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Physical, Chemical, and Microbiological Characteristics of Mineral, Mountain, and Spring Waters from Bulgaria, and Biological Properties of Identified Microorganisms

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1. Introduction

The impact of different microorganisms on the environment is the subject of several studies. However, changes in our surroundings affect the degree and nature of the interaction between humans and the outside world. The pursuit of science and practice is aimed at creating optimal opportunities to limit the adverse influence of the living environment and the more complete and rational use of natural factors for healing. In this regard, sanitary microbiology studies not only the environment's microflora and its influence on humans but also the role of individual microorganisms in self-cleansing from organic and chemical pollution, biodegradation, and biotransformation of various substances. Water plays an essential role in human activity. Natural water sources are increasingly used for drinking, technical water supply, and medicinal purposes. It is a natural habitat for various microorganisms that form complex biocenoses, determining the biological balance. Intensive water use and the ever-increasing amount of wastewater can exhaust self-purification capabilities, disrupt the natural balance in the water and make the water balance unfit for use. Together with the wastewater, pathogenic microorganisms often fall, creating a potential epidemic situation, resulting in the water playing an essential role in spreading bacterial and viral infections. The main objective in the study of drinking water (from the central and non-central water supply) is to ensure good quality water for the population, which is carried out through effective control. The main goal of studying spring water is to ensure good quality of drinking water and treatment through effective and permanent physicochemical and microbiological control. Although water is an unfavorable environment for the growth of microorganisms, pathogenic and saprophytic microflora are found in spring waters.

2. Literature Review

The following conclusions can be made from the literature review:

-Water is an environment for the emergence of life, and scientists look for water on other planets besides Earth, regardless of the different conditions - temperature, pressure, and the atmosphere content.

-The drinking water in the food industry enterprises must meet the drinking water requirements. It determines the total number of bacteria, the number of coliforms, and pathogenic bacteria, the latter being controlled by the hygienic-epidemiological laboratories.

-There are many mineral, healing, and spring waters in Bulgaria, which are not subject to physicochemical and microbiological control but are used for the drinking needs of the population. Similar water sources are located in almost all regions in the country.

-Microorganisms with valuable properties and biologically active substances have been found in many minerals, hyperthermal, thermal, and non-thermal spring waters worldwide and in our country. This opens up a new opportunity for researchers to isolate and identify new microorganisms that may apply to people's lives.

-Data on the biological activity of microorganisms isolated from different water sources are very scarce. Determining these microorganisms' enzymatic, antimicrobial, and other biological properties is a highly prospective direction for biotechnology, the food industry, medicine, agriculture, and other fields of human life.

3. Goal And Objectives

The aim of the present dissertation thesis was to study the physical, chemical, and microbiological characterization of mineral, mountain, and spring waters from Bulgaria and the biochemical analysis of the microorganisms identified.

The following tasks were set in order to achieve this goal.

1.Physical, chemical, and microbiological characteristics of mineral, mountain, and spring waters from different regions of the country.

1.1. Analysis of water from different sources

1.2. Regulators of water indexes

1.3. New sources of biologically active substances and their application to drinking water

2. Molecular-genetic identification of microorganisms isolated from water.

3. Determination of biological properties of identified strains of microorganisms.

3.1. Enzyme activity

3.2. Antimicrobial activity

3.3. The adhesive capacity to human epithelial cells

4. Materials and Methods

4.1. Materials

4.1.1. Waters: Mineral, mountain, and spring waters from 11 regions in the country – Haskovo, Stara

Zagora, Sliven, Yambol, Plovdiv, Pazardzhik, Burgas, Varna, Sofia, Lovech, and Blagoevgrad – for seven years (from 2015 to 2021) were investigated in this study.

4.1.2. Microorganisms: Reference strains of bacteria, yeasts, and molds were used, provided by NBPMKK, Sofia, as well as microorganisms from the Department of “Biological Sciences” collection at the Faculty of Agriculture of Thrace University, Stara Zagora.

4.1.3. Microbial Culture Media: Different nutrient media were used.

4.1.4. Reagents: Different reagents were used.

4.2. Methods

4.2.1. Physical, Chemical and Biological Analyses: Color on the Rublev scale; Smell; Density; pH; Oxidability; Chloride content; Nitrate content - VLM-NO3; Nitrite content - VLM-NO3; Content of ammonium ions – VLM-NH4; General hardness; Sulfate content – VLMSO4; Calcium content; Magnesium content; Phosphate content – VLM-PO4; Manganese content – VLM-Mn; Iron content – VLM-Fe; Fluoride content – VLM-F; Determination of electrical conductivity; preparation of solutions of metal ions. Preparation of dry extracts. The plant species L. minuta Kunth. was extracted for 24 h with two extractants – 96% ethanol and methanol at a hydro module of 1:4. This is followed by filtration of the obtained extracts and evaporation of the extractants on a rotary vacuum evaporator at a water temperature of 55°C. Determination of biochemical profile. API 50 CHB and API 20 E (BioMerieux SA, France) system was used to identify the isolated bacterial species based on the consumption of 49 carbon sources. The obtained results were processed with apiwebR identification software. Enzyme activity profile study. The API ZYM system (BioMerieux, France) was used for semi-quantitative determination of the enzyme profile of the studied strains. Determination of the adhesion of microorganisms. The epithelial cell line HT-29 was purchased from the American Culture Collection (ATCC) and maintained according to its instructions.

Determination of the immunomodulation of microorganisms. The epithelial cell line CaCo-2 was cultured as a monolayer in DMEM (Dulbecco’s modified Eagle’s medium, Gibco, UK) supplemented with 10% fetal bovine serum.

4.2.2. Microbiological Analyses: The methods for determining microbiological indicators have been applied according to Ordinance No 9/2001, SG No 30, and Decree No 178/23.07.2004 on the quality of water intended for drinking purposes: determination of Escherichia coli and coliform bacteria; determination of enterococci; determination of sulfite-reducing anaerobes spores; determination of the total number of aerobic and facultatively anaerobic bacteria; determination of Pseudomonas aeruginosa. Determination of coli-titer by Ginchev’s method.

Determination of antimicrobial activity against saprophytic and pathogenic microorganisms by agar-well diffusion method (d = 6 mm) with sterile zones around the wells. The diameters of the sterile zones in mm are determined.

Isolation of total DNA - PCR reactions and visualization; 16S rDNA amplification; Gene sequencing of purified 16S rRNA. All experiments in the thesis were carried out in triplicate, and the data is presented ± standard error.

The data presented in the figures were processed with the Microsoft software using statistical functions to determine the standard deviation and maximum error of estimate at significance levels $\alpha < 0.05$.

5. Results and Discussions

5.1. Physical, Chemical and Microbiological Characteristics of Mineral, Mountain and Spring Waters From Different Regions of the Country

5.1.1. Analysis of water from different sources

***Haskovo Region**

Water from the following sources was tested:

1.Thermal mineral spring “Driller No 2VP”

2.Thermal mineral spring “Driller No ZVP”

3.Thermal mineral spring “Driller No 4VP”

4.Thermal healing spring “KEI No 5”

The physical and chemical analysis results show that the studied waters differ in the values of the studied indicators, which their origin can explain. The lowest temperature is those from the thermal mineral spring “Driller No 3VP” (53.5°C), and the highest is from the thermal healing spring “KEI No 5” (58.6°C). The waters from the thermal mineral spring “Driller No 2VP” have the highest pH (7.99). The values of chlorides are lower than the maximum permissible, but those of sulfates, calcium, and fluorides are higher. Total mineralization values are comparable. The content of the studied micro-components and the values of the radiological indicators (after an expert assessment of the total indicative dose) are within the limits of the mineral waters norms. The results of the microbiological analysis show that the tested waters meet the standards, with the total number of microorganisms developing at 22°C and 37°C being four to five times lower.

Conclusion: The investigated waters from the Haskovo region

-Comply with Ordinance No 14 on resort resources, resort areas, and resorts (SG, No 79 of 1987, adopted, amended, No 70 of 2004).

-Comply with all controlled physicochemical parameters of Ordinance No 9/2001, SG No 30, and Decree No 178/23.07.2004 on the quality of drinking water.

