

# Unraveling the bacterial diversity of Cangar Hot Spring, Indonesia by Next Generation Sequencing of 16S rRNA gene

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**Abstract.** Geraldi A, Tay CC, Ni'matuzahroh, Fatimah, Hanafi WNW. 2021. Unraveling the bacterial diversity of Cangar Hot Spring, Indonesia by Next Generation Sequencing of 16S rRNA gene. *Biodiversitas* 22: 4060-4066. This study is the first attempt at using the Next Generation Sequencing (NGS) method with 16S rRNA to understand the bacterial community structure in an Indonesian hot spring. This study aims to unravel the bacterial diversity of the Cangar Hot Spring as one of the most explored natural hot springs in East Java, Indonesia. We found Proteobacteria and Bacteroidetes as the two most abundant phyla. We discovered the first occurrence of genera *Cloacibacterium* and *Methylobacillus* in the hot spring ecosystem, which was the most dominant genera at Cangar Hot Spring. We also found several potential bacteria for bioindustry and bioremediation, such as *Acinetobacter junii* and *Pseudomonas alcaligenes*. Besides that, we also observed opportunistic pathogens from genera *Comamonas* and *Vogesella*. This study result will provide valuable information for further bioprospecting of bacteria with commercial potential and the development of health and safety measures in the Cangar Hot Spring, among others. Hopefully, this report would encourage the use of NGS technology for studying other hot springs in Indonesia.

**Keywords:** Aquatic ecosystems, hot spring, bacteriome, next-generation sequencing, 16S rRNA

## INTRODUCTION

Hot springs is one of the extreme ecosystem characterized by outflowing water with a temperature above 36.7°C (Jiang et al. 2018, Pentecost et al. 2003). In the hot spring ecosystem, thermophilic microorganisms such as Archaea and bacteria can thrive well (Li et al. 2020) because of their ability to survive in high-temperature conditions. These thermophilic microorganisms are of great interest since they are able to produce thermostable enzymes and biomolecules such as biosurfactants, polysaccharides, and antibiotics, which are industrially important (Atalah et al. 2019, Di Lorenzo et al. 2014, Geraldi et al. 2019a, Kambourova 2018, Mahajan and Balachandran 2017, Sahoo et al. 2020, Teta et al. 2017). These microorganisms can also be utilized in bioremediation processes such as the biodegradation of hydrocarbons and removal of heavy metals, and also biofuel production (Castro et al. 2019, Di Donato et al. 2019, Ebaid et al. 2019, Geraldi et al. 2019b, Jardine et al. 2018, Orellana et al. 2018).

Located in the Pacific Ring of Fire, Indonesia is a country rich in geothermal sites, with 256 hot springs identified across the archipelago (Darma et al. 2010, Yohandini et al. 2015). Since 1991, the exploration and bioprospecting of thermophilic microorganisms from hot springs have been done in Indonesia, mostly using culture-based approaches (Huber et al. 1991). These explorations

have successfully isolated various thermostable enzymes (cellulase, amylase, chitinase, protease)-producing bacteria, mostly from the order Bacillales (Geraldi et al. 2019c, Lischer et al. 2020, Wang et al. 2012). Among the studied hot springs in Indonesia, Cangar Hot Spring in East Java is one of the most microbiologically explored. From the Cangar Hot Spring, bacteria strains (i.e., *Bacillus subtilis* subsp. *inaquosorum* strain and two *Bacillus licheniformis* strains) with the potential to produce thermostable biosurfactants and enzymes were isolated (Chrisnasari et al. 2018, Geraldi et al. 2019b, 2019c, Ibrahim et al. 2013, Rashid et al. 2013).

