

ANTHROPOGENIC IMPACTS ON THE BACTERIAL PROFILE OF YARIK SINKHOLE IN ANTALYA, TURKEY

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Abstract

Yarik Sinkhole is a vertical cave with a length of 1378 m and depth of 533 m. Its location was marked by The Anatolian Speleology Association in 2011, and expedition entries started in 2014. Yarik Sinkhole became the 12th deepest cave of Turkey. The aim of this study was to determine the bacterial profile of the Yarik Sinkhole using next generation metagenomic sequencing and to investigate whether the bacterial profile of the cave is affected by the activities of people living in this region. This study is important as the samples were collected during the first entrance of the exploration of the cave (up to 300 m). The samples were collected from depths of -80 m, -120 m and -300 m. A total of 33 OTUs (Operational Taxonomic Unit) contained 4 bacteria phyla. Only *Firmicutes*, *Proteobacteria* and *Actinobacteria* phyla comprised a mean abundance of more than 1% in each sample. However, 18 different species have been detected in the Yarik Sinkhole. The most predominant species are *Acinetobacter Iwoffii*, *Methylobacterium tardum*, and *Propionibacterium acnes*. Although the sampling was done during the first exploration of the Yarik Sinkhole, the fact that the majority of bacteria found in the cave are human-associated, suggest serious impacts from people living near this cave from runoff with human and animal waste and trash.

Introduction

Caves are a subsurface habitat that are not explored as much with regard to biodiversity and community composition because of some environmental and geographical limitations. Natural caves generally involve an ecosystem that has a high humidity, limited nutritional sources, and a usually stable temperature, which are stable characteristics of a natural laboratory in terms of bacteria and their metabolic processes. Such environments are habitats only to those microorganisms that are specialized for the conditions in question. Therefore, natural caves are recognized as extreme environments (Palmer, 1991; Northup and Lavoie, 2001; Tomczyk-Żak and Zielenkiewicz, 2016). Microbial communities in caves are often highly variable dependent on the microenvironment. The range of bacterial diversity and composition are determined to be related to the geochemistry of host rocks (Barton et al., 2007). Nutrients also contribute to cave microbial diversity. Organic matter and microorganisms could be carried in to caves by air currents, seepage water, floods, seasonality, and animal/human activities (Shabarova et al., 2013).

The most appropriate way to unearth the bacterial diversity of a cave is to perform sampling at the time of exploration of the cave. Thus, the original microbiological diversity of the cave can be more realistically discovered by sampling before contamination associated with the cavers. However, the conditions may vary depending on the location and surroundings of the caves in terms of human impacts. Numerous cave microbiology studies have been carried out in the world. Since each cave is unique, these studies do not lose their importance (Busquets et al., 2014; De Mandal et al., 2014; Herzog Velikonja et al., 2014; Kieraite-Aleksandrova et al., 2015; Riquelme et al., 2015; Leuko et al., 2017).

It is estimated on the basis of the studies conducted in karstic areas that there are approximately 40,000 caves in Turkey (General Directorate of Mineral Research and Exploration). Cave microbiology studies from Turkish cave include: Yücel and Yamaç (2007), investigated the antimicrobial activity of *Streptomyces* spp. isolates from 19 different caves. In addition, characterization and definition of bacteria contributing to the formation of dripstone in Yıldızkaya cave systems in Erzurum have been studied by Barış (2009). Gulecal-Pektas and Temel (2017) studied the bacterial diversity and taxonomic composition of the Oylat Cave in Bursa and the Kaklık Cave in Denizli with poor oxygen, high temperature, and sulfur conditions. Even though the number of studies on microbiology in the caves of Turkey has been increasing, these studies are insufficient when the estimated number of caves is taken into account.

The culture technique is not sufficient on its own for determining the microbiological diversity of a cave. The rate of culturable bacteria in environments that contain complex microorganisms is only 0.1–1% of the total number due to their specific nutritional requirements (Torsvik and Øvreas, 2002). With the application of molecular methods, it was revealed that nutrient-poor caves had a surprisingly rich bacterial diversity. Metagenomics is a technique to access far more microbial diversity directly from environmental samples. Next-generation sequencing is cost-effective and provi-

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des more detailed taxonomic profiles between samples to be determined (Nelson et al., 2014). It has been shown that some microorganisms isolated by using culture methods were not detected by the molecular methods, and vice versa, which it has demonstrated that cultivation methods remain critical in microbial diversity studies (Donachie et al. 2007).