-Comply with all controlled microbiological parameters of Ordinance No 9/2001, SG No 30, and Decree No 178/23.07.2004 on the quality of drinking water.

***Stara Zagora Region**

Water from the following sources was investigated:

1. Thermal healing spring “Driller No Sz-7”, Pavel banya
2. Thermal mineral spring “Driller No Sz-8”, Pavel banya
3. Thermal healing spring “Driller No X-19”, Pavel banya
4. Hyperthermal healing spring “Driller No 3”, Pavel banya
5. Hyperthermal healing spring “Driller I K-3”, in the village of Ovoshtnik
6. Thermal healing spring “Driller I Sz-37” in the village of Yagoda
7. Hypothermal spring in the village of Trakia
8. Hypothermal spring “St. Nikolay the Chudotvoretz”
9. Hypothermal spring “St. Bogoroditsa” in the municipality of Maglizh
10.”Center” hypothermal spring in Maglizh municipality
11. Hypothermal spring in the village of Koprinka in the municipality of Maglizh
12. Hypothermal spring in the town of Kazanlak
13. Hypothermal spring in the village of Kren
14. Hypothermal spring in the town of Enina
15. Ayazmo hypothermal spring
16.”Trite chuchura” hypothermal spring

The physical and chemical analysis results showed that the studied waters differ in the values of the studied indicators, which could be explained by their origin. The lowest temperature was obtained for those of all the hypothermal springs (20-21°C), and the highest results were from the hyperthermal healing spring “Driller No K-3” in the village of Ovoschtnik (78.2°C). The highest pH (8.68) value is the water from the thermal healing spring “Driller No Sz - 37” village of Yagoda. The waters from six sources – thermal healing spring “Driller No C3-7”, Pavel banya, thermal mineral spring “Driller No C3-8”, Pavel banya, thermal healing spring “Driller No X19”, Pavel banya, hyperthermal healing spring “Driller No 3”, Pavel banya, hyperthermal healing spring “Driller I K-3” in the village of Ovoshtnik and thermal healing spring “Driller I Sz-37” in the village of Yagoda have an unpleasant smell due to the presence of hydrogen sulfide. The waters from these six sources also have an increased content of fluorides, which is many times No K-3” in the village of Ovoshtnik, it is the highest in Bulgaria (24.50 mg/dm3). The waters from a hypothermal spring in the village of Trakia had a high content of nitrates, and that of manganese and iron exceeded the maximum permissible concentrations. The values for total mineralization were very different, being the lowest for the waters from the hypothermal spring “St. Nikolay Chudotvoretz”.

The content of the studied microcomponents and the values of the radiological indicators (after an expert assessment of the total indicative dose) were within the limits of the norms for mineral waters. The results of the microbiological analysis showed that the tested waters correspond to the standards, except those from the hypothermal springs. The content of coliforms and the Gram-negative bacterium Escherichia coli was found to be higher. Conclusion: the investigated waters from the Stara Zagora region

-Comply with Ordinance No 14 on resort resources, resort areas, and resorts (SG, No 79 of 1987, adopted, amended, No 70 of 2004).

-Comply with all controlled physicochemical parameters of Ordinance No 9/2001, SG No 30, and Decree No 178/23.07.2004 on the quality of drinking water, except waters with a high content of fluorides, nitrates, manganese, and iron.

-Comply with all microbiological parameters of Ordinance No 9/2001, SG No 30, and Decree No 178/23.07.2004 on water quality for drinking, except for hypothermal springs.

***Yambol Region**

Water from the following sources was investigated:

1. Hypothermal spring in the village of Okop
2. Hypothermal spring in the village of Karavelovo
3. Hypothermal healing spring “Driller Y No 33” in the village of Stefan Karadjovo

The results of the physical and chemical analysis showed that the studied water samples differ in the studied indicators, which could be explained by their origin. Water temperature (20-21.3) and pH (6.65-7.4) are comparable. The water from the hypothermal healing spring “Driller Ya No-33” in the village of

Stefan Karadjovo had an unacceptable smell due to hydrogen sulfide. These water samples also had two times higher chloride content than the other two, and the values of the three samples were significantly lower than the maximum permissible concentrations. Their calcium content exceeds the values of the two different water samples, and the maximum permitted. The values for total mineralization of the waters from the hypothermal spring from the village of Okop and the hypothermal spring in Karavelovo village were comparable. Still, they were five times lower than those from the hypothermal healing spring “Driller Y No-33” village of Stefan Karadjovo. The content of the studied microcomponents and the values of the radiological indicators (after an expert assessment of the total indicative dose) were within the limits of the mineral waters’ norms. The results of the microbiological analysis showed that the tested water samples met the standards, except for a hypothermal spring in Okop village and a hypothermal spring in “Driller No 33” in Stefan Karadjovo village. Elevated rates of coliforms, the Gram-negative bacterium Escherichia coli, and enterococci, as well as the total number of microorganisms developing at 22°C and 37°C, were found in them.

Conclusion: The investigated waters of the Yambol region

-complies with Ordinance No 14 on resort resources, resort areas, and resorts (SG, No 79 of 1987, adopted, amended, No 70 of 2004), except those from the hypothermic healing spring “Driller No 33” in the village of Stefan Karadjovo.

-Comply with all controlled physicochemical parameters of Ordinance No 9/2001, SG No 30, and Decree No 178/23.07.2004 on the water quality for drinking, except those from a hypothermal spring in the village of Okop and a hypothermal spring in “Driller No 33” in Stefan Karadjovo village.

-Comply with all controlled microbiological parameters of Ordinance No 9/2001, SG No 30, and Decree No 178/23.07.2004 on the water quality for drinking purposes, except those from a hypothermal spring in the village of Okop and a hypothermal healing spring “Driller No 33” in Stefan Karadjovo.

***Sliven Region**

Water from the following sources was investigated:

1. Thermal healing spring “Driller 22”, Sliven mineral baths
2. Thermal healing spring in the village of Banya
3. KEI “Hadzhi Dimitar” hypothermal healing spring
4.”Gunchov Izvor” hypothermal healing spring
5. Hypothermal spring in Nova Zagora

The physical and chemical analysis results showed that the studied waters differ in the values of the studied indicators, which their origin can explain. The lowest temperature was obtained for those from the hypothermal healing spring “Gunčov Izvor” (21.5°C) and the hypothermal spring in Nova Zagora (21°C). The highest temperature values were obtained from the thermal healing spring in the village of Banya (57°C). The water samples from a hypothermal spring in Nova Zagora had the highest pH (8.12). The amount of sulfate in the water from the “Driller 22” thermal healing spring from the Sliven mineral baths is almost twice as much as the maximum permissible values. Those from the “Driller 22” thermal healing spring, the Sliven mineral baths, the thermal healing spring in the village of Banya, and the hypothermal spring in Nova Zagora have an increased content of fluorides. The content of the studied microcomponents and the radiological indicators (after an expert assessment of the total indicative dose) are within the limits of the mineral waters norms. The results of the microbiological analysis showed that all the tested waters correspond to the norms in terms of microbial indicators, except the hypothermal healing spring “Gunchov Izvor,” where the content of coliforms and the Gram-negative bacterium Escherichia coli was increased.

Conclusion: The studied waters from the Sliven region

-Comply with Ordinance No 14 on resort resources, resort areas, and resorts (SG, No 79 of 1987, adopted, amended, No 70 of 2004).

-Do not comply with all controlled physicochemical parameters of Ordinance No 9/2001, SG No 30, and Decree No 178/23.07.2004 on the quality of drinking water, except those from the thermal healing spring “Driller 22”, Sliven mineral baths, regarding sulfates and fluorides, thermal healing spring in the village of Banya and hypothermal spring in Nova Zagora, regarding fluorides.

-Comply with all controlled microbiological parameters of Ordinance No 9/2001, SG No 30, and Decree No 178/23.07.2004 on the quality of drinking water, except those from the hypothermal healing spring “Gunchov Izvor.”