Since 99% of microbial species are unculturable (Amin et al. 2017, Bodor et al. 2020), culture-independent approaches are needed to explore and utilize thermophilic microorganism biodiversity from hot springs. Previous studies in hot springs at Tanjung Sakti, Gedongsongo, and Kamojang area, Indonesia (Aditiawati et al. 2009, Aminin et al. 2008, Yohandini et al. 2015), showed that Denaturing Gradient Gel Electrophoresis (DGGE) and 16S rRNA gene sequencing was successful in revealing the unculturable microorganisms. However, up to our knowledge, there is no report on the utilization of Next Generation Sequencing (NGS) technology as a cutting-edge and powerful tool for analyzing microbial communities. NGS approach on 16S rDNA amplicons obtained from Cangar Hot Spring metagenomic DNA enables the comprehensive analysis of microbial populations in the hot spring. This study is

among the first to reveal bacterial community structure in an Indonesian hot spring using NGS, including the most dominant taxa and microbe species with potential application. Results from this study would provide insight into the microbiology of Cangar Hot Springs, including useful and harmful microorganisms. Furthermore, this work would be the first step in building a database of microorganisms of hot springs in Indonesia, as well as would provide a basis for comparison with other hot springs around the world.

## MATERIALS AND METHODS

### Sampling Site and Sample Collection

The Cangar Hot Spring is a recreational hot spring in the Raden Soerjo Forest Park, Batu, East Java, Indonesia, at 112° 32' 0'' E longitude and 7° 44' 31'' S latitude. Samples of water and sediment mix were collected from several locations of the hot spring and mixed in sterile insulated water bottles. Collected samples were immediately transported to the laboratory for further analyses. The measurements of temperature and pH of the samples were done onsite and averaged at  $50.1 \pm 1.2^\circ\text{C}$  and  $6.3 \pm 0.2$ , respectively.

### 16S rRNA gene high-throughput amplicon sequencing

Samples were extracted for their total metagenomic DNA using Cetyltrimethylammonium Bromide (CTAB)-based method (Jiang et al. 2013). Extracted DNA from the samples was diluted with sterile deionized water to 1ng/ $\mu\text{L}$ . The extracted DNA sample was sent to Novogene AIT Genomics, Singapore, for high-throughput amplicon sequencing targeting the V3-V4 conserved regions in the prokaryotic 16S rRNA gene. Polymerase Chain Reaction (PCR) was conducted to amplify the V3-V4 region of 16S rRNA gene using primer 341F (5'-CCTAYGGGRBGCASCAG-3') and 806R (5'-GGACTACNNGGTATCTAAT-3') with Phusion® High-Fidelity PCR Master Mix (New England Biolabs). Sequencing libraries were generated using Ion Plus Fragment Library Kit (Thermo Fisher Scientific) according to manufacturer instructions. The library quality was assessed using Qubit® 2.0 Fluorometer (Thermo Fisher Scientific). Finally, the library was sequenced on an IonS5™ XL system platform (Thermo Fisher Scientific).

### Sequencing data processing

Based on the samples' unique barcode, paired-end reads were assigned, and then the samples were truncated by cutting off the barcode and primer sequences and were merged using FLASH v1.2.7 (Magoč and Salzberg 2011). Next, the filtration of resulting raw tags was done for assuring quality control using QIIME v1.7.0 (Caporaso et al. 2010). Chimera sequence was removed based on the comparison with Gold reference database using UCHIME algorithm (Edgar et al. 2011, Haas et al. 2011). The UPARSE software was used to analyze the obtained effective tags (Edgar 2013). The Operational Taxonomic

Units (OTU) was assigned to effective tags sequences with similarity level  $\geq 97\%$ . Representative sequences for each OTU were then annotated using MOTHUR software against the small ribosomal subunit rRNA SILVA database using a threshold of 0.8-1 (Schloss et al. 2009, Quast et al. 2012). The phylogenetic relationship of different OTUs was analyzed using MUSCLE software v.3.8.31 (Edgar 2004). Finally, the six indices (i.e., observed species, Chao1 estimator, ACE estimator, Shannon Index, Simpson Index, and the Good's coverage) related to alpha diversity were calculated using QIIME v1.7.0 and displayed using R software.

## RESULTS AND DISCUSSION

### Bacterial community structure based on next-generation sequencing analysis

The NGS analysis results revealed Cangar Hot Spring harbors a wealth of different microbial species. There were 539 species observed, with Shannon-Wiener diversity index of 5.642, and species richness estimators Chao1 and ACE of 540.615 and 540.629, respectively (Table 1). Good's coverage of the results was 100%, implying that the sequencing provided adequate classification diversity coverage. The whole Prokaryotic microorganism community structure of Cangar Hot Spring is presented in the Krona graph (Figure 1).