The Yarık Sinkhole is one of the caves which differ by its location in environmental conditions. During the summer months, the presence of people who are living there, as well as those who practice animal husbandry (approximately 30–50 people and 400 animals) are noticed. There are also tiny siphons where waters join the cave at both the main entrance of the Yarık Sinkhole and at different points deeper into the cave. In this study, we collected soil samples from three different points of the first-explored part of the cave, to both examine the bacteria profile, and, by means of the next-generation sequencing method, showed the impact of the anthropogenic effects on the cave's bacterial diversity.

Site Description

Geology

Yarık Sinkhole (GPS coordinate: UTM 448068.47 E 4036006.77 N) is located on the Sivastı Plateau that is centered 30 km north of Gazipaşa (Fig. 1) and is named with a specially-assigned geological sequence as the Sivastı formation. This formation, which is 2000-meters high, is one of the parts of the Taşeli Plateau. The study area, thought to be from the earliest Triassic age, has a complex structure with various orogenic movements such as Hershey and Alpen that undergo bending and fracturing. There are stratified schists and limestones at the Sivastı formation. Due to the different physical characteristics of the lithology, the schists are more curled and the limestones are more broken (Ulu, 1983).

A cross-sectional view of the cave map placed on the topography is presented in Figure 2. Yarık Sinkhole has a total length of 1378 m and 533 m depth. The first entrance to the cave was explored up to a depth of 300 m (Fig. 3). In 2016, the cave discovery reached a depth of 533 m. Yarık Sinkhole became the 12th deepest cave of Turkey. The entrance to the cave has a wide mouth created by fracture hence its name; Yarık in Turkish means fracture (Fig.4). The watershed of the Yarık Sinkhole is a closed valley where the main rock is limestone with little sediment on the basin of the valley. There is no vegetation except for some trees planted by villagers for shade.

Despite a wide opening, there are occasional narrow passages in the cave and a rapid downward descent is characteristic of this cave. When the cross section map of the cave is viewed after the bench, downward declination

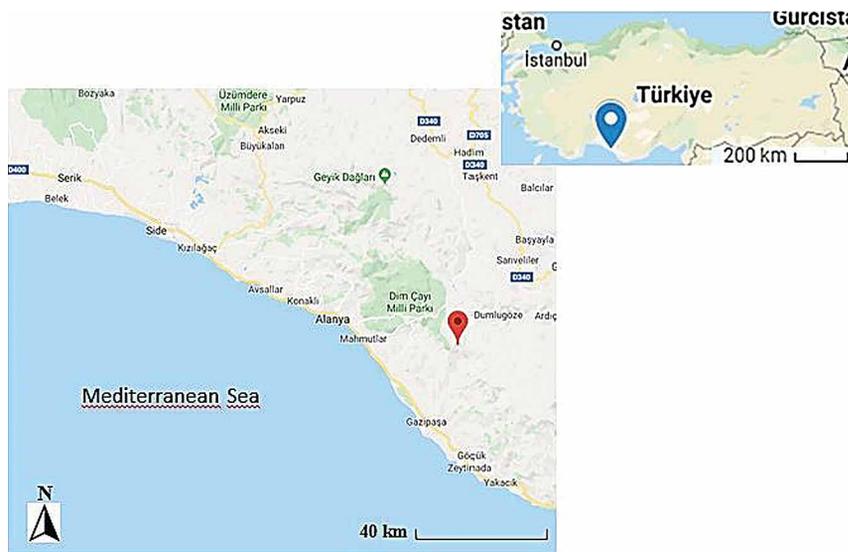


Figure 1. Yarık sinkhole location source [Google (Googlemaps, 2019)].

slowly gives away to horizontal passages with small ponds and lakes in them. The resulting bench size ranges from 5 m to 40 m on average. These benches generally formed as a result of active faults and fractures developed over time. The Yarık Sinkhole ceiling is high generally, but some of the narrow passages are difficult to pass, especially in the case of a flood when these passages will be totally blocked. Unlike most vertical caves, in the Yarık Sinkhole speleothems such as flowstones and cave pearls are found in the horizontal portion.

