Caving Impacts on Cave Microbial Diversity: Case Study of Morca Cave, Turkey

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Abstract

Some microorganisms identified in cave ecosystems have been reported to play a permanent and significant role for maintaining life in such environments. Human entrance into caves can induce some changes on cave physico-chemical parameters which ultimately affects the living organisms. In this regard, for the first time, Morca Cave was explored to evaluate the impacts that human activities may have on the microbial diversity of the cave in a limited period of time. During this expedition at a depth of 1040 m, a camp was established for four days. Before the installation and at the end of the camp, sediments and surface samples were taken from different points of the camp area and the area around it. Sequencing of 16S rRNA of each sample was performed using the next generation sequencing method. The profile of the microbial diversity before the camping revealed that Thermoplasmata dominated the archaea group and Gamma- and Alpha-proteobacteria were the most dominant bacterial group. After the camp, a decrease in the microbial diversity especially the previously mentioned classes strains is observed at the most of the sampled areas. The results also showed that Bacilli strains significantly increased after the camp and increase of Bacteroidia strains is observed at the most active sampled areas. This present study therefore highlights how microbial diversity inside a closed cave can respond to the human activities within a short period. Furthermore, it may constitute a solid basis to support efforts targeted at improving techniques for cave management and expedition for the conservation of cave nature.

Introduction

Caves are dark oligotrophic environments characterized by high humidity and a generally low but stable temperature (Tomczyk-Zak and Zielenkiewicz 2016). Cave ecosystems harbour a large number of microorganisms with different features. The way microorganisms adapt to these extreme conditions such as cave environmental conditions is more interesting due to their specific characters. Chemoautotrophic microorganisms are expected to contribute to the carbon and nitrogen cycles in their ecosystems (White and Culver 2009). In addition, caves contain important natural bioactive compounds, provided by their indigenous microorganisms, with high potential uses in different industrial and biotechnological fields (Ghosh et al. 2017; Çandıroğlu and Doğruöz Güngör 2020; Banerjee et al. 2019). Therefore, to conserve these bioactive compounds and the life inside caves, the cave microbial diversity should be protected.

However, due to anthropogenic activities such as tourism, sports and research, this fragile ecosystem is disrupted and threatened. The stability of this cave ecosystem can therefore be affected. This biodiversity may be affected in different ways by the human activities inside or outside caves. During cave expeditions, input of organic matters inside the cave; including leaving some remains of food, accidental falling-down of hair or depositing fecal materials in the cave environment, can induce the change of the ecological structure of these ecosystems (Barton 2006; White and Culver 2009). The increase of the organic materials might also induce stress of native microorganisms or even extinction of some of them.

Other studies also reported that decrease of microbial diversity in caves can be caused by anthropogenic activities inside or around the cave (Alonso et al. 2019). An increase in the levels of CO₂ inside the cave

ecosystem during expedition is reported in cave opened for tourism. Furthermore, mechanical forces applied by cavers inside the caves such as walking are reported to reduce the cave sediment oxygens (Kurniawan et al. 2017; Lavoie et al. 2006). Human flora can also be deposited inside caves by touching cave structures or other activities like leaving fecal matter. In this context, the cave microbial diversity should be investigated during the first entry to the cave in order to avoid any human related effect on this biodiversity.

Certainly investigation of the cave ecosystems for the identification of their microbial diversity is important due to their susceptible uses in medicine and biotechnological fields. However, human activities during such studies or other cave exploration activities are thought to impact on the microbial diversity. For this purpose, our study was aimed to (i) profile the microbial diversity of Morca Cave at the depth of 1040 m by using the Next Generation Sequencing method and (ii) evaluate the impacts caused by human activities (camping that was carried out during the first expedition of 1040 m of Morca Cave within a given short period of time, four days. This study will not only permit establishment of the microbial diversity of the Morca Cave for the first time and the human activities impacts on it, but it will also constitute a solid basis for future microbiological researches in this Cave.