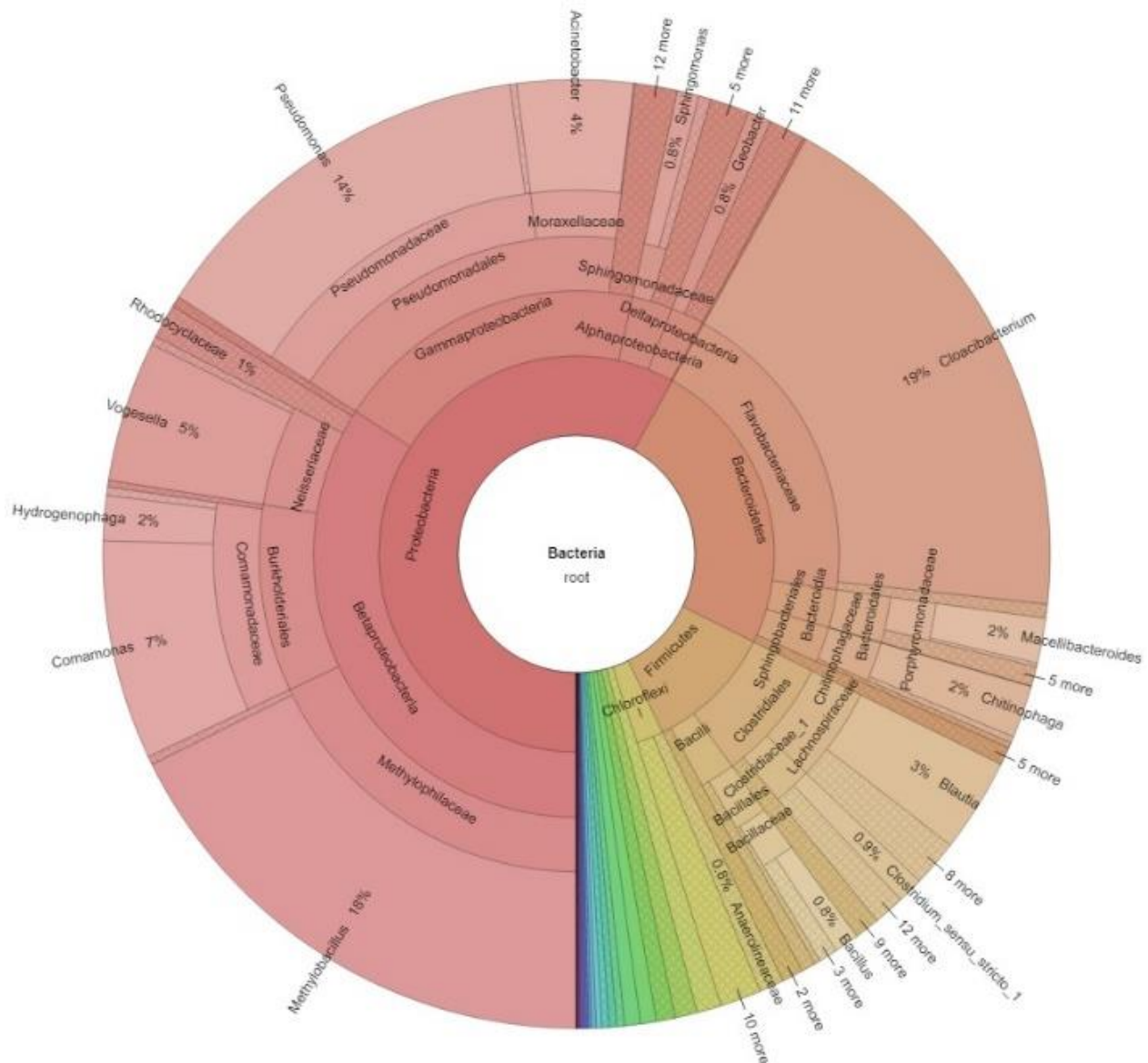
### Taxonomic composition of bacterial communities in Cangar Hot Spring

There were 28 bacterial phyla detected in the sequencing results (Figure 2). The Cangar Hot Spring bacterial community structure at phylum level was dominated by the Gram-negative phyla Proteobacteria (58.05% of total OTUs in the sample), Bacteroidetes (29.58%), and photosynthetic thermophiles Chloroflexi (2.24%). Gram-positive phyla represented mostly by Firmicutes (10.51%) and Actinobacteria (0.75%). Other phyla such as nitrifying Nitrospirae (0.54%), photosynthetic Cyanobacteria (0.24%), and Chlorobi (0.07%), iron-reducing Deferribacteres (0.16%), extremophiles Deinococcus (0.006%) were found less abundant.