However, there are not any attempts to enter the entrance of the Yarık Sinkhole by the villagers as it will be fatal since the entrance is an 80-meter shaft. Water only flows during the melting of ice in the spring. The cave camp area is 50-meters away from the entrance in a small pasture surrounded with seasonal settlements. The area is filled up with ice and snow in winter and in spring time meltwater is siphoned through a small pit with sediment at the bottom. The waste of livestock also goes along with the water.

The impact of population can be explained by the settlers in that valley. Additional side galleries within Yarık Sinkhole carry water from other watershed areas where there are additional people. Even though the impact cannot be measured, it is evident by the garbage that we have found deep in the cave which can not come only from the main entrance.

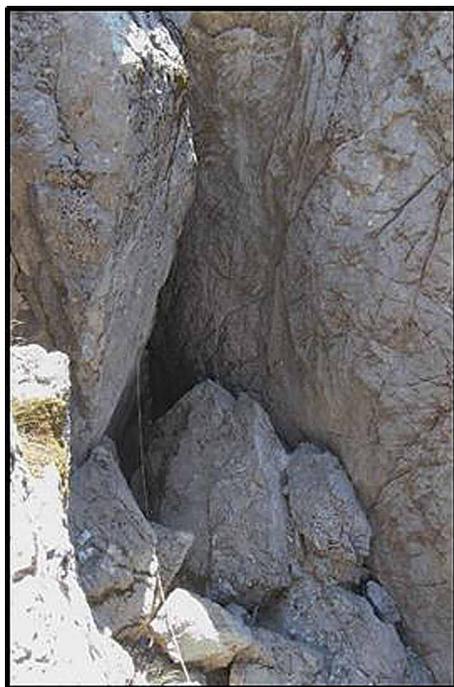


Figure 4. Yarik Sinkhole Entrance (about 10-meters long and 5-meters wide).

DNA extraction

DNA was extracted from 0.3 g (wet weight) of soil by using the Fast DNA Spin Kit for soil from Q-Biogene (Heidelberg, Germany) according to the manufacturer's instructions.

16S rRNA Metagenomic Sequencing Library Preparation and Sequencing

The microbial diversity at three depths was evaluated by using the Illumina MiSeq next generation sequencing approach (Novogene). The protocol includes the primer pair sequences for the V3 and V4 region of the 16S rRNA that create a single amplicon of approximately 460 bp (Klindworth et al., 2013). The protocol also includes overhang adapter sequences that must be appended to the primer pair sequences for compatibility with Illumina index and sequencing adapters. Illumina adapter overhang nucleotide sequences-16S rRNA specific sequences were 5'TCGTCGGCAGCGTCAGATGTG-TATAAGAGACAG-CCTACGGGNGGCWGCAG-3' for the forward primer and 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-GACTACHVGGG-TATCTAATCC-3' for the reverse primer. The first PCR was performed using Biospeedy™ Proof Reading DNA Polymerase 2x Reaction Mix (Bioeksen Ltd Co., Turkey) and 200 nm of each primer. The following program was performed on Biorad CFX Connect Instrument (Bio-Rad Laboratories, U.S.A.): 95°C for 3 minutes; 25 cycles of 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds; 72°C for 5 minutes. The PCR product was run on an agarose gel to verify the size (~550 bp) and purified using Biospeedy™ PCR Product Purification Kit (Bioeksen Ltd. Co., Turkey).

The dual indices and Illumina sequencing adapters were attached to the purified first PCR products via the second PCR that was run using the Nextera XT Index Kit (Illumina Inc., USA) and the following program: 95°C for 3 minutes; 8 cycles of 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds; 72°C for 5 minutes. The PCR products were purified using Biospeedy™ PCR Product Purification Kit (Bioeksen Ltd. Co., Turkey). The final library was run on a Bioanalyzer DNA 1000 chip to verify the size (~630 bp). The final library was diluted using 10 mM Tris pH 8.5 to 4 nM and the 5 µL aliquots were mixed for pooling the libraries. In preparation for cluster generation and sequencing, pooled libraries were denatured with NaOH, diluted with hybridization buffer (HT1), and then heat denatured before MiSeq sequencing. Illumina MiSeq v3 reagent kits were used for the runs. Each run included a minimum of 5% PhiX to serve as an internal control.