Materials And Methods

SAMPLING SITE AND SAMPLES COLLECTION

The Morca Sinkhole is located at the Middle Taurus mountains in Turkey. Sediment and surface samples were taken from around the camping site installed at 1040 metres depth during the Morca 2019 expedition (Fig. 1; Table 1). All the samples were taken under aseptic conditions: the surface samples were collected with sterile swabs preserved in sterile saline water, the sediments were collected in sterile cups using sterile spoons, and sterile gloves were worn while sampling. Samples taken before the camp were stored in ice until the end of the camp (4 days) and together with the samples collected after the camp from the same points, were transported to the laboratory where they were stored at -20°C until they were analysed.

Table 1
Details of collected samples

Sample Type	Sampling period	Sampling area
wall	Before camp	Campsite (A)
wall	After camp	Campsite (A)
Sediment	Before camp	Campsite (B)
Sediment	After camp	Campsite (B)
Sediment	Before camp	Near toilet area 1 (E)
Sediment	After camp	Near toilet area 1 (E)
Sediment	Before camp	Near toilet area 2 (F)
Sediment	After camp	Near toilet area 2 (F)
wall	Before camp	Near campsite (C)
wall	After camp	Near campsite (C)
Sediment	Before camp	Near campsite (D)
Sediment	After camp	Near campsite (D)

DNA ISOLATION

The genomic DNA from each sample was isolated using the 'FastDNA Spin Kit for Soil' kit according to the manufacturer procedure. The isolated DNA concentrations (ng/μl) were measured using a nanodrop device and run to an agarose gel under electrophoresis for the control.

ANALYSIS OF 16S rRNA AMPLICON

The V4-V5 variable region of the 16S rRNA genes from the purified DNA samples were amplified by using bacterial and archaeal universal primers 515F (5'-GTGYCAGCMGCCGCGGTAA-3') and 926R (5'-CCGYCAATTYMTTTRAGTTT-3') following the methods from literature (Walters et al. 2015). By using this library preparation kit, the PCR was run under these conditions: primary denaturation step at 94°C for 3 min following by 28 cycles of (94°C for 3 seconds, 53°C for 40 sec, 72°C for 60 sec), and the last step of elongation for 5 min at 72°C. PCR products were run in 2% of agarose gel for amplification control. Each sample product's concentration was measured and compared to equimolar volume. The united amplicons were purified by using the AMPure XP (Beckman Coulter, USA) magnetic beads present inside the kit. According to the protocol of DNA library preparation kit, the united and purified samples have been made ready for sequencing. Service procurement was carried out with an average of 50000 readings (2x300 bp) for each sample on the MiSeq device for Next Generation Sequencing – NGS service.

BIOINFORMATIC ANALYSES

Quality control of the entire sequences obtained after sequencing were carried out in the FASTQC program. 16S rRNA amplicon data were analyzed using the QIIME2 software package. First, the primer sequences used in the PCR step were removed from the sequences by cutting with the cutadapt (cutadapt trim-paired) insert. Then, the forward and reverse arrays were combined with the vsearch plugin (vsearch join-pairs). Combined fragment sequences obtained were applied "denoising" using the Deblur program (deblur denoise-16S). Chimeric fragments were also filtered at this stage, resulting in ASV (Amplicon sequence variants) sequences. At the same time, the reading amounts of each series among all samples were also revealed, and the taxonomic classification of each series was performed with the classify-sklearn plug-in (feature-classifier classify-sklearn), using the SILVA_132 database. Percentage reading rates in each taxonomic unit were calculated, and the bacterial and archaeal microbiota content of each sample was revealed. Alpha rarefaction analysis was also performed in the QIIME2 software package (diversity alpha-rarefaction): Chao1, Observed_OTUs, Shannon and Simpson metrics were calculated with the diversity alpha command.

T-TEST STATISTICAL ANALYSIS

Differences between samples taken before the camp and those taken after the camp were statistically determined through T-test statistical analysis with the critical value $\alpha = 0.05$. This test was applied on four taxonomic levels : phylum, class, and genus.

Results

OBSERVED DIFFERENCES IN THE MICROBIAL DIVERSITY BETWEEN SAMPLES COLLECTED BEFORE AND AFTER THE CAMP

A total of 1561 OTUs was identified from the total of the samples taken before camping against 1920 OTUs identified after (Table 2). Both archaea and bacteria were determined in this study area. Both before and after camping, archaea were observed with a relative abundance lower than that of bacteria. Before camping, the archaea in B, E, F, and D areas, were 0.35%, 3.77%, 0.28%, and 0.02% respectively. However, the points A and C registered an abundance rate less than 0.01% of archaea. After camping, a decrease in the abundance of archaea was observed in areas B and C while it was increased in areas D and F.

