The Chemical Characteristic and Microbial Diversity of the Hot Spring at Phusang National Park

Sureewan Bumrungthai[1*], Sureewan Duangjit[2], Buntom Somsuwan[3], and Somchai Inpeng[3]

[1]Division of Microbiology and Parasitology, School of Medical Sciences, University of Phayao, Phayao 56000, Thailand
[2]Division of Pharmaceutical Chemistry and Technology, Faculty of Pharmaceutical Sciences, Ubon Ratchathani University, Ubon Ratchathani 34190, Thailand
[3]Phusang National Park, Department of National Parks, Wildlife and Plant Conservation, Phayao 56000, Thailand

ARTICLE INFO

Received: 7 Feb 2019
Received in revised: 29 May 2019
Accepted: 12 Jun 2019
Published online: 26 Aug 2019
DOI: 10.32526/ennrj.18.1.2020.04

Keywords:
Bacterial hot spring/ Sequencing/ Metagenomic/ Phusang warm pool/ Bacterial diversity

*** Corresponding author:**
E-mail:
sureewan.b@windowslive.com

ABSTRACT

The Phusang waterfall is located in Phusang National Park in Phayao Province, Thailand. The robustness of Phusang's warm waterfall is regionally recognized as the only one with a temperature range of 35-36 °C which makes it an outstanding place to visit in Thailand. Surprisingly, Phusang waterfall originates from the Phusang warm pool (hot spring). However, data about the bacterial community and characteristics of this water are still obscure. Therefore, this study investigated the bacterial community and characteristics of water in the Phusang hot spring. Tests to determine its physical characteristics such as pH, color and turbidity were performed. Trace elements such as sodium, bicarbonates, iron, and fluoride were detected as chemical characteristics. The biological properties were verified by 16S ribosomal RNA sequencing. Illumina metagenomic analysis was directly demonstrated from the water after DNA extraction via a membrane filtration pore of 0.45 µm. The range of pH, color and turbidity of water from the Phusang hot spring was 7.33-7.53, 0.05-0.18 Pt.Co and 9.55-10.91 NTU, respectively. The biological study of microorganisms found less than 300 CFU/mL. Coliform bacteria such as *Staphylococcus aureus* and other examples such as *Aeromonas veronii*, *Acinetobacter* sp. *Neisseriaceae bacterium* were abundant. Shotgun metagenomic sequencing defined the phylum as Proteobacteria (84%), Bacteroidetes (13%), Cyanobacteria (1%), and unclassified (2%). Moreover, the amount of sodium and strontium detected was 6.00-7.52 and 1.40-1.58 mg/L respectively. These studies show that a high abundance of Proteobacteria were present in samples from this hot spring. Phusang hot spring has been classified as having low mineral content water.

1. INTRODUCTION

Terrestrial geothermal springs are distributed all over the world. They are associated with bacterial communities under temperature and chemical stress. These bacterial communities were associated with early earth environments (Purcell et al., 2007) including the hot springs in Thailand.

Phusang waterfall or Phusang warm waterfall is located in Phusang National Park, Phayao Province, Thailand (19°39'50.2"N 100°22'35.6"E). Phusang waterfall is regionally recognized as the only one with a water temperature range of 35-36 °C which makes it an outstanding place to visit in Thailand. The Phusang waterfall originates from Phusang warm pool (Phusang hot spring). The warm pool is five meters deep with a temperature range of 35-38 °C. Possibly, the water of the warm pool is derived from both a hot spring and surface water.

Hot springs are found around the world and their waters exhibit specific physical and chemical characteristics, including different pH and more levels of several trace elements than fresh or groundwater. The community of microorganisms in each hot spring depends on pH, temperature, and

Citation: Bumrungthai S, Duangjit S, Somsuwan B, Inpeng S. The chemical characteristic and microbial diversity of the hot spring at Phusang National Park. Environ. Nat. Resour. J. 2020;18(1):33-43. DOI: 10.32526/ennrj.18.1.2020.04

other physicochemical parameters of the geothermal regions (Valeriani et al., 2018; Heni and Julinar, 2015). Thus, the chemical composition, such as a high level of sulfur or hydrogen carbonate, can be associated with the natural selection and involved with the bionetwork (Valeriani et al., 2018). Several factors, including biotic and abiotic pathways in hot springs impact bacteria diversity (Valeriani et al., 2018). One study found that temperature and sulfide have a more detectable effect than any other abiotic variables (Purcell et al., 2007). In the eastern lowlands, the Elegedi hot spring in the Alid volcanic area of Eritrea has a high temperature and increased concentrations of iron and sulfates. Phylum Proteobacteria (6.2-82.3%), Firmicutes (1.6-63.5%), Deinococcus-*Thermus* (0.0-19.2%) and Bacteroidetes (2.7-8.4%) were abundant. These generacorrelate with the temperature, carbonate, iron, sodium and sulfate levels in the water (Ghilamicael et al., 2017). In India, the Bakreshwar hot spring (54 °C) in West Bengal predominantly included the bacterial phylum Firmicutes (65.85%) and Synergistetes (27.24%) and water from the hot spring (65 °C) exhibited the diversity of the phylum Firmicutes (96.10%) and Proteobacteria (3.36%) based on sequencingV3 hypervariable 16S rRNA fragments (Chaudhuri et al., 2017). The bacterial diversity in this hot spring, situated at a high altitude, is dominated by phylum Proteobacteria which has been confirmed in several studies based on 16S rRNA analysis.