Bioinformatic Analysis

The raw sequence data (concatenated forward and reverse sequence reads) were cleaned, reduced, and analyzed using Mothur Version 1.36.1. First, the barcode and the primer sequences were trimmed and then unique sequences were identified. The trimmed unique sequences were aligned to the SILVA rRNA database sequences using blastn algorithm (Pruesse et al., 2007). Before this the SILVA database sequences were trimmed to include only the V3-V4 region. The overhangs at both ends were removed via filtering the sequences and the redundancy check was carried out. For further de-nosing, the sequences were pre-clustered. The chimeras were eliminated using the implanted code UCHIME (Edgar et al., 2011). The sequences were classified by using Bayesian classifier implanted in Mothur. The reference and taxonomy files were adopted from the SILVA database (Pruesse et al., 2007). After Operational Taxonomic Unit (OTU) picking and their taxonomic assignment using the SILVA rDNA database, the OTUs were binned into phylotypes.

Sequence Accession Numbers

The raw sequencing data generated in this study were deposited into the NCBI database under accession numbers SRP123547 in the NCBI Sequence Read Archive, with the following accession numbers: SRS2657311, SRS2657312, and SRS2657313.

Results

Physical-chemical environment

The chemical characteristic of the water sample is presented in Table 1. The concentration in Ca²⁺ was found to exceed that of Mg⁺, whereby the predominant anion was determined to be HCO₃⁻. The mean air temperature and humidity inside the Yarik Sinkhole were 12.4°C and 94% respectively.

Table 1. Parameters and test methods for chemical analysis of the Yarik Sinkhole water.

| Parameter | Unit | Yarik Sinkhole Water Sample | Test Method | Reference |
|-------------------------------|-------|-----------------------------|---|-------------|
| HCO ₃ ⁻ | mg/L | 124 | SM 2320 B | APHA (1992) |
| F ⁻ | mg/L | 0.25 | SM 4500-F D | APHA (1992) |
| Ca ²⁺ | mg/L | 35.7 | EPA 200.7 | APHA (1992) |
| Cl ⁻ | mg/L | 5 | SM 4500 Cl ⁻ B | APHA (1992) |
| Mg ²⁺ | mg/L | 7.22 | EPA 200.7 | APHA (1992) |
| Na ⁺ | mg/L | 1.96 | EPA 200.7 | APHA (1992) |
| K ⁺ | mg/L | 0.74 | EPA 200.7 | APHA (1992) |
| SO ₄ ²⁻ | mg/L | 15.5 | SM 4500 SO ₄ ²⁻ E | APHA (1992) |
| pH | ... | 7.42 | TS EN ISO 10523 | TSE (1999) |
| Conductivity | µS/cm | 222 | TS 9748 EN 27888 | TSE (1996) |

Bacterial taxonomy and distribution

Taxonomic assignment of 130,703 sequencing reads (Phred score ≥ 30 ; Mean read length >300 bp) from Yarik Sinkhole was obtained by targeting the V3 and V4 region of the bacterial 16S rRNA gene. A total of 33 OTUs (based on 97% cutoff) comprising 4 bacterial phyla were found. The variation of the fractions of the phyla according to the depths was shown in (Table 2). Only *Firmicutes*, *Proteobacteria*,

and *Actinobacteria* phyla had a mean abundance of more than 1% in each sample holding more than 98% of the total sequence reads. The bacterial phylum with the highest presence percentage is *Proteobacteria* (average 80%) at all the depths. It is followed by *Actinobacteria* (average 16%). While the rate of presence of *Firmicutes* is more than 5% at -300 m, it is smaller than 1% at other depths. *Bacteroidetes* were found at less than 1% at other depths (Table 2).

At the class level, *Gammaproteobacteria* (average 17%) was present at all the depths. *Alphaproteobacteria* (average 24%), *Actinobacteria* (average 17%), *Bacilli* (average 2%) and *Bacteroidia* (average 0.3%) were also observed at all

Table 2. Relative abundance (%) of the detected species and phyla.