The ten most abundant families found in the samples collected were dominated by Gram-negative groups such as Flavobacteriaceae (19.11%), Methylophilaceae (17.97%), and Pseudomonadaceae (13.86%), while Gram-positive only represented by Clostridiaceae 1 (0.19%) (Figure 3).

**Table 1.** Number of observed species diversity index (Shannon-Weiner) and species richness (Chao1, Abundance based coverage estimator (ACE)) estimators in Cangar Hot Spring, Batu, East Java, Indonesia

Observed species	Shannon-Wiener index	Chao1	ACE	Good's coverage estimator
539	5.642	540.615	540.629	100



**Figure 1.** The whole bacterial community distribution in the Cangar Hot Spring, Batu, East Java, Indonesia is represented by the Krona graph. The relative abundance at the level of phylum, class, order, and genus was shown in the graph

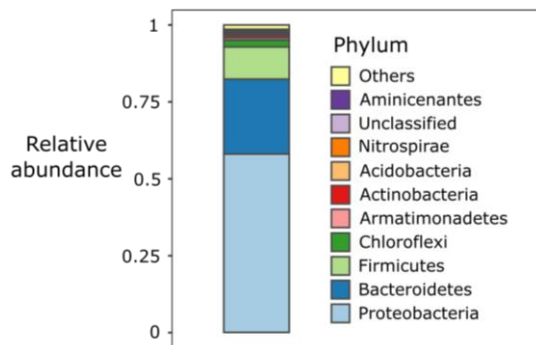
At the genus level, Gram-negative *Cloacibacterium* (18.64%), *Methylobacillus* (17.69%), and *Pseudomonas* (13.64%) were the most dominant genera in Cangar Hot Spring with *Blautia* as the only Gram-positive genus in the top ten most abundant genera (Figure 4). Some identified species were Gram-negative *Comamonas testosteroni* (3.70%), *Comamonas aquatica* (3.54%), *Acinetobacter junii* (2.37%), *Terrimonas* sp. 16-45A (1.79%), *Acinetobacter johnsonii* (0.93%), *Vogesella perlucida* (0.59%), and *Pseudomonas alcaligenes* (0.33%), and Gram-positive *Ruminococcus* sp. 5 1 39BFAA (2.86%).

## Discussion

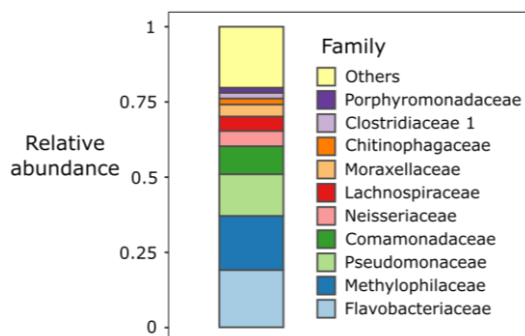
Java, the world's most populous island, is also one area with the most active volcanoes in Indonesia (Daryono et al. 2019, Purnomo and Pichler 2014). Consequently,

there are many geothermal areas on the island, including Arjuno-Welirang Volcanic Complex in East Java, where Cangar Hot Spring is located (Putra et al. 2019). Previous studies (Daud et al. 2019, Martadiastuti et al. 2017) mentioned Cangar Hot Spring has a temperature range between 39.5-53°C, and a pH ranged between 5.2-6.5 (consistent with our measurement results) with bicarbonate rich water.

