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Physiological and Molecular-genetic Characteristic of Bacteria Strains, Isolated from Mountain Spring and Mineral Waters in Plovdiv Region, Bulgaria

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Authors' contributions

This work was carried out in collaboration among all authors. Author NV designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors II and SM managed the analyses of the study. Author II managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

The present study aims to investigate the microbiological research of mineral and spring mountain waters in the region of Plovdiv, Bulgaria. In Bulgaria, there are mineral and mountain spring waters, which are not subjected to physicochemical and microbiological control by the Regional Health Inspectorate, yet they are the most widely used springs by the population as sources of drinking water. The methodology is including the determination of total number of mesophilic aerobic and facultative anaerobic bacteria. The results showed 11 rod shaped, Gram–positive and spore forming bacteria and 2 rod shaped, Gram–negative non-spore forming bacteria are isolated from spring and healing waters. The colonial and physiological characteristics of the isolated strains are defined. The biochemical profiles of the selected bacteria are determined with the system for rapid identification API 50 CH. The strains are identified by software processing of the

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obtained biochemical profiles with apiweb®. Furthermore, the tested strains are genetically identified by molecular-genetic methods – ARDRA analysis and sequencing of the gene for the 16S rRNA. The results obtained from the biochemical and molecular-genetic studies indicate that the selected strains belong to different species of genus *Bacillus*, *Aeromonas*, *Klebsiella*.
The identification strains support for registration of water sources in Plovdiv region, Bulgaria according Decree No. 9.

Keywords: Microflora; mountain spring waters; mineral waters; selection; identification; sequencing; Bacillus subtilis.

1. INTRODUCTION

The spectrum of microbial ecology got extended in the recent years, and now it includes extreme ambients such as thermal springs and sea depths. It allows analysis of the physiological condition of naturally occurring microbial habitats. Abundance of bacteria and bacterial biomass are found in the sea, inland and soil waters. The determination of bacterial count in thermal springs is scarce, and it is confined to determination of the total number of microorganisms.

Furthermore, most of the results refer to bacterial populations with low-density. Nevertheless, the influence of microorganisms over the geochemical processes in thermal springs is well known.

Therefore the determination of active bacteria in the microbial communities of thermal springs, fresh and mineral waters is of particular importance. Different methods are used for the determination of total number of cells, most of them based on color reactions. These spring waters differ in physiochemical characteristics such as water temperature, pH, oxidation reduction potential, electrical conductivity, dissolved oxygen, which defines the presence of different microbial species. The purpose of current work is isolation, selection and identification of strains of bacteria from the water of a healing spring in the region of Plovdiv.

In Bulgaria, there are mineral and mountain spring waters, which are not subjected to physicochemical and microbiological control by the Regional Health Inspectorate, yet they are the most widely used springs by the population as sources of drinking water. The springs are located in the territory of Haskovo District [1,2,3], Stara Zagora District [4,5], Varna District [6] and Burgas District [7,8], Sliven district [9], Municipality of Teteven, Lovech District [10,11]. For many of these sources physicochemical and microbiological studies have not been conducted, yet they are used for drinking and household needs [12].

2. MATERIALS AND METHODS

2.1 Microorganisms

In the work are used 13 strains, isolated from 31 healing springs in Plovdiv area, designated as N. valcheva 2-1-1 *Bacillus subtilis,* N. valcheva 16 -1 *Bacillus cereus group,* N. valcheva *13-1 Bacillus altitudinis/pumilus,* N. valcheva 3-1 *Bacillus licheniformis,* N. valcheva 2-1-1 *Bacillus subtilis,* N. valcheva 2-1-2 *Bacillus vallismortis,* N. valcheva 2-1-3 *Bacillus amyloliquefacien* N. valcheva 2-1-3 *Bacillus amyloliquefacien,* N. valcheva *Aeromonas sobria* hs – 1, N. Valcheva *Klebsiella oxytoca* LF – 1, N. valcheva *Bacillus amyloliquefaciens subsp. plantarum* SDF004, N. valcheva *Bacillus thuringiensis* KU4, N. valcheva *Bacillus cereus* S72.