***Burgas Region**

Water from the following sources was investigated:

1. Thermal healing spring Burgas mineral baths
2. Hyperthermal healing spring in the village of Shivarovo
3. Thermal healing spring in the village of Polyanovo
4.”Driller B-12” hypothermal healing spring in the village of Sadievo
5. Thermal healing spring “Driller B- No 73 Medovo”
6. Hypothermal healing spring “Driller B- No 53 Kamenar”

The physical and chemical analysis results show that the studied waters differ in the values of the studied indicators, which their origin can explain. The lowest temperature was obtained for those from the hypothermal healing spring “Driller B-No 53 Kamenar” (21.3°C) and the highest - from the thermal healing spring in the village of Polyanovo (51°C). The pH values of the water samples from the thermal healing spring Burgas mineral baths (9.95), from the hypothermal healing spring “Driller B-12” in Sadievo (9.71), from the thermal healing spring “Driller B- No 73 Medovo” (9.77) and hypothermal healing spring “Driller B- No 53 Kamenar” (9.66) exceeded the maximum permissibly. The water samples from the thermal healing spring “Driller B-No 73 Medovo” and the hypothermal healing spring “Driller B-No 53 Kamenar” were with the lowest amount of calcium (about 90 times lower compared to the maximum permissible values). All water samples had a high content of fluorides, except those from a hyperthermal healing spring in Shivarovo. The microcomponents’ content and the radiological indicators’ values (after an expert assessment of the total indicative dose) were within the limits of the mineral water norms. The microbiological analysis results showed that the tested waters, except the hypothermal healing spring “Driller B-12” in Sadievo, meet the indicators’ standards.

Conclusion: The investigated waters from the Burgas region

-Comply with Ordinance No 14 on resort resources, resort areas, and resorts (SG, No 79 of 1987, adopted, amended, No 70 of 2004).

-Do not comply with all controlled physicochemical parameters of Ordinance No 9/2001, SG No 30, and Decree No 178/23.07.2004 on the quality of drinking water, except those from the hyperthermal medicinal spring “Shivarovo” and the hyperthermal medicinal spring “Polyanovo.”

-Comply with all controlled microbiological parameters of Ordinance No 9/2001, SG No 30, and Decree No 178/23.07.2004 on drinking water quality, except the hypothermal healing spring “Driller B-12” in Sadievo.

***Varna Region**

Water from the following sources was investigated:

1. Thermal healing spring (Driller No P-83x KK “St. Constantine and Elena”
2. Thermal healing spring (P-1x “Aquarium”)
3. Thermal healing spring P-106 x “Dom Mladost”
4. Thermal healing spring P-161 x at the “Primorski” pool
5. “Lekovita Voda” thermal healing spring, Goren Chiflik village

The physical and chemical analysis results show that the studied waters differ in the values of the studied indicators, which their origin could explain. The lowest temperature values were those from thermal healing spring P-106 x “Dom Mladost” (40°C), and the highest – thermal healing spring R-161 x at the pool “Primorski” (50°C). The highest pH values were the waters from the thermal healing spring P-106 x “Dom Mladost” (9.48) and the thermal healing spring P-161 x at the pool “Primorski” (9.46). The water samples from a thermal healing spring (No P-83x KK “St. Constantine and Elena” and a thermal healing spring (P-1x “Aquarium”) had an unpleasant smell due to the presence of hydrogen sulfide. The other indicators’ values were lower than the maximum permissible ones. There is an exception for those from the thermal healing spring P-106 x “Dom Mladost” and the thermal healing spring P-161 x at the pool “Primorski,” in which the content of iron and ammonium ions was, respectively, twice the maximum permissible values. The content of the studied microcomponents and the radiological indicators (after an expert assessment of the total indicative dose) were within the limits of the mineral water norms. The microbiological analysis results show that the tested waters meet the standards except for the thermal healing spring “Lekovita Voda” in Goren Chiflik village. Elevated rates of coliforms, the Gram-negative bacterium Escherichia coli, and enterococci, as well as the total count of microorganisms developed at 22°C and 37°C for 48 hours, were found in them.

Conclusion: The investigated water samples from the Varna region:

-Comply with Ordinance No 14 on resort resources, resort areas, and resorts (SG, No 9 of 1987, adopted, amended, No 70 of 2004).

-Comply with all controlled physicochemical parameters of Ordinance No 9/2001, SG No 30, and Decree No 178/23.07.2004 on the water quality for drinking, except those from a thermal healing spring (Driller No P-83x KK “St. Constantine and Elena” and a thermal healing spring (P-1x “Aquarium”), regarding the smell of hydrogen sulfide, which is unacceptable. And thermal healing spring P-161-x at the “Primorski” pool, regarding ammonium ions.

-Comply with all controlled microbiological parameters of Ordinance No 9/2001, SG No 30, and Decree No 178/23.07.2004 on the water quality for drinking, except the thermal healing spring “Lekovita voda” in the village of Goren Chiflik.

***Plovdiv Region**

Water from the following sources was investigated:

1. Thermal healing spring “KEI Momina banya” in Hisarya is located within the regulatory limits of Hisarya, on the left bank of Tekedere
2. Thermal healing spring “KEI Momina salza” in Hisarya is located within the regulatory limits of Hisarya, between the remains of a Roman fortress
3. Subthermal spring “KEI Stublata” in Hisarya
4. Thermal healing spring “KEI Toplicata” in Hisarya
5. “Fresh” thermal healing spring in Hisarya
6. Thermal healing spring “Bistrica” in Hisarya
7. Thermal healing spring “Bancheto Miromir” in Hisarya
8. “Choban cheshma” thermal healing spring in Hisarya
9. “Chair banya” thermal healing spring in Hisarya
10. “Driller No 1” thermal healing spring in Hisarya
11. Hypothermal healing spring “Staro Zhelezare - Driller No 2” in Hisarya
12. “Driller No 3” thermal healing spring in Hisarya

13. “Driller 4” hypothermal healing spring in Hisarya
14. “Driller No 5” thermal healing spring in Hisarya
15. “Driller No 6” thermal healing spring in Hisarya
16. Thermal healing spring “Driller No 7” in Hisarya
17. Thermal healing spring “Parilkite” in Hisarya
18. “Bulgarian Rose” thermal healing spring in Banya
19. Thermal healing spring KEI “Central catchment” in Banya
20. Isothermal healing spring KEI “Male Bath” in Banya
21. Isothermal healing spring KEI “Women’s Bath” in Banya
22. Thermal healing spring “Driller 1 - Kokalche” in Banya
23. Subthermal healing spring “Driller No 8” in Dragoynovo
24. “Driller No 9” hypothermal healing spring in Dragoynovo
25. Thermal healing spring “Driller 16-Lenovo” in Lenovo
26. Hypothermal healing spring “Driller 1-Asenovgrad” in Asenovgrad
27. Hypothermal healing spring “KEI-Banski catchment” in Narechenski bani
28. “Salty Spring” hypothermal healing spring in Narechenski Bani
29. Hypothermal healing spring “Ochno Izvorche” in Narechenski Bani
30. Hypothermal spring “Bajova voda” in Bachkovo

The physical and chemical analysis results showed that the studied waters differ in the values of the studied indicators, which their origin can explain. The lowest temperature values were those from the subthermal healing spring “Driller No 8” in Dragoynovo (18.3°C), and the highest - from the thermal healing spring “Driller No 6” in Hisarya (50.9°C) and from the thermal healing spring “KEI Toplitsata” in Hisarya (51°C). The highest pH values were obtained for the water samples from the thermal healing spring “Driller 16-Lenovo” in Lenovo (9.53), above the maximum permissible values. The water samples from the Driller “Bulgarian Rose” thermal healing spring in Banya, the thermal healing spring KEI “Central catchment” in Banya, the isothermal healing spring KEI “Mazhko Banche” in Banya, the isothermal healing spring KEI “Women’s bath” in Banya, the thermal healing spring “Driller 1-Kokalche” in Banya and the subthermal healing spring “Driller No 8” in Dragoynovo had an unpleasant smell due to the presence of hydrogen sulfide. The water samples from the sources in Hisarya and Banya, the thermal healing spring “Driller 16 - Lenovo” in Lenovo, and the hypothermal healing spring “KEI - Banski Catchment” in Narechenski Bani had high concentrations of fluorides, which repeatedly exceeded the maximum permissible values. The water samples from the springs in Narechenski Bani village had twice the sulfate content compared to the maximum permissible values. The same water samples also had the highest total mineralization. The content of the studied microcomponents and the values of the radiological indicators (after an expert assessment of the total indicative dose) are within the limits of the norms for mineral waters.