Phylum Proteobacteria have been found in other hot springs (Rozanov et al., 2017) including in Thailand (Valeriani et al., 2018) along with other bacteria such as phylum Firmicutes-*Bacillus*, phylum Deinococcus-*Thermus,* etc. (Purcell et al., 2007; Kanasawud et al, 1992). In nine districts with hot spring in northern Thailand, phylum Cyanobacteria-*Synechococcus lividus* and *Synechococcus* sp. (40-80 °C) and *Phormidium boryanu*m (30-60 °C) dominated (Udomluk et al., 2005). The Bor Khlueng hot spring (Ratchaburi province, Thailand) (50-57 °C) was characterized as having phylum Acidobacteria (23%), Bacteriodetes (19%), Nitrospirae (13%), Proteobacteria (12%), Deinococcus-*Thermus* (11%) by PCR using 16S rRNA amplified and restriction fragment length polymorphism (RFLP) analysis (Kanokratana et al., 2004). From a hot spring in northern Thailand, the phylum Thermotogae-*Thermotoga* sp. strain PD524 was isolated (70-85 °C, pH 6.0-8.5, NaCl 0-10 g/L) (Kanoksilapatham et al., 2015). In a hot spring pond in the Krabi province (Thailand), the genus *Planosporangium*, a novel filamentous bacterial strain, HSS8-18 (T), was isolated from the soil (Thawai et al., 2013). Infections in humans may be caused by waterborne opportunistic pathogens hidden in a hot spring (Jardine et al., 2017). Because hot springs are common tourist attractions, authorities should be attentive to probable risks and provide measures and guidelines to ensure safety without causing undue alarm to tourists (Sukthana et al., 2005). *Escherichia coli* or *Enterococcus* are the main thermotolerant bacteria which are an indicator of the water quality. Nevertheless, the majority of environmental bacteria are unculturable. Consequently, culture methods may not be able to detect all pathogens. Illumina sequencing can be more comprehensive. A precise evaluation of ecological bacterial pathogens can be applied to improve risk valuation methods and promote public health awareness (Ghilamicael et al., 2018).

Thermophilic Actinobacteria can produce several enzymes including amylases, DNA polymerases, pullulanases, proteases, lipases and xylanases (Valeriani et al., 2018). Other bacteria such as *Bacillus licheniformis*, potential producers of thermostable enzymes such as amylase, protease, and cellulase, might be beneficial in industrial applications (Mohammad et al., 2017; Ibrahim et al., 2013). One of the key metabolites (violacein, purple pigment) of *Chromobacterium* may affect gastroente-rological diseases e.g. colorectal cancer, inflammatory gastric lesions (Giselle and Nelson, 2017), and also inhibit *Bacillus* sp. growth, Plasmodium growth (Stefanie et al., 2009) with anti-MRSA and anti-fungal properties (Mahajan and Balachandran, 2017). *Thermus*, a genus found in Thailand produced an extracellular protease (Kanasawud et al., 1992). In the Tao Dam hot spring, the TD1 bacterial strain may be a good candidate as a pectate lyase producer (Yasawong et al., 2011). A neopullulanase-like enzyme from Bor Khleung hot spring (Tang et al., 2008) and lipolytic enzymes potential can be used in industrial applications (Tirawongsaroj et al., 2008). However, the microbial communities of hot springs in Thailand have been poorly studied. In particular, the data from the hot spring of Phusang warm pool has not been studied until now.

Therefore, this study investigated the bacterial community and the characteristics of the water from Phusang hot spring. Tests to determine its physical characteristics, such as pH, color and turbidity, were

performed. Trace elements such as potassium, sodium, chlorides, bicarbonates, calcium, sulfates, strontium, iron, fluoride and etc. were detected as chemical characteristics. Biological properties were identified using a bacterial culture in nutrient agar and sequencing by 16S ribosomal RNA (16S rRNA). Illumina metagenomic sequencing analysis was performed with directly extracted DNA from warm water trapped onto membranes with a filtration pore size of 0.45 μm.