| Phyla and Species | Sampling Depth, m | | |
|--|---------------------|---------------------|---------------------|
| | -80 | -120 | -300 |
| <i>Firmicutes</i> (phylum) | 0.552 | 0.848 | 5.550 ^a |
| <i>Bacteroidetes</i> (phylum) | 0.005 | 0.006 | 0.918 |
| <i>Actinobacteria</i> (phylum) | 0.807 | 10.264 ^a | 39.484 ^a |
| <i>Proteobacteria</i> (phylum) | 98.636 ^a | 88.882 ^a | 54.048 ^a |
| <i>Alphaproteobacteria</i> (class) | 6.513 ^a | 16.552 ^a | 49.186 ^a |
| <i>Gammaproteobacteria</i> (class) | 92.124 ^a | 72.255 ^a | 4.861 ^a |
| <i>Betaproteobacteria</i> (class) | 0.000 | 0.075 | 0.000 |
| <i>Methylobacterium tardum</i> | 6.505 ^a | 16.511 ^a | 49.179 ^a |
| <i>Propionibacterium acnes</i> | 0.743 | 10.225 ^a | 38.144 ^a |
| <i>Acinetobacter lwoffii</i> | 91.621 ^a | 72.238 ^a | 4.852 ^a |
| <i>Bacillus thermoamylovorans</i> | 0.039 | 0.036 | 4.212 ^a |
| <i>Streptococcus sanguinis</i> | 0.508 | 0.002 | 1.125 ^a |
| <i>Porphyromonas</i> spp. | 0.005 | 0.006 | 0.918 |
| <i>Rothia mucilaginosa</i> | 0.015 | 0.015 | 0.908 |
| <i>Rhodococcus</i> spp. | 0.002 | 0.024 | 0.271 |
| <i>Aeribacillus geobacillus pallidus</i> | 0.000 | 0.002 | 0.203 |
| <i>Micrococcus luteus</i> | 0.000 | 0.000 | 0.159 |
| <i>Acinetobacter johnsonii</i> | 0.503 | 0.017 | 0.009 |
| <i>Bacillus</i> spp. | 0.000 | 0.000 | 0.007 |
| <i>Methylobacterium fujisawaense</i> | 0.002 | 0.015 | 0.005 |
| <i>Staphylococcus pasteuri</i> | 0.005 | 0.807 | 0.002 |
| <i>Sulfitobacter</i> sp. | 0.000 | 0.024 | 0.002 |
| <i>Solirubrobacter</i> spp. | 0.047 | 0.000 | 0.002 |
| <i>Simonsiella muelleri</i> | 0.000 | 0.075 | 0.000 |
| <i>Methylobacterium radiotolerans</i> | 0.005 | 0.002 | 0.000 |

^a Taxonomic group with an abundance higher than 1%.

the depths, except for -80 m and -300 m, where *Betaproteobacteria* were absent. In total, 18 species were identified at 3 different depths. Only 9 of these bacteria were found to constitute 5% or more. The frequency of *Acinetobacter lwoffii*, *Methylobacterium tardum*, and *Propionibacterium acnes* were the ones with the highest presence. Other species were determined to be in lower percentages (Table 2).

Discussion

Caves are special due to their formation processes and their chemical nature, are also unique in terms of microbial diversity. When exposed to human activity, caves lose their microbial richness (Lavoie and Northup, 2005; Ikner et al., 2007; Chelius et al., 2009). For this reason, studies carried out by taking samples especially during first entry and from isolated environments aim to better understand the microbiota. For cave microbiology studies, it can be said that horizontal caves are preferred because of ease of sampling compared to vertical caves. For similar reasons, the cave microbiology studies in Turkey have focused on the horizontal caves (Gulecal-Pektas, 2016; Gulecal-Pektas and Temel, 2015; Gulecal-Pektas and Temel, 2017; Candiroğlu, 2018).

This study is the first microbiological research conducted in a vertical cave in Turkey with sampling during the first

discovery of the cave. Anthropogenic impact is expected to be unlikely for the microorganism profile in the samples taken at the time of the initial exploration of vertical caves. These previously cited reasons made our study original and also important for its intended purpose. However, the area surrounding the Yarik Sinkhole area and entrance is cluttered with summer houses of villagers that live on the lower levels of the mountains. During periods of late April to late September, villagers migrate to these settlements and use the surroundings for pasture of their livestock of cows, sheep, and especially goats. These settlements do not have infrastructure for toiletry and waste water. All of the liquid waste goes into opened pits and from there seeps to the underground. They use the entrance of Yarik Sinkhole as garbage disposal which we had to intervene, warning them to stop throwing rubbish that includes baby diapers to rubber cycle tires, etc. There are several siphons connected to the sink from such areas that suggest anthropogenic impacts on the Yarik Sinkhole. Our study aimed to evaluate the bacterial diversity of the Yarik Sinkhole and to show possible anthropogenic impacts on diversity.

