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Abstract

The hot spring Vranjska Banja is the hottest spring on the Balkan Peninsula with a water temperature of 63-95°C and a pH value of 7.1, *in situ*. According to the physicochemical analysis, Vranjska Banja hot spring belongs to the bicarbonated and sulfated hyperthermal waters. The

structures of microbial community of this geothermal spring are still largely unexplored. In order to determine and monitor the diversity of microbiota of the Vranjska Banja hot spring, a comprehensive culture-independent metagenomic analysis was conducted in parallel with a culture-dependent approach for the first time. Microbial profiling using amplicon sequencing analysis revealed the presence of phylogenetically novel taxa, ranging from species to phyla. Cultivation-based methods resulted in the isolation of 17 strains belonging to the genera *Anoxybacillus*, *Bacillus*, *Geobacillus* and *Hydrogenophyllus*. Whole genome sequencing of five representative strains was then performed. The genomic characterization and OrthoANI analysis revealed that the Vranjska Banja hot spring harbors phylogenetically novel species of the genus *Anoxybacillus*, proving its uniqueness. Moreover, these isolates contain stress response genes that enable them to survive in the harsh conditions of the hot springs. The results of the *in silico* analysis show that most of the sequenced strains have the potential to produce thermostable enzymes (proteases, lipases, amylases, phytase, chitinase, and glucanase) and various antimicrobial molecules that can be of great importance for industrial, agricultural, and biotechnological applications. Finally, this study provides a basic for further research and understanding of the metabolic potential of these microorganisms.

Keywords: 16S rRNA Metagenomic analysis, Genome analysis, Thermophilic bacterial diversity, Hot spring, Enzymatic potential, Antimicrobial molecules

Introduction

Hot springs, unique and rare natural habitats present all over the world, characterized by specific geothermal and physicochemical characteristics such as temperature, water composition, pH, and redox potential [1,2]. Thermal springs, due to their extreme conditions, host a unique microbial community, with the potential phylogenetic novel microorganisms that have scientific and biotechnological significance [3]. It is estimated that only a small percentage, around 1 to 10%, of the total population in any biosphere is cultivable, including those from hot springs. To overcome the limitations of culture-dependent approaches, techniques relying on direct amplification of nearly complete sequences of the 16S rRNA gene from bulk genomes are being employed [3,4]. Culture-independent metagenomic approach offer a promising strategy to assess the phylogenetic composition, genetic diversity and functional potential of microbial communities in extreme environments [5].

Thermophilic microorganisms inhabiting hot areas can be classified into three categories: moderate thermophiles, extreme thermophiles, and hyperthermophiles with optimum growth temperatures of 50–60°C, 60–80°C, and 80–110°C, respectively [6]. These microorganisms produce biologically active compounds and enzymes, which have proven useful in various biotechnological applications.

The phenomenon of climate change is recognized as a global threat, prompting countries to adopt regulations that reinforce industrial environmental responsibilities and necessitate changes in manufacturing and consumption practices. Currently, enzymes of mesophilic origin dominate the market, but they often exhibit limited activity in the harsh conditions prevalent in industrial processes. In contrast, thermophilic enzymes are more robust, heat-tolerant, often exhibit tolerance to extreme pH, high pressure and protein-denaturing solvents-conditions commonly found in industrial settings. The utilization of enzymes, such as amylases, cellulases, chitinases, pectinases, xylanases, proteases, lipases and DNA polymerases, from thermophilic microorganisms (e.g.

Geobacillus, *Anoxybacillus*, *Thermococcus*), as biocatalysts has the potential to drive the transition from traditional to environmentally friendly "green" industrial processes [7]. One of the most significant advances in science related to the exploitation of thermostable enzymes, is the discovery of thermophilic bacterium *Thermus aquaticus* several decades ago in the hot springs of Yellowstone National Park [8]. The use of its *Taq* polymerase in the development of polymerase chain reaction (PCR) technique has revolutionized molecular biology and various scientific fields [9].

On the territory of Serbia, there are 240 geothermal springs with 60 spas [10]. The hottest springs are located in Vranjska Banja (southeastern Serbia, where the water temperature ranges from 63 to 95°C. Alongside Icelandic springs, the geothermal springs of Vranjska Banja are considered the hottest thermal springs in Europe. Vranjska Banja is located 10 km northeast of the town of Vranje and has several sources of hyperthermal water within a zone of few hundred square meters, with some reaching the temperature of even 124°C [11]. These geothermal springs of Vranjska Banja belong to magmatic waters, and are located within the zone of the Serbian-Macedonian massif and the region of the Serbian Crystalline Core. The extreme temperature of hot springs can be attributed to their location in a tectonically active zone (on the west edge in the Rhodopean Masses), characterized by young volcanism and large rafts [12].

To the best of our knowledge this study represents the first attempt to provide a comprehensive understanding of the microbial life inhabiting the hottest spring on the Balkan Peninsula, Vranjska Banja, by combining metagenomic analysis and culture-depended methods. Additionally, the relationship between microbial communities and the physicochemical parameters of thermal spring was investigated. Furthermore, the bacterial isolates were characterized, and whole-genome sequencing of the selected cultivable strains, including some novel bacterial species, was performed.

Material and methods

Sampling and Sampling sites

For the purposes of this study, water samples were collected from the "Turkish bath" cap of Vranjska Banja springs (Fig. 1), which is located at an altitude of 437 m (42°32'42"N 22°00'29"E). The water was obtained from spring A1, which is located at a depth of 25 m with a water temperature reaching up to 91°C and a capacity of 2.5 L/s [12].

In July 2020, water samples for chemical, microbiological and genetic analysis were collected using a sterile ladle and transferred into sterile 2L Erlenmeyer flask. To ensure a comprehensive representation of the overall heterogeneity, samples were taken from several points within the water source and pooled together. The collected water samples were promptly transported to the laboratory for isolation of total microbiome DNA and bacterial isolation/cultivation.