2. METHODOLOGY

2.1 Sampling methods

Water samples were collected at three different periods (Season) in a year (June 2017 to May 2018) from Phusang hot spring (35-38 ºC) located in Phusang National Park, Phayao Province, Thailand (19°39'50.2"N 100°22'35.6"E). The characteristics such as pH, color (Pt. Co), turbidity, and trace elements (such as bicarbonate, iron (Fe), fluoride (F), sulfate (SO_4), sodium (Na), potassium (K), zinc (Zn), calcium (Ca), chloride (Cl), strontium (Sr)), total hardness (as $CaCO_3$), and total dissolved solids (TDS) of water samples were analyzed twice using inductively coupled plasma (ICP, Perkin Elmer, USA) and standard methods for examination of water following the recommend-dations of the American Public Health Association (APHA) and the American Water Works Association (AWWA). For sample analysis of chemical characteristics, eight liters (L) of water were obtained under the surface of the pool near the water inlet and stored on ice. After that, the samples were placed in sterile glass containers and stored on ice for future microorganisms analysis.

2.2 DPPH assay

Three water samples of 50 mL were collected and then filtered using a 0.45 μm filter membrane. A stock solution including 1, 1-Diphenyl-2-picryl-hydrazyl (DPPH) (Sigma-Aldrich USA) was prepared in methanol and methanol/buffer with an acetic acid buffer (0.1 M, pH 5.5). The methanol/buffer was prepared by mixing 40 mL of 0.1 M acetate buffer (pH 5.5) with 60 mL methanol. The reaction tubes were kept at 30 °C for 30 min in the dark wrapped in aluminum foil (at least 5 layers). In the dim light, spectrophotometric measurements were done at 517 nm using a Spectronic Genesys 5 spectrophotometer. The data are mean ±SD. Ascorbic acid and butylated hydroxytoluene (BHT) (E. Merck, India), and propylgallate (Sisco Research Laboratories, Mumbai, India) were used.

2.3 Bacterial isolation

To isolate the bacteria, 100 μL of water was spread onto nutrient agar (NA) plates in triplicates (NA, Hi-Media Laboratories Pvt, Ltd., Mumbai, India). NA plates were incubated at 37 °C for 24 h and the total colonies were counted as CFU/mL. The growth colonies were re-streaked on NA plates and a single colony was transferred into Luria-Bertani (LB, HiMedia Laboratories Pvt, Ltd., Mumbai, India) broth for DNA extraction.

2.4 DNA extraction

Bacterial pellets or a 0.45 μm filter membrane were used for total DNA extraction. The cell lysis solution (Tris-HCl 10 mM, pH 7.8; EDTA 5 mM; SDS 0.5%; lysozyme) and proteinase K 50 μL (stock 20 mg/mL) were added and incubated at 65 °C for 1 h. 5 M potassium acetate (400 μL) was used for protein precipitation and then centrifuged at 12,000xg for 10 min at 4 °C. The supernatant was collected and an equal volume of phenol: chloroform: isoamyl (25: 24:1) was added, then centrifugation at 12,000xg for 10 min at 4 °C was done. Isopropanol was used for DNA precipitation from an aqueous phase by centrifugation at 12,000xg for 10 min at 4 °C and washed with 70% ethanol. DNA was rehydrated in TE buffer (10 mM Tris [pH 7.8] and 1 mM EDTA) and stored at -20 °C until analysis by agarose gel electrophoresis was performed. The DNA quality was checked via staining with Redsafe™ (iNtRON Biotechnology, Inc., Burlington, MA, USA) and examined using a gel documentation system (Bio-Rad, Hercules, California, USA).

2.5 Polymerase chain reaction (PCR) amplify-cation and sequencing

DNA extracted from the bacterial cells was subjected to PCR. Then 27F/1492R universal primers were used for PCR amplification (16S rRNA genes). OnePCR™ plus a master mix (GeneDireX, Las Vegas, Nevada, USA), 0.4 pmol primer pair 27F/ 1492R, 100-200 ng DNA template and Dnase/Rnase free water to 25 μL. The PCRs conditions were pre-denaturation at 96 °C for 5 min and 35 cycles of denaturation at 96 °C for 45 s, annealing at 58 °C for 45 s, extension at 72 °C for 2 min and a final extension at 72 °C for 7 min. The PCR product size was close to 1500 bps on 1.5%

agarose gel electrophoresis. DNA sequencing was used for species identification. PCR was done before sequencing by 27F/1492R primers. The result of 16S rRNA sequence was done using NCBI's (BLASTN).

2.6 Metagenomics

Shotgun metagenomic sequencing was used to analyze the bacterial biodiversity of the hot spring water. We filtered 20 L of water using a 0.45 µm filter membrane. The bacterial pellet was eluted by a PBS buffer. Phenol-chloroform was used to extract DNA followed the 2.4 DNA extraction process. The extracted DNA might represent all bacterial cells from the water sample. A high-quality and sufficient amount of DNA must be acquired for ensuing library production and sequencing. Fraction steps were checked by Agilent 2100 and Q-PCR to certify that enough targeted DNA existed. This was a range of end repairing. For tailing construction, sequencing adapters were ligated, and enrichment steps of PCR were used to produce a library. Agarose electrophoresis was used for DNA purity and integrity. The sequencing was performed using the Illumina platform after library clustering with paired-end reads. Phylum abundance data by shotgun metagenomics sequencing were based on a 16S rRNA gene sequence and included phylum data of other functional genes.