The results of the microbiological analysis showed that the tested water samples correspond to the norms, except for the hyperthermal healing water “Parilke” in Hisarya and the isothermal healing water “KEI Men’s Bath” in Banya. The content of coliforms, the Gramnegative bacterium Escherichia coli, and enterococci was increased.

Conclusion: The investigated water samples from the Plovdiv region – comply with Ordinance No 14 on resort resources, resort areas, and resorts (SG, No 79 of 1987, adopted, amended, No 70 of 2004).

-Do not comply with all controlled physicochemical parameters of Ordinance No

9/2001, SG No 30, and Decree No 178/23.07.2004 on the quality of water for drinking purposes, except the healing spring “Driller No 9” Dragoynovo village, the healing spring “Driller 1-Asenovgrad” Asenovgrad and the spring “Bajova Voda” Bachkovo village.

-Comply with all controlled microbiological parameters of Ordinance No 9/2001, SG No 30, and Decree No 178/23.07.2004 on drinking water quality, except those of hyperthermal medicinal water “Parilkite” in Hisarya and isothermal medicinal water “KEI Men’s Bath” in Banya.

***Pazardzhik Region**

Water from the following sources was investigated:

1. Hyperthermal healing spring “Driller No 3-Varvara” in Varvara village
2. Hyperthermal healing spring “Driller No 5-Varvara” in Varvara village
3. Hyperthermal healing spring “Driller No 6-Varvara” in Varvara village
4. Thermal healing spring KEI No 2 “Vetren dol” in Varvara village
5. Isothermal healing spring KEI No 1 “Bancheto” in Banya village
6. Thermal healing spring Driller No 2 “Naklonen” in Banya village
7. Subthermal spring in the town of Oborishte
8. Subthermal spring in Oborishte – Panagyurishte
9. Panagyurishte hypothermal spring – Buta
10. “Strelcha” thermal healing spring
11. Hyperthermal spring Driller No 5 “Syarna Banya-Velingrad”
12. Hyperthermal healing spring Driller No 3 “Mizinka-Velingrad”
13. Thermal healing spring” Driller No 7KG “Velova Banya-Velingrad”

The results of the physical and chemical analysis showed that the studied water samples differ in the values of the studied indicators, which their origin can explain. The lowest temperature was from a subthermal spring in Oborishte - Panagyurishte (18°C), and the highest – was from a hyperthermal healing spring “Driller No 3 “Mizinka-Velingrad” (87.7°C). The highest pH value was obtained for the water sample from the hyperthermal spring Driller No 5 “Syarna Banya-Velingrad” (9.35).

The water samples from Varvara’s thermal healing spring “Strelcha” had an unpleasant smell due to hydrogen sulfide.The water samples from the hyperthermal healing spring “Driller No 3-Varvara” in Varvara village, the hyperthermal healing spring “Driller No 6-Varvara” in Varvara had a higher content of sulfates compared to the maximum permissible values. All water samples, except those from the sources in the city of Panagyurishte, had high concentrations of fluorides, which many times exceeded the maximum permissible values. The water samples from the hyperthermal healing springs in Varvara village had the highest total mineralization. The content of the studied microcomponents and the values of the radiological indicators (after an expert assessment of the total indicative dose) were within the limits of mineral waters norms. The results of the microbiological analysis showed that the studied water samples meet the norms, except those from the subthermal spring in the town of Oborishte, the subthermal spring in Oborishte – Panagyurishte, and the hypothermal spring Panagyurishte – Buta. The content of coliforms, the Gram-negative bacterium Escherichia coli, and enterococci was increased.

Conclusion: The investigated waters from the Pazardzhik region

-Comply with Ordinance No 14 on resort resources, resort areas, and resorts (SG, No 79 of 1987, adopted, amended, No 70 of 2004).

-Do not comply with all controlled physicochemical parameters of Ordinance No 9/2001, SG No 30, and Decree No 178/23.07.2004 on the drinking water quality, due to the increased content of sulfates and fluorides, except a subthermal spring in Oborishte, a subthermal spring in Oborishte - Panagyurishte and a hypothermal spring in Panagyurishte – Buta.

-Comply with all controlled microbiological parameters of Ordinance No 9/2001, SG No 30, and Decree No 178/23.07.2004 on the quality of drinking, except those from a subthermal spring in Oborishte, a subthermal spring in Oborishte – Panagyurishte, a hypothermal spring in Panagyurishte – Buta, regarding coliforms, the Gram-negative bacteria Escherichia coli and enterococci.

***Sofia Region**

Water from the following sources was investigated:

1. Hypothermal healing spring “Driller TK No 1 “Ivanyane,” the town of Bankya
2. Isothermal healing spring “Driller No 3-Gorna Banya”
3. Hyperthermal healing spring KEI “Pchelinski Bani”
4. Thermal healing spring KEI “Sofia-Center”

The physical and chemical analysis results show that the studied water samples differ in the values of the studied indicators, which their origin can explain. The lowest temperature was the water from the hypothermal healing spring “Driller TK No 1 “Ivanyane,” Bankya (27°C), and the highest - was from the hyperthermal healing spring KEI “Pchelinski Bani” (79°C). The highest pH values were those from the isothermal healing spring “Driller No 3-Gorna Banya” (9.79), which were close to pH 10 and were natural catholyte with an antioxidant effect.

The water from KEI “Pchelinski Bani” had an unpleasant smell due to hydrogen sulfide. At the same time, the values of chlorides and calcium were higher than those of the other water samples, and the sulfate and fluoride concentrations were two and six times higher than the maximum permissible. The waters from this source also had the highest values of total mineralization. The content of the studied microcomponents and the values of the radiological indicators (after an expert assessment of the total indicative dose) were within the limits of mineral water norms. The results of the microbiological analysis showed that the tested waters correspond to the standards, with the total number of microorganisms developing at 22°C and 37°C being three to ten times lower.

Conclusion: The investigated water samples from the Sofia region

-Comply with Ordinance No 14 on resort resources, resort areas, and resorts (SG, No 79 of 1987, adopted, amended, No 70 of 2004).

-Comply with all controlled physicochemical parameters of Ordinance No 9/2001, SG No 30, and Decree No 178/23.07.2004 on the drinking water quality except those from KEI “Pchelinski Bani” and isothermal healing spring “Driller No 3 - Gorna Banya.

-Comply with all controlled microbiological parameters of Ordinance No 9/2001, SG No 30, and Decree No 178/23.07.2004 on drinking water quality.

***Lovech Region**

Water from the following sources was investigated:

1. Thermal healing spring “Driller No P-1hg-Krushuna”
2. Hypothermal healing “Driller No L-2-Shipkovo”
3. Thermal healing spring “Driller No P-1hg”

The physical and chemical analysis results show that the studied waters differ in the values of the studied indicators, which their origin can explain. The water with the lowest temperature was the hypothermal healing “Driller No L-2-Shipkovo” (31.7°C), and the highest – was from the thermal healing spring “Driller No P-1hg-Krushuna” (58°C). The water’s pH values were highest from the thermal healing spring “Driller No P3-Gorna 1hg - Chiflik” (8.22). All water samples had an unpleasant odor due to hydrogen sulfide.

The water samples from the thermal healing spring “Driller No

P-1hg-Krushuna had extremely high values of chlorides, sulfates, calcium, and fluorides compared to the maximum permissible, which also determined their highest total mineralization – 10892 mg/dm3 in Bulgaria.

The content of the studied microcomponents and the values of the radiological indicators (after an expert assessment of the total indicative dose) were within the limits of mineral water norms.

The results of the microbiological analysis showed that the tested waters correspond to the standards, and the total number of microorganisms developing at 22°C and 37°C was many times lower.

Conclusion: The investigated water samples from the Lovech region

-Comply with Ordinance No 14 on resort resources, resort areas, and resorts (SG, No 79 of 1987, adopted, amended, No 70 of 2004).