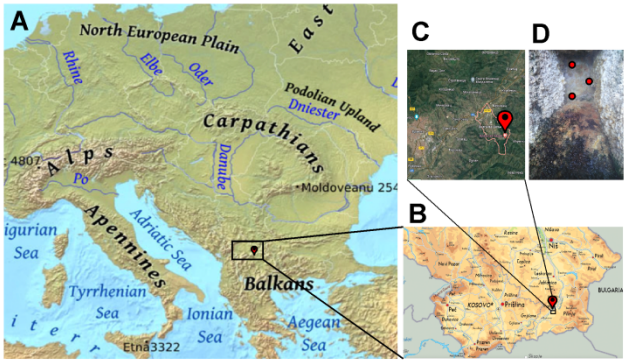

Fig. 1 Geographical location (A-C) and image of the sampling site in Vranjska Banja hot spring (D). The red location pin icon indicates the position of Vranjska Banja hot spring on the maps of Southeastern Europe and the Balkans (A), southern Serbia (B) and the Municipality of Vranje (C). The red dots on the source indicate the places from where the water samples were taken

Isolation of bacterial strains from the water of thermal spring

The isolation of bacteria was performed by the membrane filtration method on sterile filter disk membranes (Pall Europe, Hampshire, United Kingdom), with a pore diameter of 0.2 µm. The bacteria cultured on Nutrient agar (Biomèrieux, Combourg, France), prepared with filtrates from the investigated source with the addition of twice the amount of agar powder (30 g/L, Torlak, Belgrade, Serbia), were grown at a temperature of 60°C (chosen as the most suitable for isolating both non-spore and endospore-forming thermophilic bacteria). Colonies that emerged after 24 and 48 h (a total of 17 colonies) were purified using the streaking method and stored at -80°C in nutrient broth containing 15% glycerol.

Physicochemical characterization of water from the thermal spring Vranjska Banja

The temperature and pH of the spring water were determined *in situ* at the source, while the other physical and chemical properties of the thermal water were determined at the Institute for Rehabilitation, Belgrade, Serbia.

The determination of total alpha and beta activity was performed according to the procedure „ISO9696:2017 Water quality–gross alpha activity–Test method using a thick source“ and „ISO9697:2018 Water quality–gross beta activity–Test method using thick source“.

Gamma spectrometric analysis of water was performed according to „ISO10703:2007 Water quality–Determination of the activity concentration of radionuclides–Method by high-resolution gamma-ray spectrometry“.

Cultivation conditions and DNA isolation

Bacterial strains were inoculated from Petri dishes into the nutrient broth and incubated with aeration at 60°C. Genomic DNA was extracted from the logarithmic phase using the method described previously [13] with minor modifications: logarithmic phase cells were washed with TES buffer (10 mM Tris HCl, 100 mM NaCl, and 1.0 mM EDTA, pH of 7.8) and pre-treated with lysozyme (10 mg/ml, for 30 min at 37°C) before treatment with 2% SDS.

Strain identification

Taxonomic determination of isolates was performed by 16S rRNA gene sequencing. PCR amplification of the 16S rRNA gene was carried out using primers UNI16SF (5–GAGAGTTTGATCCTGGC-3) and UNI16SR (5-AGGAGGTGATCCAGCCG-3) [14]. The PCR products were sequenced by the Macrogen service (Macrogen Inc., Netherlands). For 16S rRNA gene identification, Basic Local Alignment Search Tool (BLAST, http://blast.ncbi.nlm.nih.gov/Blast.cgi) [15] was used to search against the GenBank database. Phylogenetic inferences for *Anoxybacillus* spp. based on 16S rRNA gene were obtained by MEGA 7.0 [16]. The 16S rRNA gene sequences were aligned using Clustal W with default parameters. Phylogenetic tree was constructed using the maximum likelihood (ML) method employing Tamura-Nei model. Data for the 24 representative *Anoxybacillus* species were derived from lpsn.dsmz.de database and included in this analysis. Bootstrapping of 1,000 replicates was used to infer confidence levels of ML tree.

16S rRNA gene based-metagenome sequencing and sequence data analysis

The standard 16S protocol which targets and amplifies the V3-V4 hypervariable region [17] of bacterial and archaeal 16S rRNA-encoding genes, was utilized for paired-end sequencing on the MiSeq-Illumina platform at the ID Genomics service (Seattle, WA, US). The obtained 16S rRNA amplicon library data were processed and analyzed using QIIME2 v2021.4 computing environment (https://qiime2.org/) [18]. Filtering based on joined sequence quality scores, taxonomy assignment, and diversity measurement was performed [19]. Taxonomy assignment was carried out using the q2 feature classifier, which was pretrained on the Greengenes v 13_8 database [20] with 99% OTUs (Operational Taxonomic Unit). Diversity alpha-rarefaction analysis was conducted with a sampling depth of 40,000 using QIIME2. The accuracy of the obtained taxonomic units was verified by referring to the List of Prokaryotic names with Standing in Nomenclature – LPSN (https://lpsn.dsmz.de).

Whole genome sequencing and genome analyses

The genomic DNA of five Vranjska Banja hot spring isolated strains was sequenced using Illumina HiSeq by MicrobesNG service (MicrobesNG, University of Birmingham, UK). The quality of each sequencing library was assessed using FastQC [21]. IDBA-UD with multi k-mer mode outperformed the assembly using De Bruijn Graph methods and the contigs shorter than 200 bp were eliminated [22]. Raw reads were mapped to assembled scaffolds with Burrows Wheeler Aligner-BWA [23]. Bacterial isolates were identified using the genome sequence, EzBioCloud TrueBac genome ID and GeneBank database [24]. Gene annotation and prediction of the open reading frames (ORFs) of the whole genome sequences was conducted by Rapid Annotations using Subsystems Technology (RAST) server (http://rast.nmpdr.org). The acquired data were analyzed using the SEED database in order to evaluate the metabolic potential of analyzed strains [25]. Complete genome sequences were analyzed using BAGEL4 (http://bagel4.molgenrug.nl/) and AntiSMASH tools (https://antismash.secondarymetabolites.org/#!/start) for the detection of the potential antimicrobial compounds. Virulence factor database (VFDB, http://www.mgc.ac.cn/VFs/) was applied for the identification of genes coding virulence

determinants and the presence of the antibiotic resistance markers was determined by the Comprehensive Antibiotic Resistance Database (CARD, https://card.mcmaster.ca/).

Accession numbers

The raw data of 16S libraries generated during this study are publicly available at the Sequence Read Archive (SRA) portal of NCBI (https://submit.ncbi.nlm.nih.gov/subs/sra/) under the accession number BioProject ID PRJNA813397; Sample SAMN26499420, experiment SRX14402958, run SRR18264632.
Draft genome sequences of five Vranjska Banja hot spring isolates have been deposited at the NCBI GenBank database under the following accession numbers: *Bacillus licheniformis* ST1-JAHZOI000000000; *Anoxybacillus* sp. ST4-JAHZOE000000000; *Hydrogenophilus thermoluteolus* ST11-JAHZOH000000000; *Anoxybacillus* sp. ST70-JAHZOF000000000; and *Geobacillus thermoleovorans* A70-JAHZOG000000000.