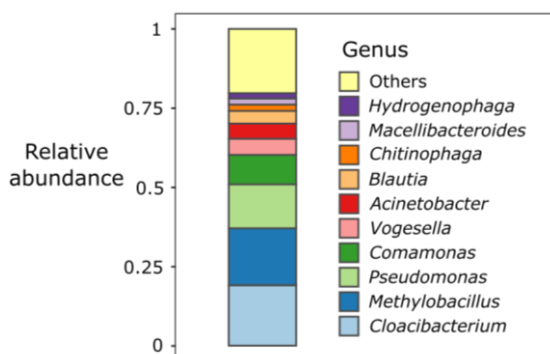
There were already several explorations on useful microorganisms collected from Cangar Hot Spring. However, there was no study on the hot spring's microbial community structure analysis using a more novel method, such as the NGS yet. In this study, NGS was employed to perform 16S rRNA metagenomic analysis for unraveling bacterial diversity in Cangar Hot Spring, especially pathogenic bacteria and bacteria with potential commercial applications



**Figure 2.** Bacterial Operational Taxonomic Unit (OTU) relative abundance at phylum level recovered from the Cangar Hot Spring, Batu, East Java, Indonesia. The total relative abundance of bacterial phyla besides the ten most abundant phyla is represented by "Others". Proteobacteria was the predominant phylum in the sample.



**Figure 3.** The bacterial OTU relative abundance at family level recovered from the Cangar Hot Spring, Batu, East Java, Indonesia. The total relative abundance of bacterial families besides the ten most abundant families is represented by "Others". Flavobacteriaceae and Methylophilaceae were the two most dominant families in the sample.



**Figure 4.** The bacterial OTU relative abundance at genus level recovered from the Cangar Hot Spring, Batu, East Java, Indonesia. The total relative abundance of bacterial genera besides the ten most abundant genera is represented by "Others". *Cloacibacterium* and *Methylobacillus* were the two most dominant genera in the sample.

In this study, the dominant components of the bacterial community structure of Cangar Hot Spring were Gram-negative bacteria, i.e., members of Proteobacteria, Bacteroidetes, and Chloroflexi phyla (>89% OTU). The dominance of Proteobacteria phylum was also reported from similar studies using 16S rRNA analysis in hot springs from a geographically distant area with moderate to high temperatures (37-95°C), such as Ayer Hangat hot spring, Malaysia (45°C, pH 7.1); Coamo hot spring, Puerto Rico (47°C, pH 8.2); Burgas hot spring, Spain (66.3°C, pH 7.6); Hammam Essalihine hot spring, Algeria (70°C, pH 7.3); and Soldhar hot spring, India (95°C) (Adjeroud et al. 2020, Chan et al. 2017, DeCastro et al. 2021, Padilla-Del Valle et al. 2017, Sharma et al. 2017). Meanwhile, the high abundance of Bacteroidetes phylum was observed in Badi Anthoni hot spring, India (55°C, pH 7.8) and Phusang hot spring, Thailand (38°C, pH 7.3) (Bumrunghai et al. 2020, Saxena et al. 2017). Bacteroidetes were less prevalent in hot springs with a temperature above 70°C (i.e., Burgas hot spring, Spain; Soldhar hot spring, India; and Sungai Klah, Malaysia (80°C) (Chan et al. 2015, DeCastro et al. 2021, Sharma et al. 2017).

Family Flavobacteriaceae and its member, genus *Cloacibacterium*, were the most dominant family and genus in Cangar Hot Spring. The high abundance of Flavobacteriaceae family members was also reported in Brandvlei hot spring, South Africa (55°C, pH 6.2), and three hot springs in India, i.e., Yumthang (39°C, pH 8.0), Chhoti Anthoni (52.1°C, pH 7.8), and Polok (76.3°C, pH 7.5), India (Najar et al. 2018, Panda et al. 2016, Saxena et al. 2017, Selvarajan et al. 2018). The presence of Flavobacteriaceae family members was also reported from other hot springs in Indonesia, i.e., Domas hot spring (90°C, pH 2) (Baker et al. 2001). Although the presence of *Cloacibacterium* in hot springs was reported elsewhere (Kashkak et al. 2016, Selim et al. 2017), to our knowledge, our result is the first observation of its abundance in the hot spring environment. Members of this genus were isolated from wastewater and human skin (Allen et al. 2006, Nouha et al. 2016). Indeed, the main human activities around Cangar Hot Spring were tourism and agriculture, and it might explain the sources of *Cloacibacterium* in Cangar Hot Spring.