3. RESULTS AND DISCUSSION

The present study provides the physical, chemical and biological characteristics of water sampling from Phusang hot spring. An environmental valuation of this hot spring was significantly influenced by risk assessment methods and the promotion of public health awareness for both the government and tourists. These intrinsic characteristics of water from Phusang hot spring may be the variables that influence the number of tourists and visitors in this location. An examination of the pH, color, and turbidity was performed to identify the physical characteristics of the water from Phusang hot spring. The temperature of the Phusang hot spring varied due to the activity of the springs, ranging from 35-38 °C (mean 36±1.5 °C). The pH of spring water was in a range of 7.2-7.5 (mean 7.3±0.1). The color and turbidity were 0.05-0.18 Pt.Co and 9.55-10.91 NTU, respectively (Table 1).

Moreover, our results suggested that the water from the Phusang hot spring has no effect on an antioxidant when compared to vitamin E (Figure 1).

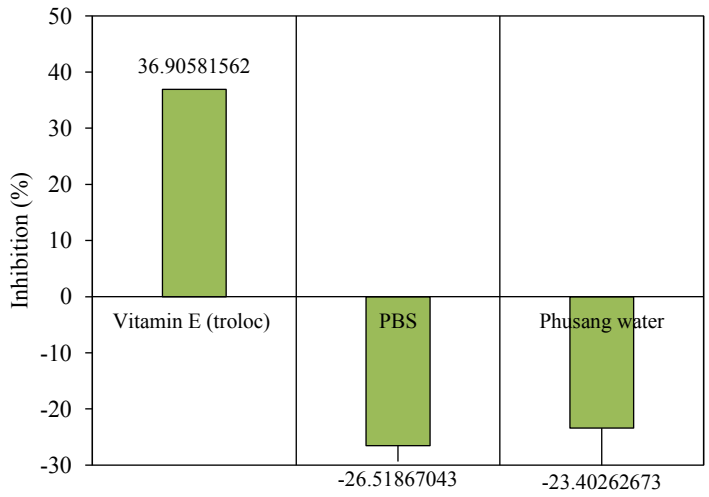

Figure 1. Antioxidant activity by DPPH assay of Phusang springs water

The total amount of solids found were <500 mg/L. Calcium, sodium, and strontium were found at 147.40, 6.00-7.52, and 1.40-1.58 mg/L, respectively. The other trace elements identified included potassium, bicarbonates, sulfates, iron, fluoride, etc. as shown in Table 1. These results indicated that Phusang hot spring should be classified as low mineral content water (Quattrini et al., 2017). The sodium is <20 mg/L and this is indicated as a low sodium definition (Quattrini et al., 2017).

The total bacterial counts of the water samples from Phusang warm water were observed on the NA medium by spread plate technique. After 20 replicates in the three periods of one year were counted, a majority of the samples showed a mean of $2.5\pm1.7\times10^2$ CFU/mL (Table 2). *Staphylococcus*

aureus and other bacteria such as *Acinetobacter* sp., *Aeromonas veronii*, *Bacillus licheniformis*, *Chromobacterium* sp., *Enterobacter* sp., *Staphylococcus* spp, *Pseudogulbenkiania* sp. were found but *Salmonella* spp. and *Clostridium perfringens* were not found by PCR and sequencing (Table 3).

Shotgun metagenomic sequencing showed these phylum abundance results: bacteria 93% of the amplicon library, unknown 7%, virus 0.02%, Archea 0.02% and Eukaryota 0.02%.

Based on these total sequences read numbers, this study found phylum Proteobacteria (84%), Bacteriodetes (13%), Cyanobacteria (1%), and unclassified (2%). The phylum Proteobacteria included, 71% from class Gammaproteobacteria, 22% from class Betaproteobacteria, 4% from class Alphaproteobacteria, 0.3% from class Deltaproteobacteria. As for the top 10 phyla found via the analysis, the most common was phylum Proteobacteria, followed by Bacteriodetes, Cyanobacterium, Firmicutes, Actinobacteria, Chloroflexi, Verrucomicrobia, Planctomycetes, Acidobacteria and Euryarchaeota. Moreover, the top ten families, genus and species are shown in Figures 2-4.

Table 1. The water turbidity, color, pH and trace elements analysis

Category	Composition (mg/L or ppm)	Unit	Phusang
Physical	Color	Pt.Co	0.05-0.18
	Turbidity	NTU	9.55-10.91
	pH	-	7.33-7.53
Chemical	Total Solids	mg/L	392.5-400
	Total Hardness (as $CaCO_3$)	mg/L	320-330
	Chloride	mg/L	6.59-7.95
	Iron	mg/L	0.75-0.94
	Strontium	mg/L	1.40-1.58
	Sulfate	mg/L	<0.0-<5.0
	Zinc	mg/L	Not-detected-<0.01
	Fluoride	mg/L	0.95-1.16
	Bicarbonate	mg/L	336.60-341.70
	Calcium	mg/L	147.40
	Potassium	mg/L	1.41-4.11
	Sodium	mg/L	7.52-8.90
Microbiological	*E. coli*	MPN/100 mL	Not-detected-<1.1
	S. aureus		Not-detected-Detected
	Salmonella spp.		Not-detected
	Cl. perfringens		Not-detected

