commonly found in biodeteriorated monuments, induce crystal formation in caves and form biofilm on the limestone.[31] The phylotypes related to *A. ferrooxidans* identified in this study were previously detected in cave walls

of the Buda thermal karst system.[32] Similarly, *Firmicutes* are frequently identified in more extreme ecosystems, and are comparatively more resistant to nutrient stress.[2,33] In particular, the bacillus group retrieved from the caves in this study has the ability to form endospores.[33] Lee et al. constructed a phylogenetic tree of 16S rRNA gene sequences retrieved from 60 caves around the world.[23] The tree showed the relative abundances of different classes of bacteria in caves, with the most abundant groups being *Proteobacteria*, *Chlorobi/Bacteroidetes*, *Actinobacteria*, and *Chloroflexi*.[24] However, proportions of these groups depended on the particular features of each cave.

Table 1 – In vitro antimicrobial activity of the cultured isolates against pathogenic bacteria and yeast.

Species	Isolates	S. typhi	S. aureus	C. albicans
Fictibacillus nanhaiensis	MY-CB9	+	+	−
Microbacterium oleivorans	MY-CA97			
Paenibacillus lautus	MY-CB14			
Pseudomonas graminis	MY-CA27			
Pseudomonas koreensis	MY-CA28, MY-CA50			
Bacillus humi	MY-CA172	+	−	−
Bacillus muralis	MY-CB146			
Bacillus simplex	MY-CB11, MY-CB152			
Bacillus tequilensis	MY-CA3			
Bacillus timonensis	MY-CB12			
Brevibacillus borstelensis	MY-CA2			
Paenibacillus lautus	MY-CB145			
Bacillus eiseniae	MY-CA109	−	+	−
Bacillus humi	MY-CA167			
Bacillus mycoides	MY-CA84			
Bacillus niacini	MY-CB144			
Bacillus thaonhiensis	MY-CA168			
Bacillus timonensis	MY-CB128			
Carnobacterium inhibens	MY-CA29			
Caulobacter vibrioides	MY-CB65			
Pseudomonas baetica	MY-CA85			
Pseudomonas cremoricolorata	MY-CA66			
Pseudomonas koreensis	MY-CA169			
Pseudomonas mosselii	MY-CA17			
Pseudomonas simiae	MY-CA99			
Staphylococcus warneri	MY-CA92			
Pseudomonas mosselii	MY-CB149	+	−	+
Brevibacillus borstelensis	MY-CA127	−	−	+

In the isolate names, CA represents Koat Maqbari Ghaar (KMG) and CB represents Smasse-Rawo Ghaar (SRG). Symbols: −, no activity; +, activity.

Several functionally important strains were identified from genera that were reported in others cave studies, including *Methylobacterium*, *Rhizobium* and isolates from the genera *Kocuria*, *Acinetobacter*, *Renibacterium* and *Bacillus*.[4,26,33,34] These bacteria can utilize a wide variety of carbon substrates, and play species-specific roles in nitrogen fixation and calcification.[5,25] The genus *Thiothrix*, detected by pyrosequencing were previously reported to make up thick mats of filamentous sulfur-oxidizing epsilon.[23,35] Rapidly digesting crystalline cellulose bacterium *C. hutchinsonii* and ionizing-radiation resistant bacteria such as *D. papagonensis* and *R. xylanophilus* were detected in both caves.[36,37] Several oligotrophic and facultative oligortophic bacteria such as *Nitrospira moscoviensis*, *Prochlorococcus* spp., *Sphingopyxis alaskensis*, *Sphingomonas oligophenolica*, *Bacillus cereus*, *Paenibacillus polymyxa*, *Streptomyces* sp., *Brevibacillus laterosporus*, *Bacillus subtilis*, *Arthrobacter* spp. were found in cultured independent pyrosequencing analysis of this study, and were previously reported from different oligotrophic environments.[38] In addition, mesophilic *Crenarchaeota* and particularly *Euryarchaeota* were detected at relatively higher abundance in the KMG that were previously found in the Lechuguilla Cave (United States)[33] and in the Altamira Cave (Spain).[39] Legatzki et al. reported the presence of an archaeal community on calcite speleothems from Kartchner Caverns, Arizona, USA.[40]

One of the limitation of this study was that only aerobic condition along with two media compositions and temperature conditions were used for culture-dependent analysis.

Overall, lower diversity was observed among cultured isolates that cannot be compared to the diversity observed with the pyrosequencing analysis. The cultured isolates from both caves were classified into the phyla *Proteobacteria*, *Actinobacteria*, and *Firmicutes*. Among the 13 genera identified by culture, a few of them were detected in the pyrosequencing analysis, including *Bacillus*, *Microbacterium*, *Pseudomonas*, and *Psychrobacter*. However, bacteria from the genera *Carnobacterium*, *Exiguobacterium*, *Paucisalibacillus*, and *Fictibacillus* were not detected in pyrosequencing data. Interestingly, among the 39 distinct cultured bacterial species, only seven species, *B. humi*, *Bacillus niacin*, *B. simplex*, *C. vibrioides*, *P. lautus*, *Psychrobacter alimentarius*, and *S. melonis* were found in the pyrosequencing data indicate that the bacteria in low abundance in a community can be only captured by cultured analysis as previously observed.[12] The phylogenetic analysis and 16S rRNA gene sequence similarity indicated that three of the cultured isolates have no close relative among the cultured type strains. These strains represent novel candidates in caves biodiversity, indicating that culture techniques provide us with the opportunity to discover new microorganisms from caves ecosystem.[30] The identification of new pathogenic bacteria and the rapid development of antimicrobial resistance highlight the importance of discovery of new antimicrobial agents.[41] Several strains isolated from the caves in this study, including *Actinobacteria*, *Myxobacteria*, *Pseudomonas*, and *Bacillus* spp. showed antimicrobial activity against two important pathogenic bacteria, *S. typhi* and *S. aureus*. In recent years, several studies highlighted that rarely studied cave microbiota

could be a potential source of new microorganism and a source for future antimicrobial agents.[42]

In conclusion, diverse microbial communities were observed within the studied caves that were corresponding to other caves at higher taxonomic level. However, at lower taxonomic level several distinct taxa were identified. In addition, nitrogen fixation, crystalline cellulose digesting and ionizing-radiation resistant related bacteria were detected in the culture-independent analysis. Cultivated isolates from the genera *Bacillus* and *Pseudomonas* were dominant in both caves and exhibited antimicrobial activity against pathogenic bacteria and yeast. There is a need for further studies to explore virgin caves systems in the world's highest mountains range in northern Pakistan, and to exploit the microbial communities of those caves for new biomolecules.

Conflicts of interest

The author declares no conflicts of interest.

Acknowledgments

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