Table 2
Alpha diversity of each sampling area.

sampling area	sampling period	chao1	observed_otus	shannon	simpson
A	NAR1	143,84	143	4,333666832	0,896421666
A	NAR2	141,0731707	141	3,212326362	0,772977034
B	NAR3	181,7142857	181	5,552910745	0,945450354
B	NAR4	342,185567	338	5,57312221	0,929612327
E	NAR5	253,4705882	248	5,290159065	0,914834489
E	NAR6	539,1818182	534	6,588622341	0,963566081
F	NAR9	438	433	6,620141902	0,9687542
F	NAR10	260,9710145	259	5,857635488	0,953874902
C	NAR11	99,32258065	99	2,721648905	0,700177309
C	NAR12	69,75	68	3,347061491	0,812636098
D	NAR13	461,0930233	457	6,3075618	0,95749345
D	NAR14	585,9482759	580	6,773397309	0,966900149
NAR1, NAR3, NAR5, NAR9, NAR11, and NAR13 represent data of the pre-camping period; NAR2, NAR4, NAR6, NAR10, NAR12, and NAR14 represent data of the post-camping period.					

Besides the archaea, bacteria identified in the samples taken before camping composed of 25 phyla dominated by Proteobacteria, Actinobacteria, Bacteroidetes, Firmicutes, Planctomycetes, Gemmatimonadetes, and Acidobacteria. It must be noted here that the indigenous microbial community of this part of Morca Cave under study was represented by the results observed on samples taken before the camping. The community composition of each specific sampling area is detailed and community differences between the two periods were evaluated on the class and genus levels.

- Camp site (surface samples) (A)

The Chao1 of the Alpha diversity which indicates the species richness was noted to be slightly high before the camping period (143.8 against 141). Shannon index, which calculates the species diversity by considering both the richness and the equitability of the species, showed a high diversity of species before the camping period (4.3 against 3.2). It is the same for the Simpson index which shown that this site had a high microbial diversity before camping (0.89 against 0.77) (Table 2). The taxonomic analysis of this site revealed that there was no archaea in the two sampling periods. Even if most bacteria in this area were unidentified at the genus level, significant differences were noted between the two periods.

Gamma-proteobacteria, Alpha-proteobacteria and Bacilli 46.29%, 29.18% and 10.59% respectively were the most dominant classes observed before the camp. Important differences were observed for Alpha-proteobacteria and Bacilli classes. While the Alpha-proteobacteria decreased by 21.7%, the Bacilli class significantly ($p = 0,003$) increased parallel to its phylum (Firmicutes) by 33.83% (Fig. 2). At genus level, *Acinetobacter* (23.25%), *Brevundimonas* (19.41%), *Massilia* (9.84%), *Aerococcus* (7.65%), *Pseudomonas* (5.34%) and *wb1-P19* (4.48%) were the most abundant ones before the camping activities.

Acinetobacteria increased by 7.37% while *Brevundimonas* and *Pseudomonas* decreased after the camping period by 17.97% and 5.34% respectively. In addition, *Exiguobacterium* belonging the class Bacilli, which was almost absent before the camping shown a significantly increase rate (33.3%).

- Camp site: sediment samples (B)

Chao1 index values for the two periods were 181.7 and 342.1 before and after camping respectively. These values shown an augmentation of species biomass after camping in this sampling area. The species diversity evaluated using Shannon and Simpson indices did not show important variation between these two periods: the Shannon index and the Simpson index before and after camping were 5.55 and 5.57, were 0.94 and 0.92 respectively (Table 2). In this area, both the Thermoplasmata (0.25%) and Nitrososphaeria (0.1%) classes of the archaea represented 0.35% of the microbial biomass. On the other hand, after the camp this microbial group was absent (Fig. 3).