The Yarik Sinkhole resembles most cave systems with its high level of humidity and stable air temperature (Riquelme et al., 2015; Lavoie et al., 2017; Leuko et al., 2017). Similar to our findings, Leuko et al., (2017) reported a higher Ca^{2+} concentration than that of Mg^{+} as a result of the chemical analysis of the water samples from the Su Betu limestone cave in Sardinia, Italy, and detected HCO_3^- as the predominant anion. Researchers concluded that the predominance of HCO_3^- found in a cave indicates a calcium-bicarbonate type cave. On the other hand, the high level of SO_4^{2-} detected in the water sample from the Yarik Sinkhole may indicate an oxidation of the sulphur minerals contained in the rocks in contact with the water. pH, presence of nutrients, light, oxygen, sulphur, and compounds of other metals all affect the growth and structure of microbial communities in a humid cave. A change in those conditions can cause the differences in the composition of species (Engel et al., 2010; Jones and Bennett, 2014).

In the current study, the phyla and their percentages present at -80 m, -120 m and -300 m depths of Yarik Sinkhole were determined with next generation sequencing (NGS). We detected *Proteobacteria*, *Actinobacteria*, *Firmicutes*, and *Bacteroidetes* phyla. The most predominant two phyla have been found to be *Proteobacteria* (average 80%) and *Actinobacteria* (average 16%). The dominant groups in caves vary by the characteristics of caves (Lee et al., 2012). The major phyla found as a result of metagenomic analysis of soil samples taken from 5 caves in the Ozark Cave system are *Proteobacteria* (27.7%), *Acidobacteria* (17.3%), *Actinobacteria* (12.2%), *Firmicutes* (8.2%), *Chloroflexi* (8.1%), *Bacteroidetes* (8%), and *Nitrospirae* (6%) (Oliveira et al., 2017). In another study, the most dominant phyla determined as a result of metagenomic analysis of sediment samples of 3 caves in Mizoram (India) were *Actinobacteria* (35.9%), *Chloroflexi* (13.9%), *Planctomycetes* (13.7%), *Acidobacteria* (11.44%), and *Proteobacteria* (6.6%) (De Mandal et al., 2014). Members of the *Proteobacteria* and *Actinobacteria* phyla, dominant in both our study and many other studies, are well-adapted to growth with limited nutrients (Jurado et al., 2010; Lee et al., 2012; Barton, 2015; Wiseschart et al., 2018).

Despite the undeniable dominance of *Proteobacteria* in cave ecosystems, the representation of the *Proteobacteria* classes varies in different environments. At the class level, *Gammaproteobacteria* was present at all the depths, as were *Alphaproteobacteria*, *Actinobacteria*, *Bacilli* and *Bacteroidia*. *Betaproteobacteria* was detected only at -120 m depth. *Proteobacteria* is a cosmopolitan bacterial group that is common and abundant (Laiz et al., 1999; Zhaou et al., 2007). Members of the *Proteobacteria* phylum have the abilities of utilizing different organic compounds, the fixation of atmospheric carbon, and nitrogen transformation (Tomczyk-Zak and Zielenkiewicz, 2016). It is also suggested that dominance of *Proteobacteria* is a result of the increasing organic input caused by cave visitors (Ikner et al., 2007). In the current study among the *Proteobacteria*, 92%, 72%, and 5% were recognized in the class of *Gammaproteobacteria*, 6.5%, 16.5% and 49% as *Alphaproteobacteria* at -80 m, -120 m and -300 m depths respectively. Similarly, it was determined that there were plenty of *Gammaproteobacteria* in soil samples taken from the Mangoo-Pee cave. 23% of the *Proteobacteria* found by 43% in soil sample of the Blowing Spring Cave was determined as *Gammaproteobacteria*, 19% as *Alphaproteobacteria*, and 1% as *Betaproteobacteria* (Barron et al., 2010).

Another dominant group, *Actinobacteria*, is known for being able to develop in environments containing limited nutrients, to degrade different humic material, and to dissolve phosphate and calcium carbonate (Ball et al., 1989; Dari et al., 1995; Laiz et al., 1999). It was reported that this phylum existed in cave walls, soil, sediment, and on speleothem surfaces, and it was suggested that it might have considerably contributed to the formation of the cave structures and the biomineralization in the cave ecosystem (Cuezva et al., 2012; Ortiz et al., 2013; Tomczyk-Zak and Zielenkiewicz, 2016).