-Do not comply with all controlled physicochemical parameters of Ordinance No 9/2001, SG No 30, and Decree No 178/23.07.2004 on drinking water quality. All mountain healing springs had a smell of sulfur.

-Comply with all controlled microbiological parameters of Ordinance No 9/2001, SG No 30, and Decree No 178/23.07.2004 on drinking water quality.

***Blagoevgrad Region**

Water from the following sources was investigated:

1. “Rupi 1” hyperthermal healing spring
2. “Rupi 2” hyperthermal healing spring

The physical and chemical analysis results show that the studied waters differ in the values of the studied indicators, which their origin can explain. The water samples had comparable values of temperature (73.5°C and 74.3°C) and pH (6.97 and 7.06). The smell of the tested waters was unpleasant due to the presence of hydrogen sulfide. The amounts of fluorides in both water samples were higher than the maximum permissible, and those of the other salts were lower. The content of the studied microcomponents and the values of the radiological indicators (after an expert assessment of the total indicative dose) were within the limits of mineral water norms.

The results of the microbiological analysis showed that the studied mineral geothermal hyperthermal springs did not meet the indicator – the total number of microorganisms at 37°C, and their number was above the permissible values for drinking water. The presence of coliforms and the Gram-negative bacterium Escherichia coli was also found.

Conclusion: The investigated waters from the Blagoevgrad region

-Comply with Ordinance No 14 on resort resources, resort areas, and resorts (SG, No 79 of 1987, adopted, amended, No 70 of 2004).

-Do not comply with all controlled physicochemical parameters of Ordinance No 9/2001, SG No 30, and Decree No 178/23.07.2004 on drinking water quality.

-Comply with all controlled microbiological parameters of Ordinance No 9/2001, SG No 30, and Decree No 78/23.07.2004 on drinking water quality except the “Rupi 2” hyperthermal healing spring, regarding coliforms, the Gram-negative bacterium Escherichia

coli and the total number of microorganisms at 37°C.

The comparative analysis of the physical and chemical parameters is important for microorganism diversity, and the synthesis of biologically active substances from them is summarized in the Table 1. The temperature, pH of the environment, salts (the species composition of cations and anions), and gases in water sources were of the most significant importance for the biodiversity of the identified microorganisms and the synthesis of biologically active substances.

***Temperature:** According to the EU qualification, waters are classified as cold, warm, and hot. Cold waters are those whose water temperature is lower than 37°C, warm with a temperature of 37°C to 60°C, and hot with a source water temperature equal to or higher than 60°C. The studied springs’ temperatures in the largest quantity were warm – 44, cold – 36, and the least hot – 10. Hot water from the studied springs is available in 10 regions, except Blagoevgrad. Cold water from the studied springs is available in seven regions, except for four - Haskovo, Varna, Pazardzhik and Blagoevgrad. Hot water from the studied springs is available only in four studied areas - Stara Zagora, Pazardzhik, Sofia, and Blagoevgrad. The mineral springs (Fig. 1) in the regions of Pazardzhik - 87.7°C “Mizinka”, Sofia - 79°C KEI “Pchelinski Bani,” Stara Zagora – 78.8°C “Ovoschtnik” and Blagoevgrad, where the geyser with the highest temperature in Europe “Separeva Banya” is located – 101.2°C and “Rupi 2”, in a geothermal zone with 74.3°C. These four springs are hyperthermal, and the microorganisms identified in them withstand extreme conditions, which is very important for their eventual application in the food industry, medicine, agriculture, biotechnology, etc.

Table 1(43): Comparative characteristics of the investigated waters in Bulgaria.

Region	Temparature OC	pH	Total Mineralization mg/dm3	Electrical Conductivity s/cm	Ca2+ ,mg/ dm3	Na+,mg/ dm3	Mg2+,mg/ dm3	SO42- ,mg/ dm3	Cl- ,mg/ dm3	HCO3– mg/dm3	H2SiO3 mg/ dm3	F-, mg/ dm3	Gases, H2S/ CO2 mg/ dm3 Ra Bq/1
Haskovo	53.50-58.6	7.23- 7.94	1667.00- 1668	2030 - 2066	176.35- 180.36	317.30- 328	3.00- 4.05	839.87- 86.27	60.80- 63.82	73.22- 136.48	74.70- 88.41	3.96- 4.05	H2S265.88- 336.26 CO220.68- 22.9
Stara Zagora	20.00- 78.2	6.70- 8.73	467.32-652	180-668	2.20- 140	112.00- 156.33	0.12 -29.16	17.69- 144	2.25- 22.68	109.70- 352.89	85.34- 94.38	0.01- 24.5	-
Yambol	20.00- 21	6.65- 7.4	1392	841- 1604	30- 193.11	873	57.76	21.00- 73.11	20,00- 2.67	873.48	25.98	0.10- 0.85	H2S 0.55 CO2 493
Sliven	21.00- 57	6.91- 8.12	392.00- 1978.41	21- 450	510.10- 110	5.30- 386	24.32- 37.21	16.05- 475.28	3.55- 45.7	254.24	35.4	0.40- 4.53	CH4
Burgas	21.30- 51	6.91- 9.95	316.15- 526	422- 633	1.24- 120	82.73- 149.4	0.12- 67	19.55-37	78.00- 8	72.00- 73.65	42.00- 52.69	0.40- 7.73	H2S <1.4 Ra 36.56
Varna	40.00- 50	7.40- 9.48	596	350- 768	41.00- 120	49.24- 53.56	21.00- 68	36.21- 77	64.41- 100	297.00- 328	25.55- 30.32	0.43- 0.71	CH4, J2
Plovdiv	18.30- 51	7.22- 9.53	249.00- 419	25.00- 1882	3.01- 82.16	59.91- 300.35	<0.12- 46	7.32- 540	7.80- 55.67	65.90- 457.64	56.59- 123.5	0.24- 5.5	H2S<0.05Ra 100-1200
Pazar dhik	59.00- 87.7	7.90- 9.39	655.00- 768	827- 1035	11.50- 53.11	132.15- 171.49	0.12- 15.86	24.00- 309.24	18.30- 5.17	79.32- 130.58	54.10- 105	3.82- 10.58	H2S 0.87
Sofia	27.00 79.00	8.15 9.79	142.85 915	169	1.4 30.86	29.24 223.05	< 0.97	19.75 440.51	2.66 18.79	78.3	37.67 102.77	0.16 9.48	H2S 7.30 CO2 32.07
Lovech	31.70 58.00	7.40 8.22	335- 10892	8,26 916	24.05 741.48	8.26 2729	27.36 231.04	33.54 2133.60	3.72 4680	32.99 219.66	18.49- 44.21	1.38- 3.49	CO2 15.48 H2S 15.48
Blagoev grad	73.50 74.30	6.97 7.06	2296	2160 2230	31.06 80.24	509.5	13.38 14.35	80.24 92.59	36.173 6.45	1495- 1526	79.74- 83.00	5.99 -6.09	CO2 < 89.16 H2S 0.53 Ra 2.62

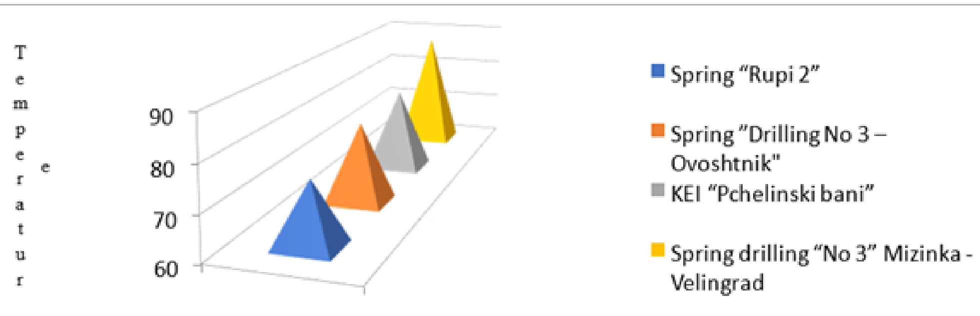

Figure 1: Highest water temperature values in the investigated waters in Bulgaria.