Table 2. Total bacterial counts using a spread plate technique

Months	No. of CFU $\times 10^2$/mL								CFU/mL)mean±SD)
	1	2	3	4	5	6	7	8	
April	4.5	2.1	1.2	1.2	0.6	0.8	2.7	3.1	$2.0\times10^2\pm1.3\times10^2$
August	4.2	1.4	4.0	2.5	1.3	1.8	-	-	$1.9\times10^2\pm1.3\times10^2$
December	6.6	2.8	5.8	2.2	3.2	2.0	-	-	$3.8\times10^2\pm1.9\times10^2$
Mean									$2.5\times10^2\pm1.7\times10^2$

*CFU/mL =colony forming unit/mL

Table 3. 16S rRNA gene sequences analysis of bacteria isolated from the Phusang hot spring.

April	August	December
Bacillus mojavensis	*Acinetobacter* sp.	*Acinetobacter* sp.
Bacillus halotolerans	*Aeromonas jandaei*	*Aeromonas veronii*

Table 3. 16S rRNA gene sequences analysis of bacteria isolated from the Phusang hot spring (cont.).

April	August	December
Bacillus licheniformis	*Aeromonas* sp.	*Chromobacterium* sp.
Bacillus mojavensis	*Aeromonas veronii*	*Neisseriaceae bacterium*
Bacillus paramycoides	*Aquaphilus dolomiae*	*Pseudogulbenkiania* sp.
Bacillus safensis	*Bacillus amyloliquefaciens*	
Bacillus tropicus	*Brevibacillus agri*	
Staphylococcus cohnii	*Brevibacillus* sp.	
Staphylococcus epidermidis	*Cellulomonas hominis*	
Staphylococcus warneri	*Chromobacterium rhizoryzae*	
Staphylococcus aureus	*Enterobacter cloacae*	
Micrococcus luteus	*Enterobacter* sp.	
	Escherichia coli	
	Flavobacterium sp.	
	Microbacterium sp.	
	Pseudogulbenkiania sp.	
	Rheinheimera sp.	
	Sphingomonas zeae	
	Staphylococcus arlettae	
	Staphylococcus epidermidis	
	Staphylococcus pasteuri	
	Staphylococcus sp.	
	Terrimonas sp.	
	Uncultured *Asticcacaulis* sp.	
	Uncultured *bacterium*	
	Uncultured *Xylella*	