2.2 Nutrient Media

LBG – agar. Composition (g/dm^3): tryptone – 10, yeast extract – 5, NaCl - 10, glucose – 10, agar – 20. pH corrected up to 7,5. Sterilization - 20 minutes at 121°C.

2.3 Methods of Analysis

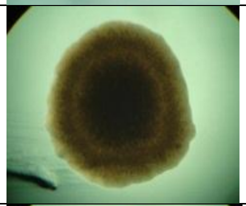
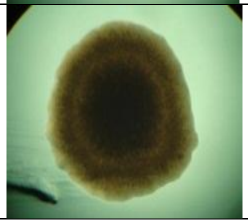
2.3.1 Determination of total number of *Mesophilic aerobic* and facultative anaerobic bacteria

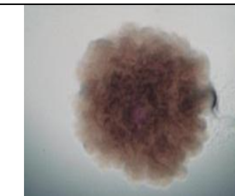
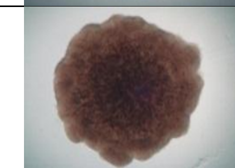
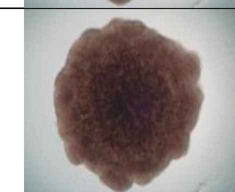
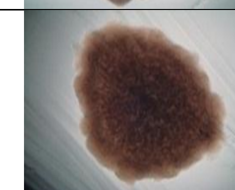
According to Bulgarian State Standard (BDS) EN ISO 6222: 2002.

2.3.2 Isolation of total DNA

The isolation of DNA is conducted by the method of Delley et al., 1990 [3].

Table 1. Colonial characteristic of isolated strains from Healing springs in Plovdiv region

Name of water source	Strain	Shape	Edges	Surface	Profile	Consistency	Color	Size	Visualization
1. Thermal healing spring, Captured natural spring (CNS) “Momina banya” in the town of Hissar with water temperature of 42°C	N. Valcheva BS2-1-1	round to elliptical	serrated	smooth	convex	dry	whitish	3 – 4 mm average	
2. Thermal healing spring “CNS Momina salza” in the town of Hissar with water temperature of 45,3°C	N. Valcheva BC16-1	irregular star pattern	serrated	smooth	crater-shaped with with solid center	dry	whitish	2 – 3 mm average	
3.Healing spring “CNS Stublata” in the town of Hissar with water temperature of 31°C	N. Valcheva BAP13-1	round, irregular shaped	wavy	rough	flat	dry, solid with white granular coating	greyish brown	3 – 4 mm average	
4. Thermal healing spring “CNS Centralen kaptazh” in the town of Banya with water temperature of 49,5°C	N. Valcheva BA3-1	round	wavy	wrinkled	crater-shaped or undulating	soft	light brown	2 – 3 mm average	
5. Thermal healing spring “Well 1 – Kokalche” in the town of Banya with water temperature of 49,5°C	N. Valcheva BS2-1-1	round	wavy	wrinkled	crater-shaped or undulating	soft	light brown	2 – 3 mm average	

Name of water source	Strain	Shape	Edges	Surface	Profile	Consistency	Color	Size	Visualization
6. Thermal healing spring "Well – Bulgarian Rose" in the town of Banya with water temperature of 48°C	N. Valcheva BV2-1-2	round to elliptical	wavy	rough	bulging	dry	white or creamy white	3 – 4 mm average	
7. Healing spring ,,CNS Mazhko banche" in the town of Banya with water temperature of 34,4°C	N. Valcheva BA2-1-3	round	wavy	rough	crater-shaped	soft, sometimes mucous	milky white, whitish	5 – 12 mm large	
8. Healing spring "CNS Zhensko banche" in the town of Banya with water temperature of 34,4°C	N. Valcheva BA2-1-3	round	wavy	rough	crater-shaped	soft, sometimes mucous	milky white, whitish	5 – 12 mm large	
9. Healing non thermal spring "Banski izvor" in the village of Narechenski bani	N. Valcheva BIAS hs – 1	round to elliptical	serrated	smooth	bulging	dry	whitish	3 – 4 mm average	
10. Healing non thermal spring "Banski izvor" in the village of Narechenski bani	N. Valcheva BIKO LF – 1	round	even	smooth, glossy	bulging	slimy	whitish	1 – 2 mm average	
11. Healing non thermal spring "Banski izvor" in the village of Narechenski bani	N. Valcheva BIBASP SDF004	round	wavy	smooth, glossy	bulging, tear-shaped	soft	whitish	2 – 3 mm average	