Results

Physicochemical characteristics of water from Vranjska Banja hot spring

Based on the physicochemical analysis presented in Table 1, Vranjska Banja hot spring belongs to the category of bicarbonated and sulfated hyperthermal waters, with bicarbonate content of 414 mg/L and sulfate content of 368 mg/L. Furthermore, due to a silicon content (H_2SiO_3) higher than 50 mg/mL [26], Vranjska Banja hot spring can also be classified as rich in silicon (90 mg/mL). On the other hand, the content of ammonium (NH_4^+), bromides (Br^-) and iodides (I^-) was below the limit of detection (data not shown).

Table 1 Results of physicochemical analysis of mineral water of Vranjska Banja hot spring

	mg/L	mmol/L	mval/L	mval % /L
Cations				
Sodium (Na^+)	291.1	12.6565	12.6565	82.1168
Potassium (K^+)	11.8	0.3025	0.3025	1.9626
Lithium (Li^+)	0.2	0.0288	0.0288	0.1868
Calcium (Ca^{++})	20.0	0.5000	1.000	6.4881
Magnesium (Mg^{++})	17.0	0.6994	1.3988	9.0755
Strontium (Sr^{++})	0.7	0.0079	0.0158	0.1025
Manganese (Mn^{++})	0.1	0.0018	0.0036	0.0233
Iron (Fe^{++})	0.04	0.0007	0.0014	0.0090
Σ			15.4128	100.0000
Anions				
Hydrogen carbonates (HCO_3^-)	414.0	6.7868	6.7868	40.3824
Chlorides (Cl^-)	62.0	1.7464	1.7464	10.3915
Fluorides (F^-)	11.5	0.6055	0.6055	3.6028
Phosphates (HPO_4^{--})	0.05	0.0005	0.0010	0.0059
Sulfates (SO_4^{--})	368.0	3.8333	7.6666	45.6174

Σ		16.8063	100.0000
Heavy metals			
Cadmium (Cd)	0.001		
Zinc (Zn)	0.003		
Lead (Pb)	0.02		
Copper (Cu)	0.01		
Nickel (Ni)	0.01		
Mercury (Hg)	0.001		
Chromium (Cr)	0.002		
Barium (Ba)	0.05		
Beryllium (Be)	0.0002		
Uranium (U)	0.2		
Mettaloids			
Arsenic (As)	0.01		
Selenium	0.01		
Weak electrolytes			
Metasilicic acid (H_2SiO_3)	90.0		
Boric acid (HBO_2)	4.2		
Sum of solid dissolved constituents	1290.7		
Air			
Free carbon dioxide (CO_2)	15.0		
Free hydrogen sulphide (H_2S)	1.5		
Specific weight	1001.50		
pH	7.1		
Dry residue (at 180˚C)	989.5		
Temperature of water	81˚C		

The total measured alpha activity of water from source A1 was <0.07 Bq/L, while the total beta activity was 0.478 + 0.052 Bq/L. The results of the gamma spectrometric analysis were: ^{137}Cs <0.003 Bq/L, ^{134}Cs <0.002 Bq/L, ^{40}K <0.49 + 0.03 Bq/L, ^{228}Ra <0.018 Bq/L, ^{226}Ra <0.011 Bq/L and ^{238}U <0, 10 Bq/L.

16S rRNA gene-based metagenome

The composition of bacterial communities in the thermal water from the Vranjska Banja hot spring was investigated using MiSeq-Illumina technology. The total number of reads passing quality filtering was 44,885 high-quality sequences. The rarefaction curve was calculated with a 3% cut-off to compare the sample, and it approached a plateau indicating an adequate and reliable sampling and sequencing effort for identifying and describing taxonomic categories/OTUs. The total number of OTUs was 494. All of the curves – Shannon, observed features and Faith's phylogenetic diversity (faith's pd) reached a stable plateau (data not shown).

Composition of bacterial communities at different taxonomic levels based on metagenomic analysis

The composition of microbial communities in Vranjska Banja hot spring at different taxonomic levels (phylum, class, order, family, genus and species) is shown in Fig. 2. Among the nine detected bacterial phyla *Aquificota* (24.34%), *Bacillota* (20.63%), and *Thermotogota* (19.12%) were the most represented (Fig. 2a). The relative abundance at the class level indicated the presence of *Aquificia* (24.34%), *Thermotogae* (19.12%), *Bacilli* (13.66%), *Deinococci* (8.38%), *Clostridia* (6.72%), *Alphaproteobacteria* (5.89%), *Thermodesulfobacteria* (4.81%),

Acidimicrobiia (3.99%), *Nitrospiria* (1.32%), and *Ktedonobacteria* (1.06%) (Fig. 2b). The dominant orders present in the analyzed sample with a percentage greater than five were *Aquificales* (24.34%), *Thermotogales* (19.12%), *Caryophanales* (11.35%), *Thermales* (8.37%) and *Rhodospirillales* (5.40%) (Fig. 2c). At the family level the most abundant taxonomic categories were *Aquificaceae* (24.34%), *Thermotogaceae* (19.12%), *Thermaceae* (8.37%), *Paenibacillaceae* (6.78%), *Acetobacteraceae* (5.35%), *Thermodesulfobacteriaceae* (4.81%), *Acidimicrobiaceae* (3.99%), *Syntrophomonadaceae* (3.42%), *Bacillaceae* (3.19%), *Streptococcaceae* (1.64%), and *Thermogemmatisporaceae* (1.06%) (Fig. 2d). *Aquifex* and *Fervidobacterium* were the most prevalent genera in the Vranjska Banja hot spring sample with a presence of more than 15%, following the genera *Thermus* (7.74%), *Paenibacillus* (6.13%), *Acidisphaera* (5.22%), *Geothermobacterium* (4.78%), *Acidimicrobium* (3.97%), *Caldicellulosiruptor* (3.42%), *Geobacillus* (2.80%), *Lactococcus* (1.31%), *Thermodesulfovibrio* (1.18%), and *Thermogemmatispora* (1.06%) (Fig. 2e). Additionally, at the species level *Fervidobacterium islandicum* had the highest abundance in the Vranjska Banja hot spring sample with the presence of 17.26%. Other identified species were present in a significantly lower amounts, including – *Acidimicrobium ferrooxidans* (3.67%), *Thermus scotoductus* (3.34%), *Parageobacillus thermoglucosidasius* (2.76%), *Paenibacillus filicis* (2.65%), *Thermodesulfovibrio aggregans* (0.83%), *Thermus oshimai* (0.77%), *Sulfobacillus yellowstonensis* (0.60%), *Thermus antranikianii* (0.58%), and *Thermobaculum terrenum* (0.58%) (Fig. 2f).