* The number in parentheses corresponds to the dissertation

***pH:** Spring waters are considered neutral when the concentration of hydrogen cations is from 6.8 to 7.2. They are acidic at pH lower than 6.8, at pH higher than 7.2 – alkaline, and pH 7 – neutral. The investigated common, mountain, and mineral spring waters are primarily alkaline; in second place are neutral, and there are only two – with an acidic reaction in Yambol and Stara Zagora regions. There are neutral and alkaline water samples in five regions – Haskovo, Stara Zagora, Yambol, Sliven, and Burgas. There are alkaline waters in Varna, Pazardzhik, Sofia, and Lovech. There are only neutral waters in Blagoevgrad – Rupite region. The highest and lowest pH values in the studied waters are summarized in Fig 2. Neutral and alkaline water samples are most favorable for developing bacteria and synthesizing biologically active substances. The ability to adapt to high or low-temperature values, pH, the concentration of salts, and gases, which are extreme conditions for bacteria, is critical. Alkaline waters have an antioxidant effect, and acidic waters have a bactericidal effect. Some of the ice springs are natural catholytes, anolytes because the pH of the medium is about 10 or about 6.5. Under such extreme conditions, spore-forming bacteria of the Bacillus mainly develop and multiply.

***Salt and Gas Content:** None of the studied 90 ordinary, mountain, and mineral springs from 11 regions had the same physicochemical composition according to the controlled parameters. All areas were unique in their salt and gas content. The warm neutral, alkaline spring water, and the great variety of salts and gases in the studied springs, lead to the great biodiversity of microorganisms and the synthesis of diverse, vitally important biologically active substances, established in the studies of several authors described in the literature. In the conditions of a favorable epidemiological situation, foods, including water, are mainly tested for Salmonella and Staphylococcus aureus. The rest of the microorganisms are studied by the so-called hygiene-indicative (indicator) microorganisms. The causative agents of harmful food infections and toxicoses have an intestinal origin and enter the digestive tract from fecal masses. The group of coliforms and the group of fecal enterococci are used as hygienic-indicative microorganisms for fecal contamination. Coliforms are Gram-negative, rod-shaped, non-spore-forming, motile, facultatively anaerobic microorganisms. They develop at temperatures of 30, 35, and 37°C and digest lactose with gas formation. They include four genera: Escherichia, Citrobacter, Enterobacter, Klebsiella, and a new genus, Raoultella (which attacks the lungs and causes pneumonia), was separated from the last genus. All these genera have the same morphological and cultural properties but differ in biochemical, serological, and biological properties. The most important among all of them is Escherichia coli. The separation of these four genera is carried out biochemically using the IMVIC complex: (I – reaction for indole; M – reaction with methylrot; A – formation of acetoin from glucose (Vogges – Proskauer reaction); C – absorption of citrates). Fecal enterococci are Gram-positive, coccoid, non-spore-forming, motile, facultatively anaerobic microorganisms. They may grow at temperatures 10 to 45°C, pH 9.6, at 6.5% NaCl, and withstand 40% bile salts. They could be destroyed at a temperature of 60°C for more than 30 min. The summarized experimental data presented in Table 3, show that the coli titer is less than 100 in 14 of the 90 studied spring water samples. The content of Coli - bacteria in the water indicates the presence of a source of fecal pollution. All indicators characterizing mineral, mountain, and spring water samples are determined by membrane filtration and the smallest amount of water in which pathogenic bacteria are not detected.

***Conclusion:**Results from the 90 mineral, mountain, and spring water samples in the territory of 11 regions – Stara Zagora, Yambol, Sliven, Burgas, Varna, Plovdiv, Pazardzhik, Sofia, Lovech, and Blagoevgrad, indicated that 91 unknown types of microorganisms were isolated, which were subjected to of identification, according to modern conventional and molecular-genetic methods.

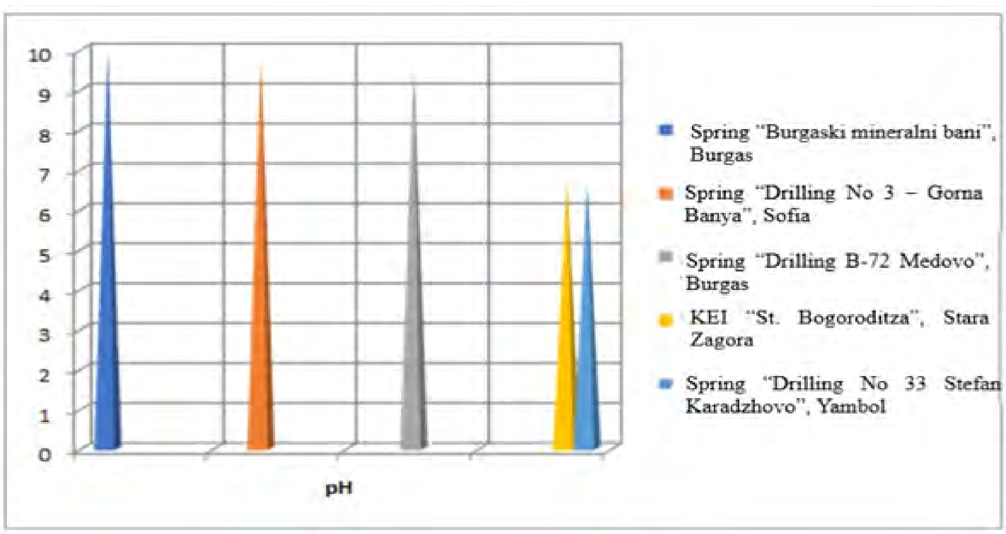

Figure 2: Highest and lowest water pH values of the investigated waters in Bulgaria.

Table 2(44): Comparative examination of ivestigated water types depending on the physical and chemical indicators by area.

Region	Type of the studied water samples according to the physicochemical characteristics
Type of the studied water samples according to the physicochemical characteristics	- thermal sulfate-calcium-sodium, silicon water containing fluoride, hydrogen sulfide, carbon dioxide.
Stara Zagora	- hypothermal, thermal and hyperthermal, bicarbonate-sodium, fluoride, and silicon water with low concentrations of hydrogen sulfide and carbon dioxide; - thermal, hydrocarbonate-sulfate, sodium, fluoride, and silica water.
Yambol	hypothermal, hydrocarbonate-calcium-magnesium water containing hydrogen sulfide and carbon dioxide.
Sliven thermal,	bicarbonate-sulfate, sodium-calcium water containing fluoride.
Burgas thermal,	chloride-hydrocarbonate, sodium, and silica water, containing fluoride and hydrogen sulfide; - subthermal, hydrocarbonate, sodium, containing fluoride; - hypothermal, hydrocarbonate, sodium, siliceous, containing fluoride.
Varna	- thermal, mineralized, hydrocarbonate, calcium, sodium, and magnesium; some contain methane.
Plovdiv	- thermal, hydrocarbonate-sulfate-sodium, and siliceous, containing fluoride and hydrogen sulfide; - thermal, sulfate-hydrocarbonate, sodium, silicon, and fluoride water containing hydrogen sulfide; - hypothermal, hydrogen carbonate sodium-calcium, and siliceous water containing hydrogen sulfide.
Pazardzhik	- hyperthermal, sulfate-sodium, and silicon water, containing fluoride and hydrogen sulfide; - hyperthermal, hydrocarbonate-sulfate, sodium, silica water, containing fluoride and hydrogen sulfide; - isothermal, mineralized, sulfate-sodium-calcium, containing fluoride and hydrogen sulfide; - hypothermal, sulfate-hydro carbonate-sodium, silica water containing fluoride and hydrogen sulfide.
Sofia	- hyperthermal, chloride-sulfate, sodium-calcium-magnesium, silica water containing fluoride, hydrogen sulfide, and carbon dioxide; - hypothermal, isothermal, hydrocarbonate-sulfate, sodium water containing hydrogen sulfide and carbon dioxide.
Lovech	- hypothermal mineralized, calcium-magnesium sulfate hydrocarbonate, containing hydrogen sulfide and carbon dioxide.
Blagoevgrad	- hyperthermal, slightly mineralized, hydrocarbonate sodium-calcium and magnesium water, containing hydrogen sulfide and carbon dioxide.

Table 3(45): Determining the presence of coliforms in the investigated waters on Endo medium.