In addition, the bacterial group before camping was dominated by Gamma-proteobacteria with a relative abundance rate of 47.01%, followed by Actinobacteria (16.64%), and Alpha-proteobacteria (13.12%). However, after the camping period, it was observed that while Gamma-proteobacteria had increased (11.98%), Actinobacteria shown a decrease of (11.25%) (Fig. 3). Genera *Pseudomonas* (17.04%) and *Acinetobacter* (14.18%), *Wb1-P19* (6.03%), *Variovorax* (2.06%), *Sphingorhabdus* (8.70%), *Nocardioides* (2.36%), *Gaiella* (1.61%), *Paeniglutamicibacter* (1.31%) and *Pir4_lineage* (1.01%) were the most abundant species before camping. At the end of camping, *Pseudomonas*, *Acinetobacter*, and *Sphingorhabdus* decreased by 15.3%, 5.08%, and 6.59% while *wb1-P19* and *Limnobacter* increased at the end of the camping period 11.59% and 15.68% respectively.

- Near the toiletarea 1 (E)

According to Chao1, Shannon and Simpson indices microbial biomass and diversity increased in this area after the camping (Table 2). This area shown the highest archaea biomass (3.7%) among all the sampling sites (Fig. 4). The following classes were identified Thermoplasmata (2.96%), Woesearchaeia (0.06%) and Nitrososphaeria (0.75%) classes with *Candidatus Nitrososphaera* being the sole genus. The highest rate of archaea remained uncultured archaeon. After the camping period, the abundance of each class decreased (Fig. 4).

The classes Actinobacteria (39.03%) and Gamma-proteobacteria (25.09%) dominated this site before the camping. Other classes like NC10 (7.47%), Alpha-proteobacteria (3.02%) and Planctomycetacia (1.71%) were also identified. However, after the camping it observed that Actinobacteria as well as Gamma-proteobacteria classes decreased by 34.03% and 10.34 respectively. On the other hand, a significant

increment in Bacilli and Clostridia classes 44.21% and 8.49% respectively were noted after the camping (Fig. 4). It is also important to mention that there was a decrease in Nitrospira strains (0.28%) in this area. Genera *Nocardioides*, *Variovorax*, *Alkanindiges*, *Acinetobacter*, and *Massilia* of unidentified genus belonging the family Micrococcaceae of phylum Actinobacteria were identified before the camping with the respective rates of 19.58%, 8.22%, 2.93%, 2.49% and 2.09%. 19.08%. At this level, *Nocardioides* and *Variovorax* decreased by 19.58% and 8.22% respectively after the camping period. Furthermore, *Exiguobacterium*, *Brevibacillus*, and *Bacillus* also increased by 14.76%, 12.46%, and 4.72% respectively in the post-camping period with the respective increase rates.

- Near the toilets area 2 (F)

The Table 2 show a lowering of species richness, through the Chao1 index values, in this area. Behind that, the Simpson and Shannon indices shown a drop of the microbial diversity after the camp with 0.96 and 6.58 before the camping period against 0.95 and 5.85 at the camping end, respectively. These values shown that this sampling area (before the camp) housed the most microbial diversity among all the studied points (Table 2). Archaea strains identified in this site before the camp period were composed of Thermoplasmata (0.03%), Woeseearchaeia (0.03%) and Nitrososphaeria (0.21%) classes. Contrary to the diversity, the biomass of archaea (0.76%) observed before the camping was increased at the end of the camping. Thermoplasmata strains increased by 0.64% while strains of Woeseearchaeia and Nitrososphaeria decreased after the camp with the respective decrease rates (0.03%) and (0.12%) (Fig. 5). In addition, the bacterial group composed of 20 phyla with a highly diversified class level dominated by Gamma-proteobacteria (52.72%) at the pre-camping period. The Bacilli (9.61%), Alpha-proteobacteria (7.37%), NC10 (6.02%), Actinobacteria (2.66%), and Planctomycetacia (2.36%) have also identified at abundance rates >1%. Important variations were noted between the two sampling periods on Gamma-proteobacteria and Bacilli classes which shown respectively a decrease rate of 12.61% and an increase rate of 11.49% (Fig. 5). In this second point near the toilet area, the camping end was also marked by a decrease of members of Nitrospira strains (0.18%). In addition, genera *Pseudomonas* (9.99%), *wb1-P19* (9.17%), *Massilia* (4.48%), *Bacillus* (4.45%), *Candidatus Methyloirabilis* (3.43%), *Acinetobacter* (2.95%) and *Brevibacillus* (2.76%) were identified before the installation of the camp. The significant change at this level was observed on the genus *Acinetobacter* which shown an increase rate of 13.5% after the camp.