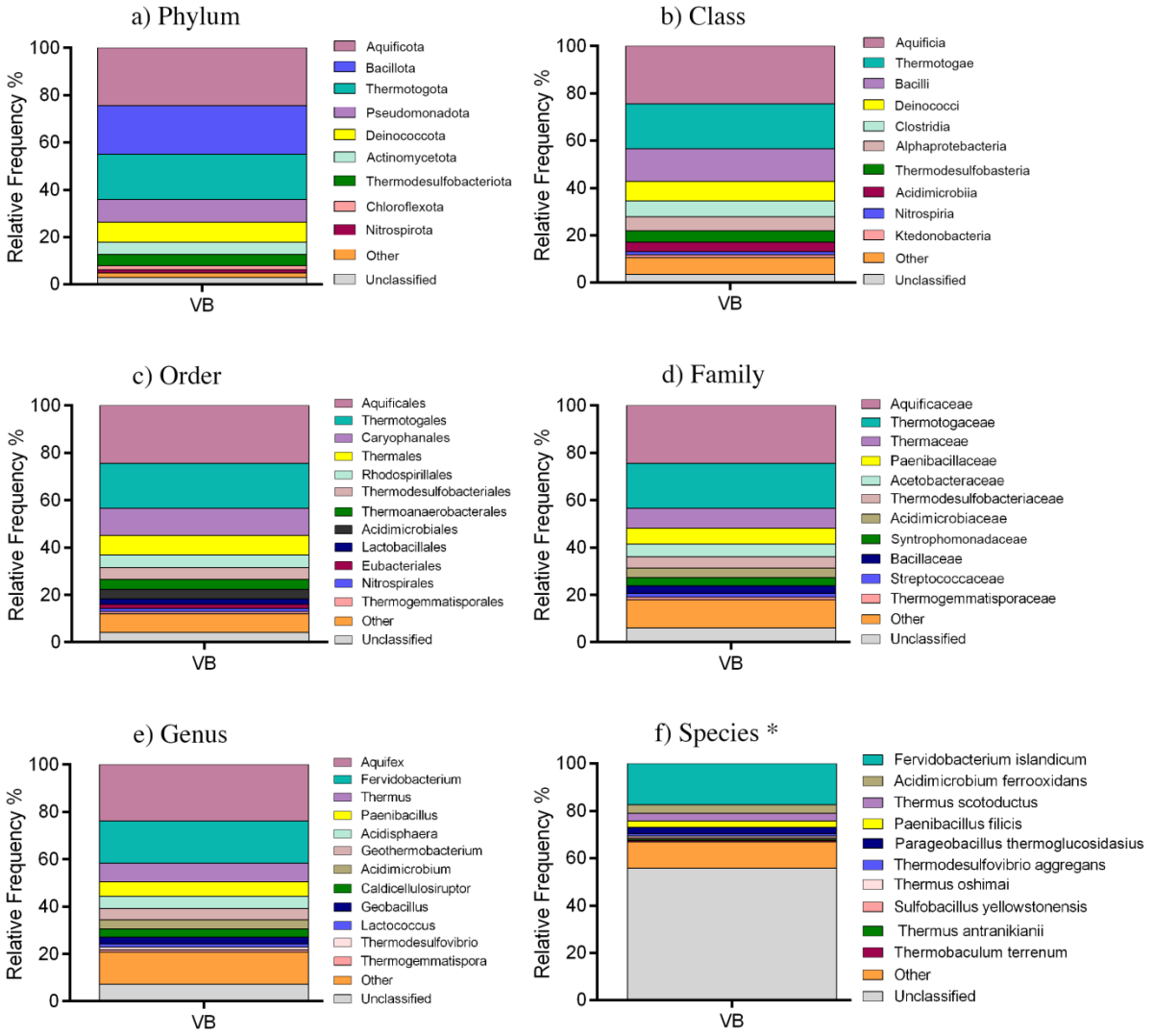

Fig. 2 Taxonomic diversity and relative abundance of bacteria in the Vranjska Banja hot spring water were assessed at various taxonomic levels: phylum (a); class (b); order (c); family (d); genus (e); species (f) level of bacteria in the Vranjska Banja hot spring water. * It should be noted that the species level classification accuracy of Qiime2 may not be entirely reliable.

Isolation and characterization of cultivable bacteria from Vranjska Banja spring water

The applied cultivation approach led to the isolation of 17 isolates. All cultivable isolates from Vranjska Banja hot spring water were re-purified and identified by 16S rRNA gene sequencing. Using NCBI BLAST alignment tool it was determined that these isolates belong to four different genera: *Bacillus* (ST1, ST2, and ST3), *Anoxybacillus* (ST4, ST5, ST15, ST16, ST17, ST18, and ST70), *Hydrogenophilus* (ST11, ST12, and ST13) and *Geobacillus* (A70, MF70, P70, and C70) (Table S1). Among these, the five cultivable isolates, representing each group, were selected for whole genome sequencing: – ST1, ST4, ST11, ST70, and A70.

Whole genome sequence analyses

Feature	Bacterial strains from Vranjska Banja hot spring				
	ST1	**ST4**	**ST11**	**ST70**	**A70**
Size (bp)	4,569,607	2,834,644	2,217,026	2,855,946	3,499,025
GC content (%)	45.6	42.3	61.7	41.5	52.2
N50	635,816	92,610	211,349	217,741	117,442
L50	3	8	4	4	9
Number of contigs	98	127	58	57	138
Number of subsystems	341	297	259	302	308
Number of coding sequences	4,713	3,059	2,120	2,931	3,362
Number of RNAs	115	135	57	127	138
rRNAs (5S, 16S, 23S)	9, 13, 8	11, 23, 16	3, 1, 4	10, 16, 15	9, 16, 18
tRNAs	80	81	45	82	90
ncRNAs	5	4	4	4	5
Pseudo genes (total)	102	75	20	57	140
CRISPR Arrays	/	1	2	1	3

The genomic DNA of the five Vranjska Banja hot spring selected strains was sequenced using Illumina HiSeq 2500 platform. Detailed characteristics of all five genomes (ST1, ST4, ST11, ST70, and A70) are described in Table 2. The genome sizes of the analyzed strains ranged from 2.2- 4.5 Gb in length. *Hydrogenophilus thermoluteolus* ST11 had the smallest genome size among the sequenced strains, while *Bacillus licheniformis* ST1 had the largest. The total GC content varied from 41.5% for *Anoxybacillus* sp. ST70 to 61.7% for *Hydrogenophilus thermoluteolus* ST11. *Bacillus licheniformis* ST1 had the highest number of subsystems (341) and coding sequences (4,713). The number of RNAs ranged from 57 for *Hydrogenophilus thermoluteolus* ST11 to over 100 for all the other analyzed strains, with *Geobacillus thermoleovorans* A70 having the highest number (138). *In silico* analysis revealed the absence of CRISPR Arrays only in *Bacillus licheniformis* ST1, while other strains possess one (*Anoxybacillus* sp. ST4, *Anoxybacillus* sp. ST70) to three CRISPR Arrays (*Geobacillus thermoleovorans* A70).