Water saurce	Coliforms
Thermal mineral spring "Driller No 2VP", Haskovo region	−
Thermal healing spring "Driller No 3VP", Haskovo district	−
Thermal healing spring of "KEI No 5", Haskovo region	−
Thermal healing spring "Driller No Sz-7" Pavel banya, Stara Zagora region	−
Thermal healing spring "Driller No Sz-8 Pavel banya, Stara Zagora district	−
Thermal healing spring "Driller No X-19" Pavel banya, Stara Zagora region	−
Hyperthermal healing spring "Driller No 3-Pavel banya, Stara Zagora district	−
Hyperthermal healing spring "Driller No K-3-Ovoshtnik", Stara Zagora region	−
Thermal healing spring "Driller No Sz-37-Yagoda", Stara Zagora district	−
Hypothermal spring in the village of Trakia, Stara Zagora region	+
"St. Nicholas Chuditvoretz" hypothermal spring, Stara Zagora region	+
Hypothermal spring "St. Bogoroditsa" in Maglizh municipality, Stara Zagora region	+
Center hypothermal spring in Maglizh municipality, Stara Zagora district	−
Hypothermal spring Koprinka village in Maglizh municipality, Stara Zagora district	+
Hypothermal spring in the town of Kazanlak in the city of Maglizh, Stara Zagora region	−
Hypothermal spring in Kren – Enina, Stara Zagora region	−
Hypothermal spring in the town of Enina, Stara Zagora region	+
Ayazmo hypothermal spring, Stara Zagora region	−
Hypothermal spring "Trite chuchura," Stara Zagora region	−

Hypothermal spring in the village of Okop, Yambol region	+
Hypothermal spring in the village of Karavelovo, Yambol region	–
Hypothermal healing spring "Driller No-33, Stefan Karadzhovo village", Yambol region	–
Thermal healing spring "Driller 22 - Sliven mineral baths, Sliven district	–
Thermal healing spring, Banya village, Sliven region	–
Hypothermal healing spring KEI "Hadji Dimitar," locality "Toplata voda", city of	–
Shivachevo, district of Sliven	
Hypothermal healing spring "Gunchov Izvor," Sliven region	+
Hypothermal spring in Nova Zagora, Sliven region	–
Thermal healing spring Burgas mineral baths, Burgas region	–
Hyperthermal healing spring in the village of Shivarovo, Burgas region	–
Thermal healing spring, Polyanovo village, Burgas region	–
Hypothermal healing spring "Driller B-12" in the village of Sadievo, Burgas region	+
Thermal healing spring "Driller B- No 73 Medovo", Burgas region	–
Hypothermal healing spring "Driller B- No 53 Kamenar", Burgas region	–
Thermal healing spring Driller No P-83x KK "St. Konstantin and Elena", Varna region	–
Thermal healing spring P-1x "Aquarium," Varna region	–
Thermal healing spring "R-106 x "Dom Mladost", Varna region	–
Thermal healing spring "R-161x Varna at Primorski pool", Varna region	–
Thermal healing spring "Lekovita voda" Goren Chiflik village, Varna region	+
Thermal healing spring "KEI Momina Banya" in the town of Hisarya, Plovdiv region	–
Thermal healing spring "KEI Momina Salza" in the town of Hisarya, Plovdiv region	–
Subthermal spring "KEI Stublata" in the town of Hisarya, Plovdiv region	–
Thermal healing spring "KEI Toplitsata" in the town of Hisarya, Plovdiv region	–
Thermal healing spring "Fresh" in the town of Hisarya, Plovdiv region	–
Thermal healing spring "Bistrica" in the town of Hisarya, Plovdiv region	–
Thermal healing spring "Bancheto Miromir" in the town of Hisarya, Plovdiv region	–
Thermal healing spring "Choban Cheshma" in the town of Hisarya, Plovdiv region	–
Thermal healing spring "Chair Banya" in the town of Hisarya, Plovdiv region	–
Thermal healing spring "Driller No 1" in the town of Hisarya, Plovdiv region	–
Hypothermal healing spring "Staro Zhelezare-Driller No 2" in the town of Hisarya,	–
Plovdiv region	
Thermal healing spring "Driller No 3" in the town of Hisarya, Plovdiv region	–
Hypothermal healing spring "Staro Zhelezare - Driller No 4" in the town of Hisarya,	–
Plovdiv region	
Thermal healing spring "Driller No 5" in the town of Hisarya, Plovdiv region	–
Thermal healing spring "Driller No 6" in the town of Hisarya, Plovdiv region	–
Thermal healing spring "Driller No 7" in the town of Hisarya, Plovdiv region	–
Thermal healing spring "Parilkite" in the town of Hisarya, Plovdiv region	–
Thermal healing spring "Bulgarian Rose" Driller in the town of Banya, Plovdiv region	–
Thermal healing spring KEI "Central Catchment" town of Banya, Plovdiv region	–
Isothermal healing spring KEI "Men's bath" Banya, Plovdiv region	+
Thermal healing spring KEI "Women's Bath" Banya, Plovdiv region	–
Thermal healing spring "Driller 1-Kokalche" in the town of Banya, Plovdiv region	–
Subthermal healing spring "Driller No 8" in the village of Dragoynovo, Plovdiv region	–
Hypothermal healing spring "Driller No 9" in the village of Dragoynovo, Plovdiv region	–
Thermal healing spring "Driller 16-Lenovo" in the village of Lenovo, Plovdiv region	–
Hypothermal healing spring "Driller 1-Asenovgrad" in the town of Asenovgrad,	–
Plovdiv region	

Hypothermal healing spring "KEI-Banski Catchment" in the village of Narechenski	—
Bani, Plovdiv region	
Hypothermal healing spring "KEI-Banski Catchment" in the village of Narechenski	—
Bani, Plovdiv region	
Hypothermal healing spring "Salty Spring" in the village of Narechenski Bani, Plovdiv region	—
Hypothermal spring "Bajova voda" in the village of Bachkovo, Plovdiv region	—
Hyperthermal healing spring "Driller No 3-Varvara" in the village of Varvara,	—
Pazardzhik region	
Hyperthermal healing spring "Driller No 5-Varvara" in the village of Varvara,	—
Pazardzhik region	
Hyperthermal healing spring "Driller No 6-Varvara" in the village of Varvara,	—
Pazardzhik region	
Thermal healing spring KEI No 2 "Vetren dol" in the village of Varvara, Pazardzhik region	—
Isothermal healing spring KEI No 1 "Banchero" in the village of Banya, Pazardzhik region	—
Thermal healing spring driller No 2 "Naklonen" in the village of Banya, Pazardzhik region	—
Subthermal spring in the town of Oborishte, Pazardzhik region	+
Subthermal thermal spring in Oborishte - Panagyurishte, Pazardzhik region	+
Panagyurishte hypothermal spring - Buta, Pazardzhik region	+
Thermal healing spring "Strelcha," Pazardzhik region	—
Hyperthermal spring driller No 5 "Syarna Banya-Velingrad," Pazardzhik region	—
Hyperthermal healing spring driller No 3 "Mizinka-Velingrad," Pazardzhik region	—
Table 3 (45) continued.	
Thermal healing spring "Driller No 7KG" "Velova Banya-Velingrad," Pazardzhik region	—
Hypothermal healing spring "Driller TC No 1 "Ivanyane" town of Bankya, Sofia region	—
Isothermal healing spring "Driller No 3-Gorna Banya", Sofia region	—
Hyperthermal healing spring KEI "Pchelinski Bani," Sofia region	—
Thermal healing spring KEI "Sofia-Center," Sofia region	—
Thermal healing spring "Driller No P-1hg-Krushuna", Lovech region	—
Hypothermal healing spring "Driller No L-2-Shipkovo", Lovech region	—
Thermal healing spring "Driller No P-1hg-Chiflik", Lovech region	—
Hyperthermal healing spring "Rupi 1", Blagoevgrad region	—
Hyperthermal healing spring "Rupi 2", Blagoevgrad region	+

+ presence of coli - bacteria; — the absence of coli - bacteria.

5.1.2. Regulators of Water Indexes: The influence of various regulators has been investigated. The obtained results confirmed the data from the literature that metal ions/EVOdrop, electromagnetic waves and zeolite could be used as the regulators of water indexes.