The second most dominant family and genus found were Methylophilaceae and its member, genus *Methylobacillus*. Family Methylophilaceae is a member of Betaproteobacteria phylum, which includes obligate and facultative methylotrophs Gram-negative rod-shaped bacteria. These bacteria were capable to metabolize C<sub>1</sub> compounds (i.e., compounds containing no carbon-carbon bonds), mainly methanol or methylamine as a sole source of carbon and energy (Yang et al. 2020, Zhang et al. 2020). Methylophilaceae plays a crucial role in the global carbon cycle in aquatic ecosystems, including hot springs. *Methylobacillus* was observed in two hot springs in India, i.e., Sholdar (1.88% of total OTUs) and "Pilgrim Terrace" (1.09%) (Beck et al. 2014, Chistoserdova et al. 2007, Ghosh et al. 2015, Sharma et al. 2017). Our finding is also the first observation of *Methylobacillus* abundance in the hot spring ecosystem.

In previous studies, several species of the genus *Bacillus* with industrial potential were isolated from Cangar Hot Spring (Chrisnasari et al. 2018, Geraldi et al. 2019b, Ibrahim et al. 2013, Rashid et al. 2013). However, this study suggests that the genus *Bacillus* was only representing a minor fraction of the total bacteria collected from the Cangar Hot Spring. Compared to *Bacillus*, there were other potentially useful microorganisms found more abundant in this study. For instance, the second most dominant genus, *Methylobacillus*, is known as a potential platform used in producing recombinant proteins, amino acids, and other value-added compounds due to their high efficiency in carbon conversion and ability to grow in a relatively cheap and abundant carbon source (methanol) (Agafonova et al. 2018, Beck et al. 2014, Si et al. 2016, Yadava and Maitra 2017). Genus *Chitinophaga* (1.79% of total OTUs) is a potential source for chitinase and enzymes related to lignocellulosic material degradation (Kishi et al. 2017, Larsbrink et al. 2017, Sharma et al. 2020). Another potential genus is *Hydrogenophaga* (1.53%), with some species members known for their potential in heavy metal oxidation, polycyclic aromatic hydrocarbons, biodegradation, and bioplastic production activities (Fan et al. 2019, Koller et al. 2019, Reddy et al. 2016). The NGS analysis results also identified other useful species, such as biosurfactants-producer *Acinetobacter junii* and value-added chemicals-producer *Pseudomonas alcaligenes* (Dong et al. 2016, Ohadi et al. 2017, Oliveira et al. 2009, Wang et al. 2020). These useful microorganisms present in Cangar Hot Spring are most likely thermotolerant, which is a beneficial characteristic for their application in bioprocess and bioremediation (Nigam 2013).

Besides beneficial microorganisms, NGS analysis results also revealed pathogenic bacteria in the Cangar Hot Spring. Recreational hot springs are known to harbor pathogens associated with human diseases such as *Clostridium* and *Legionella* strains (Chan et al. 2015, Ghilamicael et al. 2018). Our results suggested the occurrence of opportunistic pathogens causing bacteremia or the presence of bacteria in the human blood, such as *Comamonas testosteroni* (3.70%), *Comamonas aquatica* (3.54%), and *Vogesella perlucida* (0.59%) (Farooq et al. 2017, Farshad et al. 2012, Kaeuffer et al. 2018, Tartar and Tartar 2020, Tiwari and Nanda 2019, Yu et al. 2020). Furthermore, genus *Blautia* (3.21%) and *Ruminococcus* (2.86%), which are related to fecal contamination, were also observed (Devane et al. 2020, Koskey et al. 2014, Staley et al. 2016). The information related to pathogens can develop better health and safety monitoring and risk assessment methods in Cangar Hot Spring.

This study provided insight into the bacterial community structure of the Cangar Hot Spring. The bacterial community composition in the hot spring was dominated by Proteobacteria and Bacteroidetes phyla as other hot springs with moderate temperatures elsewhere. Among others, this study is the first that reported the abundance of genera *Cloacibacterium* and *Methylobacillus*. The presence of useful bacteria with the potential to produce enzymes and value-added chemicals, and to involve in the bioremediation process, was also observed.

Likewise, the occurrence of opportunistic pathogens, which is important for the development of health and safety measures in the hot spring, was also reported. As the first effort on using next-generation sequencing in unraveling the microbial diversity in Indonesian hot springs, we expect this study to encourage further exploration in other hot springs in Indonesia.

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