Table 2 Genomic features and *in silico* analysis of five bacterial strains originated from Vranjska Banja hot spring, based on draft genome sequences obtained by the NCBI Prokaryotic Genome Annotation Pipeline

N50 - a length of the shortest contig for which longer and equal length contigs cover at least 50% of the assembly;
L50 - a count of smallest number of contigs whose length sum makes up half of genome size.

According to 16S rRNA gene sequence analysis *Anoxybacillus* sp. ST4 and ST70 showed the greatest similarity with *Anoxybacillus karvacharensis* MK418417 and *Anoxybacillus gonensis* AY122325, respectively (Fig. 3). The OrthoANI values for the genomes of ST4 and ST70 as well

as the available genomes of related members of the *Anoxybacillus* genus, ranged from94.98-85.57% and 94.99-86.32%, respectively (Table S2).

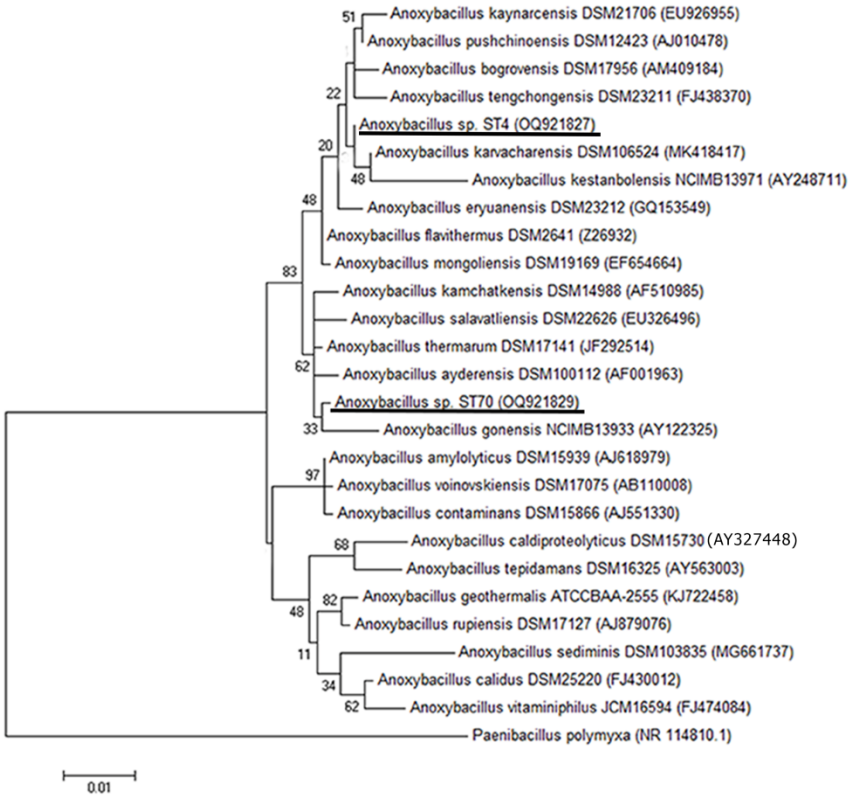

Fig. 3 Maximum likelihood phylogenetic tree was constructed based on 16S rRNA gene sequences available from the GenBank database. The dendrogram was constructed using the maximum likelihood method with the Clustal W program using MEGA 7.0 software package (1,000 bootstrap replicates). Scale bar, 0.01 represents substitutions per nucleotide position

The distribution of functional categories in all five analyzed genomes revealed a predominance of genes belonging to the following categories: metabolism; cofactors, vitamins, prosthetic groups and pigments; motility and chemotaxis; dormancy and sporulation; respiration and stress response (Table S3). Genome annotations of ST4 and ST70 revealed slight differences in genome architecture. These strains exhibited distinct gene clusters related to cell wall and capsule; phages, prophages, transposable elements and plasmids; iron acquisition and metabolism; DNA and protein metabolism; motility and chemotaxis; respiration and carbohydrates. The prevalence of stress response genes was highest for genes involved in oxidative stress, detoxification and sigma B stress response regulation in the analyzed strains. Additionally, the number of hfl operon and bacterial hemoglobin genes was highly abundant in ST4 and ST70 (Fig. 4). The genome analysis also identified the presence of glycopeptide antibiotic genes (*vanY*, *vanT*, *vanW*) and genes encoding disinfecting agents and antiseptics (*qacJ*, *qacG*) in all strains except for ST11 (Table S4). Among the virulence factors, the presence of genes encoding for capsule, pyochelin, different proteases, endopeptidases, flagellar and chemotaxis proteins was observed. Notably, *Hydrogenophilus thermoluteolus* ST11 has the highest number of virulence factors genes (Table S5).