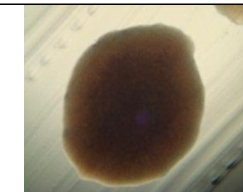
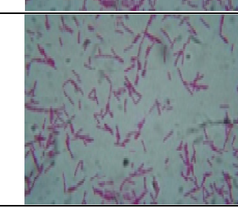
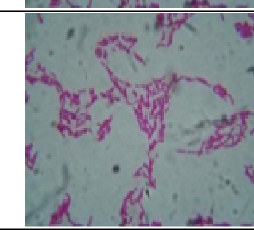
Name of water source	Strain	Shape	Edges	Surface	Profile	Consistency	Color	Size	Visualization
12. Healing spring "Soleno izvorche" in the village of Narechenski bani with water temperature of 21,5°C	N. Valcheva SIBT KU4	round	wavy	smooth, glossy	bulging, tear-shaped	soft	whitish	2 – 3 mm average	
13. Spring "Badzhova voda" in the village of Bachkovo with water temperature of 20°C	N. Valcheva BVBC S72	round to elliptical	serrated	smooth	slightly bulging	dry	whitish	3 – 4 mm average	

Table 2. Morphological characteristic of isolated strains from Healing springs in Plovdiv region

Name of water source	Strain	Shape	Edges	Spatial arrangement	Mobility	Spore formation	Coloring per gram	Visualization
1. Thermal healing spring "CNS Momina banya" in the town of Hissar with water temperature of 42°C	N. Valcheva BS2-1-1	round to elliptical	serrated	smooth	convex	dry	whitish	
2. Thermal healing spring "CNS Momina salza" in the town of Hissar with water temperature of 45,3°C	N. Valcheva BC16-1	irregular star pattern	serrated	smooth	crater-shaped with solid center	dry	whitish	
3. Healing spring "CNS Stublata" in the town of Hissar with water temperature of 31°C	N. Valcheva BAP13-1	round, irregular shaped	wavy	rough	flat	dry, solid with white granular coating	greyish brown	

Name of water source	Strain	Shape	Edges	Spatial arrangement	Mobility	Spore formation	Coloring per gram	Visualization
4. Thermal healing spring, “CNS Centralen kaptazh” in the town of Banya with water temperature of 49, 5°C	N. Valcheva BA3-1	round	wavy	wrinkled	crater-shaped or undulating	soft	light brown	
5. Thermal healing spring “Well 1 – Kokalche” in the town of Banya with water temperature of 49,5°C	N. Valcheva BS2-1-1	round	wavy	wrinkled	crater-shaped or undulating	soft	light brown	
6. Thermal healing spring, “Well – Bulgarian Rose” in the town of Banya with water temperature of 48°C	N. Valcheva BV2-1-2	round to elliptical	wavy	rough	bulging	dry	white or creamy white	
7. Healing spring “CNS *Mazhko banche*” in the town of Banya with water temperature of 34,4°C	N. Valcheva BA2-1-3	round	wavy	rough	crater-shaped	soft, sometimes mucous	milky white, whitish	
8. Healing spring “CNS *Zhensko banche*” in the town of Banya with water temperature of 34,4°C	N. Valcheva BA2-1-3	round	wavy	rough	crater-shaped	soft, sometimes mucous	milky white, whitish	