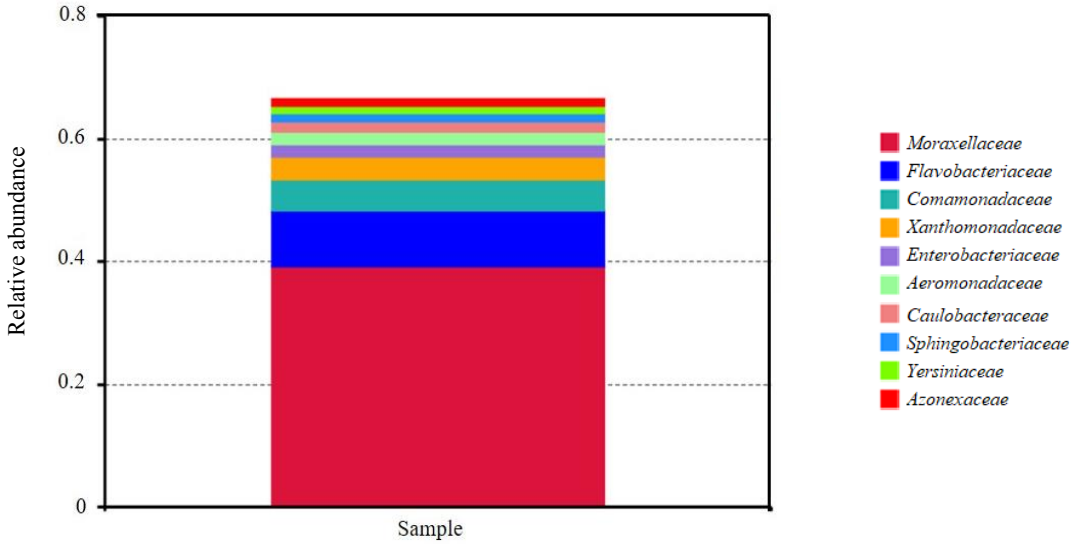

Figure 2. Top ten families found by shotgun sequencing

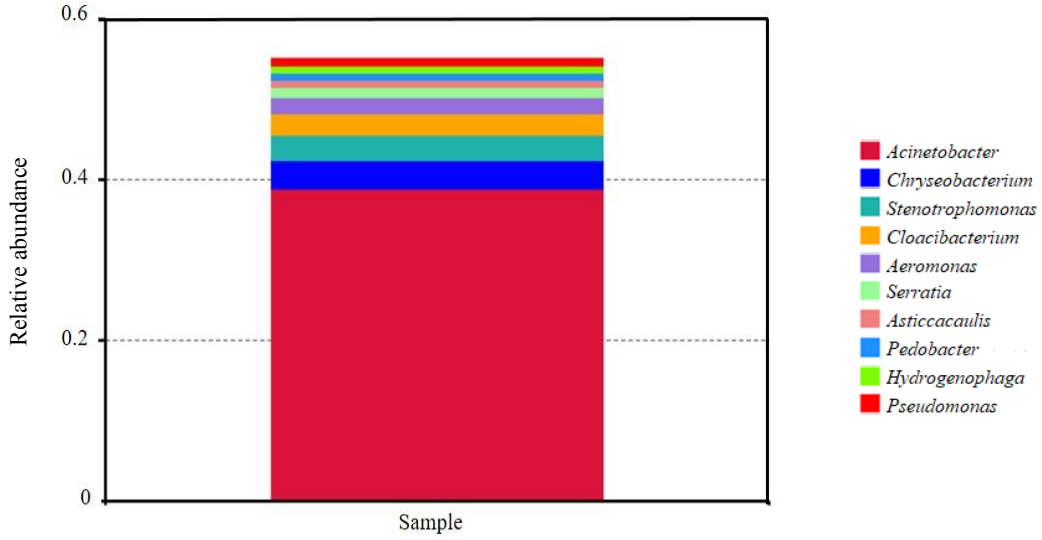

Figure 3. Top ten genus found by shotgun sequencing

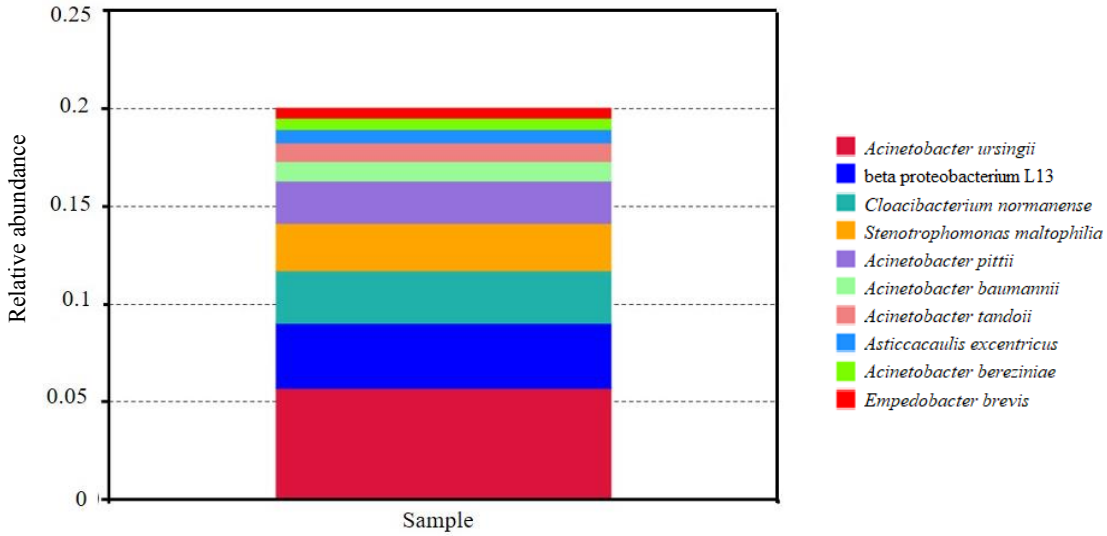

Figure 4. Top ten species found by shotgun sequencing

In Figure 5, an antibiotic mixture of two or more antibiotics is labeled in the form of an acronym. The relative abundance in unit ppm is the result of magnifying 10^6 times of the original abundance data. For antibiotic analysis, the most common was bacitracin, followed by CA, tetracycline, and sulfonamide as shown in Figure 5.

The water from Phusang hot spring ranged from neutral pH to alkaline pH. The water samples showed no evidence of antioxidants when compared to vitamin E. The Phusang water could be applied for use as a commercial cosmetic product such as a moisturizer or mineral aqua for skin care with no antioxidant claim.

The low amount of sodium found in the water (<20 mg/L) might be useful for drinking in cases of hypertension (Quattrini et al., 2017; Ha, 2014). Reducing dietary salt consumption to decrease deaths ratio from hypertension, cardiovascular disease and stroke has been recommended by the World Health Organization (WHO) (Ha, 2014). Tap water exhibited a large range of strontium concentration (0-1.9 mg/L) in the United States (US) (Lesley et al., 2012). Strontium was detected in 650 different European natural water samples (<0.0002-22.8 mg/L) (Voerkelius et al., 2010).