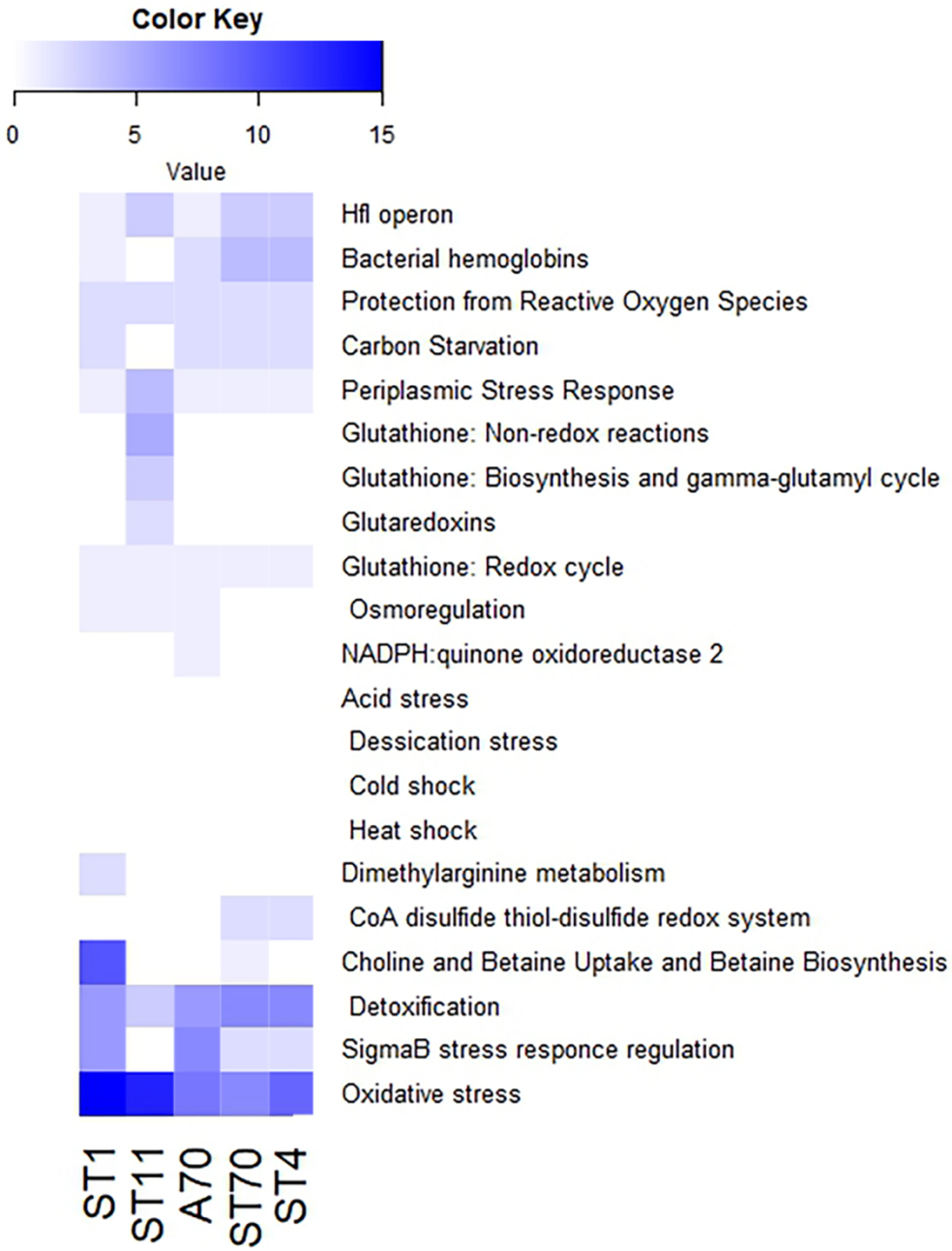

Fig. 4 Stress response genes prevalence in the five bacterial strains originated from Vranjska Banja hot spring. The heat map scale represents the relative abundance of the genes encoding stress response elements.

To assess the presence of genes encoding various enzymes in the analyzed genomes from Vranjska Banja, *in silico* analysis was conducted (Table 3). The results revealed that *Bacillus licheniformis* ST1 had the highest number of genes encoding hydrolytic enzymes, followed by *Geobacillus thermoleovorans* A70 and *Anoxybacillus* sp. ST4 and ST70. In contrast *Hydrogenophilus thermoluteolus* ST11 had the lowest number of such genes.

Table 3 Number of genes encoding selected enzymes per genome for the sequenced strains

Strain / Enzymes	*Bacillus licheniformis* ST1	*Anoxybacillus* sp. ST4	*Hydrogenophilus thermoluteolus* ST11	*Anoxybacillus* sp. ST70	*Geobacillus thermoleovorans* A70
Amylase	2	2	0	2	1
Glucanase	5	0	0	0	0
Chitinase	1	0	0	0	0
Lipase	5	1	0	2	4
Phytase	1	0	0	0	1
Protease	24	20	15	18	20

Furthermore, the genome sequences were examined for the presence of genes encoding antimicrobial molecules. The analysis revealed that isolates carry several genes that could encode the various antimicrobial molecules (ribosomaly and non-ribosomaly synthetized peptides; lipopeptides, lanthipeptides, lassopeptides, sactipeptides, sonorensin). Complete data on the identified molecules with antimicrobial activity can be found in Table 4.

Table 4 Genome-based identification of molecules with antimicrobial activity, which was determined using BAGEL4 and AntiSMASH tools

Isolates	Region	Type	Start	End	Similarity
		Bagel4			
ST1					
	NODE_3.2	Sactipeptides	309,776	329,776	
	NODE_1	225.2;UviB	140,6118	142,4217	
	NODE_8	492.1;Competence	30,410	50,503	
	NODE_9	Sactipeptides	65,138	85,138	
	NODE_2.3	Sactipeptides	462,707	482,707	
ST4	NODE_7	Sactipeptides	21,392	41,392	
ST11	not found				
ST70	NODE_8	Sactipeptides	34,688	54,688	
	NODE_4	Sactipeptides	180,692	200,692	
	NODE_25	Lanthipeptide_class_II	-8,842	11,158	
	NODE_16	24.1;Enterocin_W_beta	-4,387	20,340	
A70	NODE_23	37.1;Geobacillin_I_like	31,493	44,042	
	NODE_9	Sactipeptides	53,147	73,147	
	NODE_27	Lanthipeptide_class_I	19,112	39,112	
	NODE_25	Sactipeptides	-1,384	18,616	
		AntiSMASH			
ST1	NODE_1.1	Type 3 polyketide synthase	502,210	543,307	
	NODE_1.2	terpene (carotenoid)	733,015	754,904	
	NODE_1.3	Betalactone (fengycin)	852,796	881,310	53%
	NODE_2.1	lantipeptide class II	248,507	275,468	100%
	NODE_2.2	lassopeptide	339,701	362,162	
	NODE_2.3	non-ribosomal peptide synthetase cluster (bacillibactin)	467,158	514,315	53%
	NODE_3.1	Thiopeptide (butirosin A, butirosin B); sonorensin	107,676	148,969	7%
	NODE_3.2	siderophore	268,845	284,311	
	NODE_4	non-ribosomal peptide synthetase cluster (lichenysin)	184,864	250,304	100%
	NODE_6	tRNA-dependent cyclodipeptide synthases	46,407	67,156	
ST4	NODE_5	terpene (carotenoid)	121,154	142,005	83%
	NODE_25	Betalactone (fengycin)	1	21,791	46%
	NODE_30	Type 3 polyketide synthase	1	25,073	
	NODE_34	RRE- element containing cluster	6,983	18,357	
ST11	NODE_1.1	Thioamide, non-ribosomal peptide synthetase cluster	200,943	242,903	
	NODE_1.2	ribosomally synthesised and post-translationally modified peptide product (RiPP) cluster	373,259	384,143	
	NODE_6	betalactone	1	24,731	
ST70	NODE_1.1	ribosomally synthesised and post-translationally modified peptide product (RiPP) cluster	457,785	478,606	
	NODE_1.2	terpene (carotenoid)	566,954	587,805	66%
	NODE_5	ribosomally synthesised and post-translationally modified peptide product (RiPP) cluster	60,340	73,675	
	NODE_6	Type 3 polyketide synthase	26,491	67,558	
	NODE_11	Betalactone (fengycin)	11,053	35,171	46%