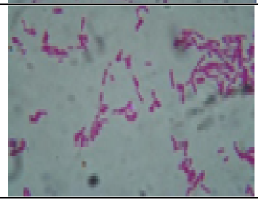
Name of water source	Strain	Shape	Edges	Spatial arrangement	Mobility	Spore formation	Coloring per gram	Visualization
9. Healing non thermal spring "Banski izvor" in the village of Narechenski bani	N. Valcheva BIAS hs – 1	round to elliptical	serrated	smooth	bulging	dry	whitish	
10. Healing non thermal spring ,,Banski izvor"in the village of Narechenski bani	N. Valcheva BIKO LF – 1	round	even	smooth, glossy	bulging	slimy	whitish	
11. Healing non thermal spring "Banski izvor" in the village of Narechenski bani	N. Valcheva BIBASP SDF004	round	wavy	smooth, glossy	bulging, tear-shaped	soft	whitish	
12. Healing spring "Soleno izvorche" in the village of Narechenski bani with water temperature of 21,5°C	N. Valcheva SIBT KU4	round	wavy	smooth, glossy	bulging, tear-shaped	soft	whitish	
13. Spring "Badzhova voda" in the village of Bachkovo with water temperature of 20°C	N. Valcheva BVBC S72	round to elliptical	serrated	smooth	slightly bulging	dry	whitish	

Table 3. Growth of isolates from the healing springs in Plovdiv region in liquid growing medium for 24 – 48 h, at temperatures 3°C – 50°C

Name of water source	Strain	Liner	Turbidity	Sediment	Relation to oxygen
1. Thermal healing spring “CNS Momina banya“ in the town of Hissar with water temperature of 42°C	N. Valcheva BS2-1-1	+	+	+	Facultative anaerobes
2. Thermal healing spring “CNS Momina salza” in the town of Hissar with water temperature of 45, 3°C	N. Valcheva BC16-1	+	+	+	Facultative anaerobes
3. Healing spring “CNS Stublata” in the town of Hissar with water temperature of 31°C	N. Valcheva BAP13-1	+	—	+	Facultative aerobes
4. Thermal healing spring “CNS Centralen kaptazh” in the town of Banya with water temperature of 49, 5°C	N. Valcheva BA3-1	+	—	+	Facultative aerobes
5. Thermal healing spring “Well 1 – Kokalche” in the town of Banya with water temperature of 49, 5°C	N. Valcheva BS2-1-1	+	+	+	Facultative anaerobes
6. Thermal healing spring “Well – Bulgarian Rose” in the town of Banya with water temperature of 48°C	N. Valcheva BV2-1-2	+	—	+	Facultative aerobes
7. Healing spring “CNS Mazhko banche” in the town of Banya with water temperature of 34, 4°C	N. Valcheva BA2-1-3	+	—	+	Facultative aerobes
8. Healing spring “CNS Zhensko banche” in the town of Banya with water temperature of 34, 4°C	N. Valcheva BA2-1-3	+	—	+	Facultative aerobes
9. Healing non thermal spring “Banski izvor” in the village of Narechenski bani	N. Valcheva BIAS hs – 1	+	+	+	Facultative anaerobes
10. Healing non thermal spring “Banski izvor” in the village of Narechenski bani	N. Valcheva BIKO LF – 1	+	+	+	Facultative anaerobes
11. Healing non thermal spring “Banski izvor” in the village of Narechenski bani	N. Valcheva BIBASP SDF004	+	+	+	Facultative anaerobes
12. Healing spring “Soleno izvorche” in the village of Narechenski bani with water temperature of 21, 5°C	N. Valcheva SIBT KU4	+	—	+	Facultative aerobes
13. Spring “Badzhova voda” in the village of Bachkovo with water temperature of 20°C	N. Valcheva BVBC S72	+	—	+	Facultative aerobes

2.3.3 16S rDNA amplification

All PCR reactions are carried out using the PCR test - PCR VWR, volume of 25 µl in Progenecycler (Techne, UK) according to the manufacturer's instructions, as in each reaction are used 50 ng of total DNA of the tested strain and 10 pmol of primers. DNA of the assessed strain is amplified by using of universal primers for 16S rDNA - 27f (5'AGAGTTTGATCMTGGCT CAG3') and 1492r (5'ACCTTGTTACGACTT3').

The amplification program includes: Denaturation - 95°C for 3 min, 40 cycles - 93°C for 30 s, 55°C for 60 s, 72°C for 2 min, extreme elongation - 72°C for 7 min.

The PCR product obtained by amplification of 16S rDNA is visualized by 2% agarose gel, coloured by ethidium bromide solution (0.5 µg/ml), of UVP Documentation System (U.K.).