Even though microorganism diversity differs by the method used (culture-depend or culture-independent), by the sampling area, and by the sample type (soil, cave wall, speleothem surface, etc.), the core phyla reported in previous cave studies are *Proteobacteria* and *Actinobacteria* (Groth et al., 1999; Tomczyk-Zak and Zielenkiewicz, 2016). Besides these, the presence frequency of the *Firmicutes*, *Acidobacteria*, *Bacteroidetes*, *Chloroflexi*, and *Planctomycetes* phyla in caves is at a considerable rate (Youssef and Elshahed, 2008; Jurado et al., 2010; Lee et al., 2012; Barton, 2015; Wiseschart et al., 2018).

The evaluation of the NGS results of our study shows low diversity (only 18 species were identified through metagenomics) compared to other studies. The ingress of the waters contaminated by human/animal wastes into the Yarik Sinkhole can be one of the reasons. The microorganisms and organic substances that enter the cave from outside via contamination might negatively influence the cave's ecosystem leading in turn to the irreversible loss of its native biodiversity (Ikner et al., 2007; Chelius et al., 2009).

On the other hand, in the study conducted by Yasir (2018), 13 strains were identified by the culture method and a few genera, including *Bacillus*, *Microbacterium*, *Pseudomonas*, and *Psychrobacter*, were determined by the pyrosequencing analysis. However, in the pyrosequencing data *Carnobacterium*, *Exiguobacterium*, *Paucisalibacillus* and *Fictibacillus* were not detected. In addition, studies have shown that low abundance bacteria can be captured by culture methods (Lagier et al., 2012; Shade et al., 2012; Stefani et al., 2015). For this reason, the bacterial diversity of the environment should be determined more accurately by combining the culture methods with series based studies such as 16S rRNA gene analysis and metagenomics. Although there are some commonalities among the groups detected by using culture and molecular techniques, microorganism groups obtained through molecular results are richer since they also contain nonculturable groups as well. The results change in accordance with the characteristics of each cave (Engel et al., 2010; Jurado et al., 2010; Jones et al., 2012; Lee et al., 2012; Barton, 2015). However, when results are evaluated on the basis of species, variations in bacterial diversity of each cave become more obvious.

In the present study, *Methylobacterium*, *Acinetobacter*, *Propionibacterium*, and *Bacillus* were found more than other genera. It is known that these bacteria can utilize a wide variety of carbon sources and play an important role in calcification (Hiraishi et al., 1995; Cacchio et al., 2004; Portillo et al., 2008; Busquets et al., 2014). *Propionibacterium acnes* and *Acinetobacter lwoffii* can really get an advantage *in vivo* from polyphosphate as an energy reserve and they may use it during periods of starvation or unfavorable conditions (Van Groenestijn et al., 1987; Chen, 1999). *A. lwoffii*, *P. acnes*, and *Streptococcus sanguinis* that were found at 1% or higher in at least one of the sampled depths cause diseases such as bacteremia, pulmonary infections, meningitis, sepsis, pneumonia, bacterial endocarditis, and periodontal diseases (Doughari et al., 2011; Baker et al., 2018; Achermann et al., 2014). Also *A. lwoffii* is a normal flora of the oropharynx and the skin in approximately 25% of healthy individuals (Regalado et al., 2009). According to research conducted by the Human Microbiome Project the bacteria of the genera Consortium *Lactobacillus*, *Propionibacterium*, *Streptococcus*, *Bacteroides*, *Corynebacterium*, *Staphylococcus*, *Moraxella*, *Haemophilus*, *Prevotella*, and *Veillonella* are of human origin (Huttenhower et al. 2012). Leuko et al., (2017) detected a high level of *P. acnes* in their study conducted in the Su Bentu limestone cave in Sardinia, Italy, and associated that result with human contamination. To our knowledge, all of the bacteria that were found in the samples collected from Yarik Sinkhole were previously found in caves, except for *Sulfitobacter* (Busquets et al., 2014; De Mandal et al., 2014; Herzog Velikonja et al., 2014; Kieraitė-Aleksandrova et al., 2015; Riquelme et al., 2015; Leuko et al., 2017).

The achievement of sampling in parallel to the discovery of the Yarik Sinkhole separates this study from other cave microbiology studies to a significant extent. Analysis of the samples showed that the bacterial diversity is limited and the detected bacteria are generally originated from humans. These results show that anthropogenic activities around a vertical cave such as the Yarik Sinkhole cause contamination of the cave.

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