	NODE_15	non-ribosomal peptide synthetase cluster (bacillibactin)	1	37,905	46%
A70	NODE_4.1	Betalactone (fengycin)	1	23,496	46%
	NODE_4.2	Type 3 polyketide synthase	29,342	70,409	
	NODE_5.1	RRE- element containing cluster	1	11,819	
	NODE_5.2	terpene	115,722	137,578	
	NODE_8	ribosomally synthesised and post-translationally modified peptide product (RiPP) cluster	90,074	100,928	

Discussion

Thermophilic microorganisms as a source of thermostable enzymes, important for biotechnology and commercial use, have been the focus of research since ancient times, and especially in recent decades [27]. Thermophilic bacteria are microbes that mostly live in hot springs and survive in temperatures above 70°C. As a result of growth at high temperatures and unique macromolecular properties, thermophilic bacteria can have high metabolism, physically and chemically stable enzymes, and lower growth but higher final product yields than mesophilic species [28]. The hot springs of Vranjska Banja are one of the hottest springs in Europe. According to the genealogical and balneological classification of mineral waters of Serbia, the formula of the chemical composition of the spring water from source A1 in Vranjska Banja [26] is:

$$M_{1,3}\frac{HCO_{50}^{3}SO_{42}^{4}Cl_{8}}{Na + K_{94}Ca_{6}}Q > 60,0$$

Interest in the study of thermophilic microorganisms living in them arose at the beginning of the twentieth century. The first findings on thermophilic bacteria of the genus *Bacillus* (*Bacillus thermophilus vranjensis*) from this source date back to 1910 [29,30]. After more than 100 years, we conducted a pilot study of the microbial community composition of the Vranjska Banja hot spring using high throughput metagenomic sequencing and microbial cultivation approaches. Due to global warming and climate changes, which have the greatest impact on biodiversity, it is important to preserve habitats, or at least to have information about them and their changes over the time [31]. Global warming does not have a large impact on thermal springs as extreme and isolated habitats, but the impact of human activities on biodiversity is evident [32]. For this reason, in addition to the biodiversity analysis, culturable strains were isolated from the water of this thermal spring for a more in-depth genomic analysis and possible application of the strains and/or their enzymes in biotechnology. Metagenomics has become an indispensable tool for studying the diversity and metabolic potential of environmental microbes, most of which are not yet culturable [33]. It has been employed to assess and exploit the biodiversity of many habitats including those of extremophiles [2,5,34,35]. The 16S rRNA metagenomic sequencing of bacterial communities from the water of the Vranjska Banja hot spring revealed a wide range of thermophilic and mesophilic microorganisms. Additionally, in the present study we isolated bacteria from extremely hot spring water (*Bacillus licheniformis*, *Anoxybacillus* sp., *Geobacillus thermoleovorans* and *Hydrogenophilus thermoluteolus*), including potentially new ones, and sequenced complete genomes of five strains. All isolated strains belonged to the phyla *Bacillota* (family *Bacillaceae*)

and *Pseudomonadota* (family *Hydrogenophilaceae*). The results showed that most of them have the potential to produce thermostable enzymes (proteases, lipases, amylases, phytase, chitinase and glucanase) and various antimicrobial molecules that can play a key role in industrial, agricultural and biotechnological applications [36-38].

Water from the thermal spring of Vranjska Banja belongs to the category of pH neutral bicarbonated (414 mg/L) and sulfated (368 mg/L) hyperthermal waters. Similar physicochemical parameters were previously described for the Tato Field thermal spring in Pakistan (bicarbonates and sulfates values of 525-610 mg/L and 410-460 mg/L, respectively) [4]. Since the Vranjska Banja hot spring is classified as a natural mineral hyperthermal water, the microbiological community consisting of thermophilic Gram-positive and Gram-negative bacteria (dominant phyla are:*Aquificota*, *Bacillota* and *Thermotogota*) is expected [39,40]. Based on metagenomic analysis, those three phyla account for approximately 65% of all isolated phyla, confirming the data that microbial richness decreases with the increase in environmental temperature. The predominant detection of the phylum *Aquificota* could reasonably be attributed to its high silica content (metasilicic acid 90 mg/L). The same observations on the dependence of the distribution of *Aquificales* on silica content have been previously reported for Yellowstone National Park [41], hot springs in Pakistan [4] and hot springs on the Tibetan Plateau [42]. The detection of phylum *Thermotogota*, as the third most dominant phylum, can be attributed to the high temperature of the hot spring of Vranjska Banja. Similarly, cultures of the genus *Fervidobacterium* from the order *Thermotogales* were isolated from the thermal springs in Thailand [43] and China [44], where the average temperature of sampling sites exceed 80°C. In addition, thermophilic and hyperthermophilic bacteria from the phylum *Aquificota*, *Thermodesulfobacteriota*, *Thermotogota*, and some members of the phylum *Bacillota* and *Pseudomonadota* were predominantly isolated from hot springs with temperatures above 75°C, while a moderately thermophilic environment (<70°C) favoured photosynthetic bacteria from the phylum *Chloroflexota* and *Cyanobacteriota* [4,42], which is in accordance with the results of this study. Moreover, phyla such as *Deinococcota* and *Chloroflexota* were negatively correlated with phosphorus, while the presence of Si and elemental S had a positive effect. Further, geochemical factors such as temperature, Ca, Cl, and dissolved SiO_2 positively influenced the presence of *Bacillota*, while *Pseudomonadota* was most strongly correlated with total sulphide content [45]. At the genus level, the increase in *Thermus* was positively influenced by total Mg, silicate-silicon and total hardness [46]. When comparing the obtained results with similar hot springs in the region, the high percentage of overlapping in detected phyla between hot springs in Bulgaria (Levunovo (82°C) and Vetren Dol (68°C)) and Vranjska Banja was observed. In particular, *Bacteroidota*, *Pseudomonadota*, *Cyanobacteriota*, *Chloroflexota*, *Bacillota*, *Actinomycetota*, *Deinococcota*, and *Nitrospirota* were the dominant phyla found in these hot springs [47]. On the other hand, the similarities in the detected phyla between Vranjska Banja and hot springs from the geothermal regions in Romania, depended mainly on their temperature and water chemistry. The most common phyla detected in the Chiraleu hot spring (40 to 53°C) were *Cyanobacteriota*, *Pseudomonadota*, *Chloroflexota*, and *Nitrospirota*, phyla slightest present in Vranjska Banja. Another hot spring Mihai Bravu (53 to 65°C), showed the highest similarity in physicochemical characteristics of water (regarding concentration of HCO_3^- and SO_4^{2-} ions) and detected phyla (*Chloroflexota*, *Pseudomonadota*, *Deinococcota*, and *Aquificota*) with Vranjska Banja [48]. The presence of sequences of some mesophilic bacteria, which cannot survive and grow in such nutrient-limited and hot water, can be explained by the contamination of the hot spring water with bacteria from the environment (soil or mesophilic bacteria from surface waters) [39].