- Near the camp site: Surface samples (C)

Results of alpha diversity analyses shown that this site has the lowest microbial count and diversity (Table 2). The Chao1 index before the camp was 99.3 while after the camp was 69.75. Contrary to that, the diversity of this area increased at the end of the camp. The Shannon and Simpson indexes were respectively 2.72 and 0.70 before the camp period against 3.34 and 0.81 at the end of the camp (Table 2). Before as well as after the camp, archaea strains were absent (0.0%) in this site. The bacterial community was dominated by strains of Gamma-proteobacteria (69.51%) and Alpha-proteobacteria (37.92%). However, their abundances decreased by respectively 31.92% and 18.47% at the end of the

camping. Strains of Bacilli class became dominants in this area by showing an increase rate of 49.01% (Fig. 6). Furthermore, our results revealed that *Acinetobacter* (56.37%), *Brevundimonas* (23.36%) and *Massilia* (7.71%) dominated the area before the camping period. At the end of the camping period, genera *Acinetobacter* and *Brevundimonas* were observed with respective decrease rates of 28.11% and 22.65% contrary to the genera *Exiguobacterium* and *Sphingomonas* which shown significant increase rates of respectively 36.54% and 5.24%.

- Near the camp site: Sediment samples (D)

Compared to all the other studied points, this site shown the highest species richness at the two sampling periods. The Chao1 index, of the Alpha diversity, was 461.09 before camping and 585.9% at the end of the camping period (Table 2). Both Shannon and Simpson indices shown an augmentation of the diversity after the camping period (Table 2). Before the camp, the abundance of archaeal community in this site was weak and almost composed by strains of Nitrososphaeria class (0.02%). The increase of diversity and biomass of this community after the camp period is marked by the increase of Nitrososphaeria strains (0.34%) and the appearance of Thermoplasmata (0.10%). On the other hand, the bacterial class level at the pre-camping period, is largely dominated by Gamma-proteobacteria (54.61%) followed by Alpha-proteobacteria (9.88%). However, at the post-camping period, the class Gamma-proteobacteria decreases by 37.58% while Bacilli and Actinobacteria classes increased significantly with (28.86%) and (8.3%) as respective rates (Fig. 7). The genera *wb1-P19* (14.26%), *Massilia* (8.70%) and *Acinetobacter* (5.76%) are the most dominant at the pre-camping period. *Pseudomonas*, *Sphingomonas*, *Brevundimonas*, *Bacillus*, *Terrimonas* are also identified at the genus level with relative percentage rates between 5% and 1%. At the post-camping period, *Massilia* and *Acinetobacter* were nearly absent (0.0%) and (0.03%) while *Bacillus* and *Micromonospora* shown increase rates of 8.18% and 6.13% respectively.

Discussion

To our knowledge, this study is the first microbiological study run in the Morca Cave. It was run in a new explored part of the cave where it was the first time that cavers entered. In this fact, the samples taken before the camp may constitute the profile of microbial community of this part of the cave.

In this study, the abundance rate of archaea observed in samples taken before the camp was extremely lower than that of bacteria observed in the same samples. Even if every cave ecosystem presents its own and proper microbial community, our observation of archaea in this Morca cave part is consistent to most of studies conducted in other cave ecosystems (Kimble et al. 2018; Jurado et al. 2020). The genus *Candidatus Nitrososphaera* belonging phylum Thaumarchaeota is the unique identified archaea's genus in our study. It is well known as ammonium oxidizer (Kimble et al. 2018; Marques et al. 2018; Baskaran et al. 2020). Hence, this archaeon is reported to be one of the major chemolithotroph in oligotrophic environment such as cave ecosystem. Zhao et al. (2017) showed the dominance of Ammonia Oxidizing Archaea in Ammonia Oxidizing communities (especially Thaumarchaeota) within Alkaline Cave

sediments. This archaeon may have a vital function in the Morca Cave due its role in the nitrogen cycle as ammonium oxidizer.