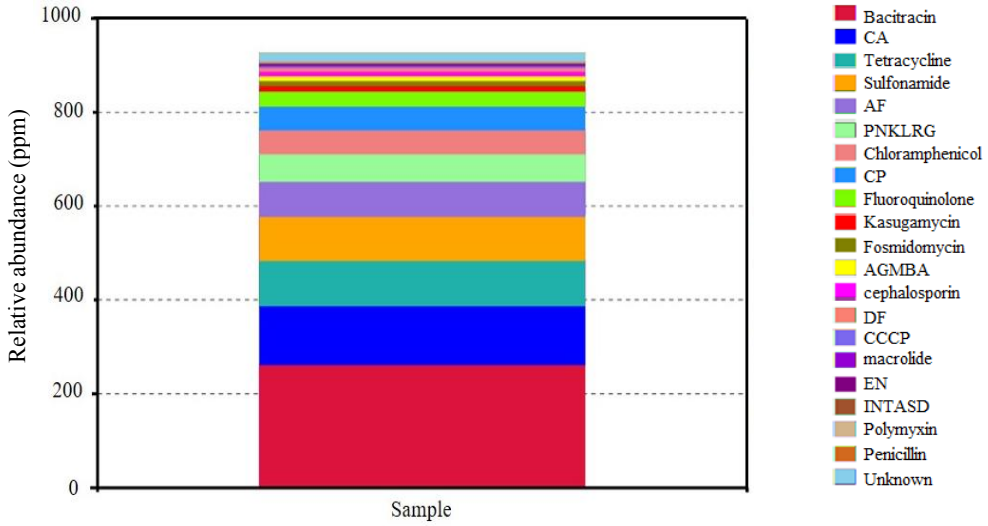

Figure 5. The abundance of the antibiotics in each sample

In this study, the amount of strontium may be beneficial to the elderly but not to children. Risk of osteoporotic vertebral and nonvertebral fractures might be reduced by strontium, seemingly through a decrease of resorption and an increase of bone formation (Voerkelius et al., 2010; Meunier et al., 2004; Reginster et al., 2005; Seeman et al., 2006; Meunier et al., 2009; Roux, 2008). A smell of sulfur is not present in Phusang hot spring according to the result of this study (<5.0 mg/L).

Phylum Proteobacteria and Chloroflexi were most commonly found in samples from Tato Field, Tatta Pani, and Murtazaabad according to this study (Ghilamicael et al., 2017; Chaudhuri et al., 2017; Amin et al., 2017). In the Murtazaabad hot springs (90-95 °C), phylum Thermotogae was favored with the higher temperature, whereas the phylum Proteobacteria has favored growth in the Tatta Pani thermal spring (60 °C) (Amin et al., 2017). The Bakreshwar hot springs (65 °C) showed an abundance of the phylum Firmicutes (96.10%) and Proteobacteria (3.36%) (Chaudhuri et al., 2017). The Bor Khlueng hot spring (Ratchaburi Province, Thailand), contains sulfide-rich mineral water (50-57 °C) and Proteobacteria was found at levels of 12% (Kanokratana et al., 2004). Phylum Chloroflexi were dominant at locations with low silica and high temperature (Amin et al., 2017). Therefore, the community structures are dependent upon temperature (Purcell et al., 2007; Amin et al., 2017) and sulfide rather than by any other abiotic (Valeriani et al., 2018; Purcell et al., 2007). This study supports the idea that temperature has an effect on predominately Proteobacteria in low-temperature hot springs such as the Phusang hot spring. The presence of domain Archaea (Chaudhuri et al., 2017) in the hot springs was confirmed at 0.02% including *Halorubrum kocurii*, *Haloferax natronorubrum*, *Halorubrum aethiopicum*. Similarly, a study of the Bor Khlueng hot spring (Ratchaburi Province, Thailand) found Crenarahaeota (Kanokratana et al., 2004). A significant correlation with abundant salinity occurring was found between Halobacteria, Actinobacteria and Cyanobacteria in Garbanabra and Gelti (Ghilamicael et al., 2017). The occurrence of some Cyanobacteria was highly dependent on temperature (Strunecky et al., 2018). Therefore, this study could be a biological basis to confirm that the Phusang warm pool was derived from hot spring water.

This study found 1% of the bacteria could not be unassigned an identity using shotgun sequencing, suggesting many undiscovered and unexplored microbiota were present. The novel insights of nature ecology interactions and bacterial communities that were found in this study might help in determining future study courses in these locations (Amin et al., 2017). In Bor Khlueng (Thailand), the majority of the detected prokaryotic sequences were unknown (approximately 80%) (Kanokratana et al., 2004). For example, Kanoksilapatham et al. (2015) studied a hot spring in northern Thailand where a strain of hyperthermophilic *Thermotoga* sp. (strain PD524) was isolated. Some studies at a hot spring pond in the Krabi Province (Thailand) found a novel filamentous bacterial strain HSS8-18 (T) genus *Planosporangium* that was isolated from the soil (Thawai et al., 2013) and a strain CC-KL-3^{T} showed the highest sequence

of *Hydrogenophaga bisanensis*. This is similar to the result of the current study that found *Hydrogenophaga* sp. T4, *Hydrogenophaga flava*, *Hydrogenophaga taeniospiralis*, etc. and unassigned *Hydrogenophaga* that need to be evaluated in a further study (Lin et al., 2017).