The obtained sequence data indicates that *Fervidobacterium islandicum* is the dominant species in the hot spring of Vranjska Banja, with a proportion of 17.26%, followed by *Acidimicrobium ferrooxidans*, *Thermus scotoductus*, *Parageobacillus thermoglucosidasius*, *Paenibacillus filicis*, *Thermodesulfovibrio aggregans*, *Thermus oshimai*, *Sulfobacillus yellowstonensis*, *Thermus antranikianii*, and *Thermobaculum terrenum* (with less than 5% abundance), but a variety of genera and species thrive in this habitat. The high proportion of unclassified and unspecified species (more than 65%) indicates the great diversity and uniqueness of the microbiome of this thermo mineral source.

It is worth noting that not even one of the highly represented species (identified by metagenome analysis) was isolated under the conditions and on the media used for cultivation in this study. These results are consistent with previous findings that dominant taxa in the environment often cannot be obtained by microbial cultivation [3]. On the other hand, most species isolated, but not found in the amplicon sequencing data belonged to *Bacillus* or related genera whose cells might have been present in resistant forms (spores) in the environment and therefore could have been missed by sequencing analysis [49]. Moreover, *Bacillus*-like genera are ubiquitous in nature and represent the majority of microbes isolated from thermal springs due to their high cultivability [3]. The results of several research groups supported this statement. A comprehensive study conducted in Bulgaria revealed that of the sixty-seven strains isolated from 18 thermal springs, sixty-six belonged to four genera of the *Bacillus* group: *Anoxybacillus*, *Geobacillus*, *Brevibacillus* and *Bacillus* [50]. Some species of the genus *Anoxybacillus* were not only predominant, but also novel bacteria, such as *Anoxybacillus bogrovensis* [51] and *Anoxybaillus rupiensis* [52]. Another study conducted in Turkey found that the highest percentage (89.2%) of isolated strains (cultured in the temperature range from 40 to 90°C) from seven different geothermal hot springs (in the regions of Eastern and Southeastern Anatolia Regions) belonged to endospore-forming bacteria [53]. The sequenced genomes of isolates designated as ST4 and ST70 had OrthoANI values <95%, which is considered a threshold for species demarcation [3] and are therefore presented as new species. Based on the genomic information, both isolates encoded few antibiotic resistance genes, confirming that the Vranjska Banja hot spring is a human-protected environment. Analysis of the presence of stress response genes that enable these microorganisms to reduce, adapt to, and repair damage caused by extreme environmental conditions, revealed that the most abundant gene is the oxidative stress gene cluster. Oxidative stress is one of the most damaging stresses, which causes both molecular and metabolic cellular damage, facing thermophiles during industrial processing to isolate secondary metabolites such as enzymes, antimicrobial molecules, and others. [54]. Therefore, thermophilic bacteria that possess repair mechanisms for oxidative damage are of great biotechnological value. Further analysis revealed that most of the gene clusters for stress response were common to both strains, with the exception of choline and betaine uptake and betaine biosynthesis, which were only found in strain ST70. Based on the genome characterization, we propose strains ST4 and ST70 to represent members of a novel species of the genus *Anoxybacillus* in the family *Bacillaceae*, phylum *Bacillota*.

Considering the uniqueness of the microbiological composition and the importance of certain bacterial strains in the community of Vranjska Banja thermal water, future work should be directed towards more detailed analyses and periodic isolation of microorganisms using various microbiological substrates and cultivation conditions. All this with the aim of studying phylogenetically novel strains and discovering strains with interesting biotechnological properties, with special emphasis on enzymes active under extreme conditions.

We expect that future sampling and analysis of the springs in Vranjska Banja will contribute to the discovery of new genera and species. Therefore, our next goal will be comprehensive sampling from different microhabitats (water in the pond from different depths, walls of the water channel, mud and the immediate surroundings of the spring) to document the overall diversity and the abundance of microorganisms. This unique hot spring has been recognized for centuries for its specific geographical-tourist, cultural and spa-medicinal values, while its biological value is almost unexplored. This source is protected from intensive and constant anthropogenic influences, and therefore represents significant scientific and biotechnological value.

Statements and Declarations

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Competing Interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Figure Captions

Fig. 1 Geographical location (A-C) and image of the sampling site in Vranjska Banja hot spring (D). The red location pin icon indicates the position of Vranjska Banja hot spring on the maps of

Southeastern Europe and the Balkans (A), southern Serbia (B) and the Municipality of Vranje (C). The red dots on the source indicate the places from where the water samples were taken

Fig. 2 Taxonomic diversity and relative abundance of bacteria in the Vranjska Banja hot spring water were assessed at various taxonomic levels: phylum (a); class (b); order (c); family (d); genus (e); species (f) level of bacteria in the Vranjska Banja hot spring water. * It should be noted that the species level classification accuracy of Qiime2 may not be entirely reliable.

Fig. 3 Maximum likelihood phylogenetic tree was constructed based on 16S rRNA gene sequences available from the GenBank database. The dendrogram was constructed using the maximum likelihood method with the Clustal W program using MEGA 7.0 software package (1,000 bootstrap replicates). Scale bar, 0.01 represents substitutions per nucleotide position

Fig. 4 Stress response genes prevalence in the five bacterial strains originated from Vranjska Banja hot spring. The heat map scale represents the relative abundance of the genes encoding stress response elements.