From isolated total DNA is multiplied the gene for 16S rRNA by PCR – reaction by using of universal primers for multiplication of 16S rDNA. The obtained amplification products are purified and sequenced by „Macrogen Europe Laboratory", Holland. The comparative analysis of the sequences of the genes for 16S rRNA is conducted through the software CLC Sequence Viewer.

3. RESULTS AND DISCUSSION

Via plating on LBG-agar medium is determined the colonial characteristic of the selected strains, and through colored microscopic sections – their morphology (Table 1 and Table 2). It is defined their ability to form spores, as well as being able to develop under different temperature levels (Table 1, Table 2 and Table 3). Total DNA is isolated from 13 strains, the gene 16SpRNA is multiplied through PCR-reaction by using of universal primers for multiplication of 16S rRNA, and the obtained amplification products are purified and sequenced from "Macrogen Europe Laboratory", Holland.

The trial results reveal that 11 of the isolated strains are Gram-positive, spore-forming, and 2 are Gram-negative, non spore-forming. They develop at temperatures 3°C – 45°C, as the best growth rate is reported at temperature of development 30°C. Total DNA is isolated from 13 strains, the gene 16SpRNA is multiplied through PCR-reaction by using of universal primers for multiplication of 16S rRNA, and the obtained amplification products are purified and sequenced from „Macrogen Europe Laboratory", Holland. The comparative analysis of sequences of genes for 16S rRNA is conducted therough the software CLC Sequence Viewer. 13 strains differ by the sequence of the gene for 16S rRNA. The obtained sequences are processed by BLASTn software, and their species genus identification of strains is confirmed (Tables 4, 5) [13,14]. The following studies are base of research in our project. The research of *Bacillus subtilis* DR8806 was performed in Hot Mineral Spring [15]. For Cytotoxic Aeromonas Hydrophila Strains there was study in Italia [16]. Isolation and Characterization of a High H2-producing Strain *Klebsiella oxytoca* HP1 was performed in Hot Spring [17]. In Deep-subsurface Geothermal Water was studied Genes Coding for rRNA [18].

Table 4. Species of genus identity of the strains isolated from healing and spring waters in Plovdiv region, after processing the sequences of genes for 16S rRNA with BLASTn

Strain	Species of genus identity (Reference strain)	Trueness, %
1. N. Valcheva BS2-1-1	*Bacillus subtilis*	99%
2. N. Valcheva BC16-1	*Bacillus cereus group*	99%
3. N. Valcheva BAP13-1	*Bacillus altitudinis/pumilus*	99%
4. N. Valcheva BL3-1	*Bacillus licheniformis;*	100%
5. N. Valcheva BS2-1-1	*Bacillus subtilis*	99%
6. N. Valcheva BV2-1-2	*Bacillus vallismortis*	98%
7. N. Valcheva BA2-1-3	*Bacillus amyloliquefacien*	93%
8. N. Valcheva BA2-1-3	*Bacillus amyloliquefacien*	98%
9. N. Valcheva BIAShs – 1	*Aeromonas sobria hs – 1*	97%
10.N. Valcheva BIKOLF – 1	*Klebsiella oxytoca LF – 1*	99%
11.N. Valcheva BIBASP SDF004	*Bacillus amyloliquefaciens subsp. plantarum SDF004*	99%
12.N. Valcheva SIBT KU4	*Bacillus thuringiensis KU4*	100%
13.N. Valcheva BVBC S72	*Bacillus cereus S72*	99%

Table 5. Species of genus identity of the strains isolated from healing and spring waters in the regions of Haskovo and Stara Zagora, after processing the sequences of genes for 16S rRNA with BLASTn