The order Methanomassiliicoccales belonging the class Thermoplasmata (phylum Euryarchaeota), which is the most abundant class of archaea in this part of the cave, is composed of mainly uncultured methanogen archaea (Borrel et al. 2014). In the present study, these archaea are observed in the two sites near the toilet sites. Furthermore, they are composed of only uncultured archaeon starting from the family level. This order is among the seven orders belonging Euryarchaeota that are reported as most biogenic methane producers of the deep biosphere (Sar et al. 2019; Doğruöz-Güngör 2020). In general, methane producer archaea produce methane under anaerobic conditions if inorganic matters like oxygen, nitrate, and sulphate are limited (Evans et al. 2019). The presence of such microorganisms in cave ecosystems may be related to the oligotrophic and low oxygen characteristics of these ecosystems. Since methane present in the environment plays a role in the carbon cycle and contribute to the environmental nutrient as well as the weather of the environment (Evans et al. 2019), such microorganisms identified in this studied cave may played an important vital role in this cave ecosystem. In other hand, the class Woesearchaeia belonging the phylum Nanoarchaeota is also identified at the pre-camping period in area E and F. Even if information about the function of these archaea strains in cave ecosystems is still limited, they were previously reported from different cave environments and other soil samples (Jurado et al. 2020; Wang et al. 2020).

Some Proteobacteria are known for their ability to decompose organic material present in cave ecosystems (Tomczyk-z_ Ak and Zielenkiewicz 2016; Barton and Jurado 2007). These microorganisms largely represented by Gamma- and Alpha-proteobacteria classes dominated all the studied areas before the camp except area E dominated by Actinobacteria. Accordingly, genera like *Acinetobacter*, *Pseudomonas*, *Wb1-P19*, *Brevundimonas*, and *Massilia* were the most dominants. In the formation of karstic caves and cave speleothems, the role of some bacteria through different metabolic ways is evident. When atmospheric CO₂ interacts with the water molecules from the water which fall into the cave, they form carbonate ions as one of their products. These ions will further be used by some bacteria in the presence of cave calcium minerals to form the precipitated product 'calcium carbonate' which is the main composed material of karstic caves (Mengmeng et al. 2019; Gan et al. 2020; Rusznyák et al. 2012).

Acinetobacter and *Pseudomonas* species isolated from some karstic caves have been *in-vitro* determined with their ability to precipitate calcium carbonate (Türkgenç and Doğruöz-Güngör 2021). The identification of these bacteria in our study may respond to their principal role in such karstic ecosystem. On the other hand, *Massilia* are found to utilize large number of carbohydrates as well as other organic compounds as carbon and energy sources with a simultaneous acid production (Tomczyk-z_ Ak and Zielenkiewicz 2016; Doğruöz-Güngör 2020). Its presence in caves is reported to participate in rock dissolving (Tomczyk-z_ Ak and Zielenkiewicz 2016). Through its metabolic activities, it may also play significant role in the nutrient and carbon cycle of this ecosystem. *Brevundimonas* species are belonging to the family Caulobacter which is reported to be able to adapt extreme environments such as

oligotrophic caves and supporting for the growth of other microorganisms present in the same poor nutrient ecosystem (Tomczyk-Z. Ak and Zielenkiewicz 2016; Li et al. 2004).

Actinobacteria is among the most reported cave bacterial community. In this current study, its most dominant genus is *Nocardioides*. Species of this genus such as *N. cavernae* sp. nov., and *N. speluncae* sp. nov are previously isolated from karst caves, characterized as oligotrophic and able to support growth from wide range of substrates (Han et al. 2017; Fang et al. 2019; Growth et al. 1999; Yoon et al. 1999). It is also reported that strains of this genus have inorganic compounds and carbon monoxide oxidation ability (Lee et al. 2021). Another interesting genus identified in this study is the methanotroph *Candidatus Methyloirabilis* belonging phylum Rokubacteria. This is an autotroph with carbon dioxide fixation potential. In addition, species of the genus *Ca. Methyloirabilis* isolated from some caves are reported to denitrifying organic compounds to generate oxygen for the oxidation of methane present in the environment (Rasigraf et al. 2014; Bendia et al. 2020). We suggest a vital role of this bacterial strains in this ecosystem. In addition, its presence might be considered for further studies in the context of methane presence possibility in this ecosystem.