The family Moraxellaceae (genus *Acinetobacter*) constitutes 42% of the total bacteria. Family Enterobacteriaceae (genus *Enterobacter* 0.5%, *Klebsiella* 0.5%, *Shigella* 0.09%, *Salmonella* 0.09%, *Escherichia* 0.08%, *Cronobacter* 0.08%) constitutes 2% of total bacteria. Family Yersiniaceae (genus *Serratia*) constitutes 1% of the total bacteria. In hot springs, human pathogens can survive and grow. The main thermotolerant bacteria frequently used to estimate a load of pathogenic bacteria in water are *Escherichia coli* or *Enterococcus*. (Thorolfsdottir and Marteinsson, 2013; Signorelli et al., 2006; McClung et al., 2017; Rebellon et al., 2018). *Legionella* was a pathogen found in samples from 57.8% (48/83 samples) (Viroj, 2005) of the studied places at natural hot springs in northern, southern eastern and central Thailand. This study found *Listeria monocytogenes* (0.002%). Because there can be possible risks of exposure to hazardous pollutants in public waters, and since spas and natural springs are popular tourist attractions, authorities should be aware of possible hazards and provide tactful measures and guidelines to ensure safety without causing undue alarm to tourists (Sukthna et al., 2005; Rebellon et al., 2018; Hayasaka et al., 2018). This location should be managed in a way that is similar to the practice near the Khao-Than hot spring, Surat Thani Province, southern Thailand, that found high natural radium radiation (Bhongsuwan and Auisui, 2015). Therefore, this study suggests that the water from Phusang must be treated before use.

Emerging waterborne opportunistic pathogens that can cause infections in humans may harbor in hot spring water. This study investigated the diversity and antimicrobial resistance of emerging and opportunistic bacterial pathogens. Isolates were found to have resistance to ten antibiotics (bacitracin, CA, tetracycline, sulfonamide, AF, PNKLRG, chloramphenicol, CP, fluoroquinolone, kasugamycin etc.). This study suggests that a potential reservoir for emerging opportunistic pathogens are hot springs. Multiple antibiotic-resistant strains highlight the occurrence of unknown populations of emerging and possible waterborne opportunistic pathogens in the environment (Jardine et al., 2017).

Moreover, the results suggest that Actinobacteria might be producing antimicrobial molecules and biopharmaceuticals more than other bacteria. Thermophilic Actinobacteria was a producer of several enzymes such as DNA polymerases, amylases, xylanases, lipases and proteases (Valeriani et al., 2018; Ghilamicael et al., 2017) or lipolytic enzymes from Jae Sawn hot spring (Thailand), which lead to the isolation of a novel esterase (Est1) and patatin-like phospholipase (PLP) with potential use in industrial applications (Tirawongsaroj et al., 2008). This study found *Bacillus licheniformis* is a potential producer of a thermostable enzyme such as amylase, cellulose, gelatins, lecithin, and protease. This supports future studies to explore further environmental and industrial applications (Mohammad et al., 2017; Ibrahim et al., 2013). Their quick reaction time, thermostability, and decreased risk of contamination are an adeptness of thermophilic microorganisms and their enzymes. Thus, a good source for isolating efficient biomass degrading thermophiles and thermozymes was a hot spring (Lee et al., 2018).

Moreover, *Chromobacterium* was found in this study and further study of it might evaluate the function of this metabolite to inhibit cancer and other bacterial growth (Giselle and Nelson, 2017; Stefanie et al., 2009; Mahajan and Balachandran, 2017).

4. CONCLUSION

These studies conclude that a high abundance of Proteobacteria and Chloroflexi exist in samples from the hot springs in the Phusang zone was present. The most common was Family Moraxellaceae, followed by Enterobacteriaceae and Yersiniaceae. Phusang hot spring was classified as having low mineral content water. Strontium and low sodium might be beneficial to the elderly and hypertension patients, respectively.

ACKNOWLEDGMENT

We thank the Thalassemia Unit, University of Phayao for use of the PCR machine and gel documentation system. Department of National Parks, Wildlife and Plant Conservation. Phusang National Park. Assist. Prof. Dr. Bhundit Innawong, Department of Food Technology, Faculty of Engineering and Industrial Technology, Silpakorn University for idea thinking. Chamnan Sangkeo (Director) and the Adventure team, University of Phayao. Student from Special problem class (2016-

2018), University of Phayao for preparing materials, spread plate and DNA extraction. This work was supported by funding grant no. RDG59A0013-02-03 from Area-Based Collaborative Research (ABC)/ University of Phayao; grant no. 601007 (2017) from the School of Medical Science, University of Phayao; and grant no. RD62057 from University of Phayao.

CONFLICT OF INTEREST

The authors declare no conflict of interest associated with this manuscript.

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