Strain	Species of genus identity (Reference strain)	Trueness, %
1. N. ValchevaHMB1(46/H1)	*Bacillus subtilis* 0 - 2	99%
2. N. ValchevaHMB2(40/H2)	*Bacillus cereus* S74	99%
3. N. ValchevaHMB3(56/H3)	*Bacillus thuringiensis* B62	99%
4. N. ValchevaHMB4(32/H4)	*Bacillus cereus* XA5 - 11	100%
5. N. ValchevaHMB5(49/H5)	*Bacillus cereus* S74	99%
6. N. ValchevaHMB6(44/H6)	*Bacillus cereus JN267*	98%
7. N. ValchevaGI1(52/GI1)	*Bacillus cereus KH2*	93%
8. N. ValchevaGI2(55/GI2)	*Bacillus cereus* WIF15	98%
9. N. ValchevaGI3(54/GI3)	*Bacillus cereus* S433Ba - 98	97%
10. N. ValchevaGI4(41/GI4)	*Bacillus cereus* S74	99%
11. N. ValchevaGI5(50/GI5)	*Bacillus thuringiensis* B62	99%
12. N. ValchevaGI6(36/GI6)	*Bacillus cereus* A7 - 5	100%
13. N. ValchevaYA1(47/YA1)(BM47)	*Bacillus methylotrophicus* PY5	99%
14. N. ValchevaYA2(48/YA2)	*Aeromonas hydrophila* 14 H 12	99%
15. N. ValchevaD1(45/D1)	*Bacillus thuringiensis* B62	99%
16. N. ValchevaR1 (53/R1)	*Bacillus cereus* LH1	99%
17. N. ValchevaR2(51/R2)	*Bacillus thuringiensis* B62	99%
18. N. ValchevaPB (37/PBGK)	*Bacillus thuringiensis* B62	99%
19. N. ValchevaPBBGFRA(42/BGFRA)	*Bacillus cereus* A7-5	97%
20. N. ValchevaSMB(43/SMB)	*Bacillus thuringiensis* B62	99%

The waters in the report are not drinking waters and they are dangerous for digestive system. They a lot of water sources in Bulgaria with drinking waters which correspond of Decree No. 9 for mountain spring water and Decree No. 14 for mineral water in Bulgaria, European Union.

4. CONCLUSION

The experimental research allowed the determination of species of genus identity of the strains isolated from the waters of mountain spring and mineral sources in Plovdiv region, Bulgaria. The isolated strains belong to spore forming bacteria of genus *Bacillus,* and non spore forming of genus *Aeromonac* and genus *Klebsiella.* The species of genus identity of the isolated 20 strains of microorganisms from the region of Haskovo and Stara Zagora are defined in Valcheva, N., Microflora of spring mountain and mineral waters in the regions of Haskovo and Stara Zagora.

Selected are strains with high proteolytic and amylolytic activity. The highest amylolytic activity show *Bacillus thuringiensis* – 24,5 mm (GI5(50/GI5)), *Bacillus methylotrophicus* (YA1(47/YA1) (BM47)), Bacillus cereus – 24 mm (PBBGFRA (42/BGFRA)), which makes the above representatives of genus Bacillus a perspective option as producers of amylases in the biotechnological production. The highest proteolytic activity show *Bacillus thuringiensis* – 26,5 mm (PB(37/PBGK)), Bacillus cereus – 24,5 mm (GI6(36/GI6)), Bacillus cereus – 23,5 mm (GI3(54/GI3)), which makes these representatives of genus Bacillus a perspective option as producers of proteases in the biotechnological production (Valcheva N., 2014).

It is established antimicrobial activity of the strains *Bacillus* sp., against saprophytic and pathogenic microorganisms: *Penicillium* sp., *Fusarium moniliforme*, *Rhizopus* sp., *Aspergillus* niger*, Aspergillus oryzae, Aspergillus awamori*, *Mucor* sp. and *Enterococcus faecalis*, which is probably due to the produced bacteriocins in the process of development and growth of the four species Bacillus – *Bacillus cereus*, *Bacillus thuringiensis*, *Bacillus subtilis*, *Bacillus methylotrophicus*, as the most active strains can be pointed out *Bacillus methylotrophicus* (YA1(47/YA1) (BM47)), Bacillus cereus (R1(53/R1)), *Bacillus cereus* (GI2(55/GI2)) и *Bacillus thuringiensis* (HMB3(56/H3)), *Bacillus subtilis* (HMB1(46/H1)),which makes these representatives a perspective option as producers of bacteriocins.

Each strain reveals a set of activities that allows application in the biotechnology and for protection of plant species.