MICROBIAL DIVERSITY AND DISTRIBUTION RELATED TO SAMPLE TYPES

The diversity and distribution of microbial community is affected by the ecological factors. Different sample types (sediment, rocks, air ...) contain different chemicals and may show different physical properties. In addition, the different environmental conditions observed in every cave which are related to the geological position of the cave ecosystems caused that every cave is unique. The microbial diversity in caves is previously shown that is dependent to the sample type (sediment, rock, air, water ...) as well as the cave type (Zhu et al. 2019; Dong et al. 2020). In our study, wall samples (areas A and C) were observed with low microbial diversity than the sediment samples (Table 2). Similarly, Zu et al. (2019) observed that the higher microbial diversity and abundance was in sediment samples than in rock samples. However, Dong et al. (2020) shown completely different results in which they obtained a high microbial diversity in rocks than in soil samples. This explains that the microbial community composition and distribution of cave ecosystems is not only related to the sample type but also to the biotic and abiotic factors of the cave sampling area.

HUMAN ACTIVITIES IMPACTS ON MICROBIAL BIOMASS AND DIVERSITY

The decrease of the microbial diversity of the underground habitats such as caves are thought to be caused by anthropogenic effects (Ikner et al. 2007). In addition, low archaeal diversity is observed in more entropized caves (Alonso et al. 2019). In our study, decrease of microbial diversity is observed in the campsite (A) and the toilet area2 (F) after the camp. Besides, diminution of archaea diversity is noted near the toilet area 2 (E) and the campsite (B) after the camping period. This might be explained by the fact that these areas were suggested to be the most impacted areas during the camping period.

IMPACTS OF CAMPING ON MICROBIAL TAXONOMY

- Analysing our results, it is observed that Proteobacteria dominated the studying ecosystem before the camp while Gram (+) bacteria were the most dominants in this ecosystem after the camp. It is suggested that high number of visitors inside caves may induce the increase of the Gram (+) bacteria and the decrease of Gram (-) ones (Kukla et al. 2018). It could be speculated that the number of cavers in this camp have negatively impacts on the ecosystem bacterial community.

The dominance of Firmicutes due to anthropogenic impacts was observed in different studies. In the Kartchner Caverns, Ikner et al. (2007) found through culture based methods that high abundance of Firmicutes was in low and medium tourist impacted cave areas. Similar results are observed in the medium touristic Cave of Bats through sequencing the 16S rRNA of cave samples (Urzi et al. 2010; Tomczyk-z_ Ak and Zielenkiewicz 2016). Furthermore, the high abundance of Firmicutes in the disturbed zone of sediments from coastal area was related to the anthropogenic activities in that zone (Su et al. 2018). The statistical analyses in our study show significant increase of Firmicutes strains ($p = 0.003$; $r < 0.5$) after the camp period. According to the previous studies this observation may be related to the human impacts in this environment.

Accordingly, all sampling sites shown important decrease of Proteobacteria (Gamma- and Alpha-proteobacteria) except the campsite (B). It is important to note that this site was highly active during the emplacement of the camp as well as during the camping period as explained in the report of Usuloğlu et al. (2019). Furthermore, we should note that this point was the entrance of the tent pitching for the camp. Consequently, the increase of Proteobacteria in this area is thought to be resulted from the mixing of microorganisms from different areas of this studying part of Morca Cave. Even if a high dominance of Proteobacteria strains was reported as the result of high organic compound input, this conclusion was based on culture-dependent method (Ikner et al. 2007). In this actual study, due to the used method (culture-independent method) and the results obtained from all the remain studied sites, the increase of Gamma- and Alpha-proteobacteria in this site cannot reflect to only a high impute of organic materials. However, studying the organic composition of samples parallely may be recommended in the future studies for a definite conclusion. On the other hand, previous studies demonstrated that Proteobacteria are usually act as primary producers in the food chain of cave ecosystems (Tomczyk-z_ Ak and Zielenkiewicz 2016). Their abundance in all samples collected before the camp may indicate their important position as a basic living source in this part of the Morca Cave. Consequently, the decrease of these strains as previously demonstrated may affect the life in this ecosystem.