It is created a spore concentrate from Bacillus for application in protection of plants species from fungal diseases.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Valcheva N, Denkova Z, Denkova R. Physicochemical and microbiological characteristics of spring waters in Haskovo. Journal of Food and Packaging Science Technique and Technologies. 2013;2:21–25.
2. Valcheva N, Denkova Z, Nikolova R, Denkova R. Physiological, biochemical, and molecular – genetic characterization of bacterial strains isolated from sping and healing waters in region of Haskovo. Food, Sciense, Engineering and Technologies, Plovdiv. 2014;LX:940–946.
3. Valcheva N, Denkova Z, Nikolova R, Denkova R. Physiological - biochemical and molecular - genetic characteristics of bacterial strains isolated from spring and healing waters in the Haskovo region, N.T. at UCT. 2013;LX.
4. Valcheva N, Denkova Z, Denkova R, Nikolova R. Characterization of bacterial strains isolated from a thermal spring in Pavel Banya, Stara Zagora Region, N.T. at UCT. 2014;LXI.
5. Valcheva N. The microflora of medicinal and spring waters in haskovo and Stara Zagora Region, Dissertation, University of Food Technology. 2014;1–142.
6. Valcheva N, Ignatov I. Physicochemical and microbiological characteristics of thermal healing spring waters in the district of Varna. Journal of Medicine, Physiology and Biophysics. 2019;59:10-16.
7. Valcheva N. Physicochemical and microbiological characteristics of thermal healing spring waters in the district of Burgas, European Reviews of Chemistry. 2019;2.
8. Valcheva N. Physicochemical and microbiological characteristics of thermal healing spring waters in the districts of Varna and Burgas, Black Sea Region, Bulgaria. European Journal of Medicine. 2019;7(2):120-130.
9. Valcheva N, Ignatov I, Dinkov G. Microbiological and physicochemical research of thermal spring and mountain spring waters in the district of Sliven, Bulgaria. Journal of Advances in Microbiology. 2020;20(2):9-17.
10. Ignatov I, Mosin OV, Velikov B, Bauer E, Tyminski G. Longevity factors and mountain water as factor. Research in Mountain and Fields Areas in Bulgaria, Civil and Environmental Research. 2014; 30(4):51-60.
11. Ignatov I, Valcheva N. Microbiological and physicochemical research of mountain spring waters in Vasiliovska Mountain, Municipality of Teteven, Bulgaria, Chemical Science International Journal. 2020;29(3):16-23
12. Tumbarski Y, Valcheva N, Denkova Z, Koleva I. Antimicrobial activity against some saprophytic and pathogenic microorganisms of bacillus species strains isolated from natural sping waters in Bulgaria. British Microbiology Research Journal. 2014;4(12):1353–1369.
13. Tumbarski et al. Isolation, characterization and amino acid composition of a bacteriocin produced by *Bacillus methylotrophicus* strain BM47, Food Technology & Biotechnology. 2018;56(4): 546-552.
14. Velichkova K, Sirakov I, Rusenova N, Beev G, Denev S, Valcheva N, Denev T. *In vitro* antimicrobial activity on *Lemna minuta*, *Chlorella vulgaris* and *Spirulina* Sp. extracts. Fresenius Environmental Bulletin. 2018;27(8):5736-5741.
15. Asoodeh A, Lagzian M. Purification and Characterization of a new gluco amylo pullulanase from thermotolerant alkaliphilic *Bacillus subtilis* DR8806 of a Hot Mineral

Spring. Process Biochemistry. 2012;47(5): 806-815.

16. Biscardi, et al. The occurrence of cytotoxic aeromonas hydrophila strains in italian mineral and thermal waters. Sci Total Environ. 2002;292(3):255- 63.
17. Minnan, et al. Isolation and characterization of a high h2-producing strain *Klebsiella oxytoca* HP1 from a Hot Spring, Res. Microbiol. 2005;156(1):76-81.
18. Takai K, Horikoshi K. Molecular phylogenetic analysis of archaeal intron-containing genes coding for rRNA obtained from a deep-subsurface geothermal water pool, Appl. Environ. Microbiol. 1999;65: 5586–5589.

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