An important indicator of human impact is the increase of Bacteroidetes and decrease of Nitrospirae strains (Ikner et al. 2007; Alonso et al. 2019). In this actual study, the increase of Bacteroidia strains and lowering of Nitrospira strains after the camp are observed in the sites near the toilet areas (Fig. 4 and Fig. 5). These sites are suspected to be the most impacted among all the sampling points. Human related bacteria such as *E. coli*, *Staphylococcus aureus*, *Propionibacterium*, and the soil thermotolerant *Bacillus* sp. are reported as human impact indicators (Lavoie and Northup 2006; Leuko et al. 2017; Bercea et al.

2019; Alonso et al. 2019). Surprisingly, none of these indicators are observed in all the studied samples collected after the camp. This would simply indicate that the period of the study was not enough for the establishment (adaptation) of these *ex-situ* microorganisms. Further studies for different sampling periods should be run for a definitive conclusion. In addition, cave microorganisms are reported to be able to remediate the cave ecosystems by compete the *ex-situ* organisms introduced through anthropogenic activities (Barton and Hershey 2019). However, samples should be taken after the rest period of the cave and before a new period of activities to set such conclusion in the case of our study topic.

However, high abundance of Clostridia class in the area near the Toilet 1 (E) after the camp is observed in our study. Belonging this class, the family Clostridiaceae is also observed at the same abundance in this same sampling area after the camp. This group of bacteria is considered as one of the two major groups colonizing the human faeces (Wéry et al. 2010). Accordingly, this observation is suggested to be resulted from the anthropogenic activities in this area because strains of this class are observed in the Dzou Cave sediments from tourist campsites or near the cave entrance (Mazina et al. 2020).

Conclusion

Summary, our study aimed to evaluate the human impacts on microbial diversity of a newly explored cave in a short period of time. The results provided in this actual study revealed that the microbial diversity in this studied part is composed of low archaea abundance and a dominance of Gamma- and Alpha-proteobacteria before the camping. However, at the post-camping period, a decrease of these Proteobacteria classes is observed while the increase of Bacilli and Clostridia is noted. In addition, Increase of Bacteroidia and decrease of Nitrospira have been observed near the toilet areas. These alterations may indicate that human activities have negatively impacted on the microbial diversity of this area. In our advanced studies, samples taken from these areas after one year (cave rest period) are planning for studying possible recovery of their microbial diversity rather than the establishment of human related species.

Declarations

- ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable

- CONSENT FOR PUBLICATION

Not applicable

- AVAILABILITY OF DATA AND MATERIAL

The generated raw sequencing data of the 12 studied samples were deposited into the NCBI database under these accession numbers: SAMN18256317-28.

- **COMPETING INTERESTS**

Nahdhoit AHAMADA RACHID and Nihal DOĞRUÖZ GÜNGÖR declare that they have no conflict of interest.

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- **AUTHORS CONTRIBUTIONS**

M.Sc Nahdhoit AHAMADA RACHID : Conceptualization, Methodology, Investigation, Vizualization, Writing.

Dr Nihal DOĞRUÖZ GÜNGÖR : Project administration, Conceptualization, Writing

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- **AUTHORS' INFORMATION**

Not applicable

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Figures

Figure 1

Studying area (10, 11)

Figure 2

Observed differences at class level between samples taken before and after the camping at the area A. NAR1: before camping; NAR2: after camping

Figure 3

Observations at class levels for samples taken before and after the camping at the area B. NAR3: before camping; NAR4: after camping

Figure 4

Significant differences observed at class level between samples taken before and after camping in the sample area E. NAR5: before camping; NAR6: after camping

Figure 5

Significance differences observed at class level between samples taken before and after the camping at the area F. NAR9: before camping; NAR10: after camping

Figure 6

Observation of significance difference at class level between samples taken before and after the camping at the area C. NAR11: before camping; NAR12: after camping

Figure 7

Significance differences observed at the class level between samples taken before and after the camping at the area D. NAR13: before camping; NAR14: after camping