5.1.3. New Sources of Biologically Active Substances and their Application to Drinking Water

The interdisciplinary approach can be the basis for future developments of new commercial products, including the studied biologically active substances - common duckweed extract (L. minuta Kunth.), rose oil (R. damascena Mill.), and Rhodope haberlea (H. rhodopensis Friv.) and their relation to water.

5.2. Molecular-Genetic Identification of Microorganisms Isolated from Water

Once an unknown species is isolated from nature, physiological-biochemical and molecular studies should be carried out to reveal its properties, which are necessary for species determination. The results obtained in our studies were compared with the reference strains' properties in identifying the unknown strain.

The strain identification was carried out by physiological-biochemical tests and molecular-genetic methods for the samples from the Haskovo region.

VITEK® MS /Mass Spectrometric System for Microbial Identification/ was used for the strains isolated from waters in the remaining ten areas.

***Haskovo Region**

Four microbial strains isolated from the following sources were identified:

-Thermal mineral spring “Driller No 2VP”

-Thermal healing spring “Driller No 3VP”

-Thermal healing spring of “KEI No 5”

-Thermal healing spring Haskovo mineral baths

The results showed that the strains had comparable indicators; all were Grampositive; as two are facultative anaerobes, one is an obligate aerobe, and one is an aerobe. The ability of microorganisms to absorb carbon is presented in Table 4. The species affiliation of the strains is presented in Table 5. The data show a very high level of reliability in their identification.

***Stara Zagora Region**

Sixteen microbial strains isolated from the following sources were identified:

-Thermal healing spring Driller No Sz-7 Pavel banya

-Thermal healing spring Driller No Sz-8 Pavel banya

-Thermal healing spring Driller No X-19 Pavel banya

-Hyperthermal healing spring Driller No 3 Pavel banya

-Hyperthermal healing spring “Driller No K-3-Ovoshtnik”

-Thermal healing spring “Driller No S3- 7-Yagoda”

-Hypothermal spring in the village of Trakia

-”St. Nicholas Chudotvorets” hypothermal spring

-”Holy Virgin” hypothermal spring in Maglizh municipality

-Hypothermal spring “Center”

-Hypothermal spring in the village of Koprinka in the municipality of Maglizh

-Hypothermal spring in the town of Kazanlak in the municipality of Maglizh

-Hypothermal spring in Kran – Enina

-Hypothermal spring in the town of Enina

-Ayazmo hypothermal spring

-Hypothermal spring “Trite chuchura”

The results showed that the strains had comparable indicators, as all were Grampositive; six of them were facultative anaerobes, and the remaining 10 were aerobes. The species affiliation of the strains is presented in Table 6. The data show a very high level of reliability in their identification.

***Yambol Region**

Four microbial strains isolated from the following sources were identified:

-Hypothermal spring in the village of Okop

-Hypothermal spring in the village of Karavelovo

The results showed that the strains had comparable indicators as all were Grampositive; three of them were facultative anaerobes and one aerobe. The species affiliation of the strains is presented in Table 7. The data showed a very high level of reliability in their identification.

***Sliven Region**

Four microbial strains isolated from the following sources were identified:

-Thermal healing spring, Driller 22-Sliven mineral baths

-Thermal healing spring in the village of Banya

-KEI “Hadzhi Dimitar” hypothermal healing spring, “Toplata voda” area, the town of

Shivachevo

-Hypothermal healing spring “Gunchov Izvor”

-Hypothermal spring in Nova Zagora

The results showed that the strains had comparable indicators as all were Grampositive; two of them were facultative anaerobes, and three were aerobes.

The species affiliation of the strains is presented in Table 8. The data show a very high level of reliability in their identification.

***Burgas Region**

Six microbial strains isolated from the following sources were identified:

-Thermal healing spring Burgas mineral baths

-Thermal healing spring in the village of Shivarovo

-Thermal healing spring in the village of Polyanovo

-Hypothermal healing spring “Driller B - 12” in the village of Sadievo

-Thermal healing spring “Driller B- No 73 Medovo”

-Hypothermal healing spring “Driller B- No 53 Kamenar”

The results show that the strains had comparable indicators: one was Gram-negative, and the other five were Gram-positive; four strains were facultative anaerobes, one obligate aerobe, and one aerobe.

The species affiliation of the strains is presented in Table 9. The data show a very high level of reliability in their identification.

***Varna Region**

Four microbial strains isolated from the following sources were identified:

-Thermal healing spring (Driller No P-83x KK “St. Constantine and Elena”)

-Thermal healing spring (P-1x “Aquarium”)

-Thermal healing spring P-106 x “Dom Mladost”

-Thermal healing spring P-161x Varna at the “Primorski” pool

The results showed that the strains had comparable indicators as all were Grampositive; three of them were facultative anaerobes and one aerobe. The species affiliation of the strains is presented in Table 10. The data show a very high level of reliability in their identification.

Table 4(58): Uptake of carbon sources strains isolated from waters in Haskovo region.

Carbon source	Strain N. Valcheva P1	Strain N.Valcheva P2	Strain N. Valcheva Y1	Strain N. Valchev Y2
Glycerol	+ (90 – 100 %)	0.5	0.5	0.5
Erythriol	-	-	-	-
D-arabinose	-	-	-	-
L-arabinose	+ (90 – 100 %)	-	-	-
Ribose	+ (90 – 100 %)	+ (90 – 100 %)	+ (90 – 100 %)	+ (90 – 100 %)
D-xylose	-	-	-	-
L-xylose	-	-	-	-
Adonitol	-	-	-	-
b-methyl-D-xyloside	-	-	-	-
Galactose	-	-	-	-
D-glucose	+ (90 – 100 %)	+ (90 – 100 %)	+ (90 – 100 %)	+ (90 – 100 %)
D-fructose	+ (90 – 100 %)	+ (90 – 100 %)	+ (90 – 100 %)	+ (90 – 100 %)
D-mannose	+ (90 – 100 %)	-	-	-
L-sorbose	-	-	-	-
Rhamnose	-	-	-	-
Dulcitol	-	-	-	-
Inositol	+ (90 – 100 %)	-	-	-
Manitol	+ (90 – 100 %)	-	-	-
Sorbitol	+ (90 – 100 %)	-	-	-
a-methyl-Dmannoside	-	-	-	-
a-methyl-D-glucoside	+ (90 – 100 %)	-	-	-
N-acetylglucosamine	-	+ (90 – 100 %)	+ (90 – 100 %)	+ (90 – 100 %)
Amigdalin	-	0.5	-	-
Arbutin	+ (90 – 100 %)	+ (90 – 100 %)	+ (90 – 100 %)	+ (90 – 100 %)
Esculin	+ (90 – 100 %)	+ (90 – 100 %)	+ (90 – 100 %)	+ (90 – 100 %)
Salicin	+ (90 – 100 %)	+ (90 – 100 %)	+ (90 – 100 %)	+ (90 – 100 %)
Cellobiose	+ (90 – 100 %)	0.5	0.5	0.5
Maltose	+ (90 – 100 %)	+ (90 – 100 %)	+ (90 – 100 %)	+ (90 – 100 %)
Lactose	+ (90 – 100 %)	-	-	-
Melibiose	+ (90 – 100 %)	-	-	-
Saccharose	+ (90 – 100 %)	+ (90 – 100 %)	+ (90 – 100 %)	+ (90 – 100 %)
Trehalose	+ (90 – 100 %)	+ (90 – 100 %)	+ (90 – 100 %)	+ (90 – 100 %)
Inulin	-	-	-	-
Melezitose	-	-	-	-
D-raffinose	+ (90 – 100 %)	-	-	-
Amidon	+ (90 – 100 %)	+ (90 – 100 %)	+ (90 – 100 %)	+ (90 – 100 %)
Glycogen	+ (90 – 100 %)	+ (90 – 100 %)	+ (90 – 100 %)	+ (90 – 100 %)
Xylitol	-	-	-	-
b-gentiobiose	-	-	-	-
D-turanose	-	-	-	-
D-lyxose	-	-	-	-
D-tagarose	-	-	-	-
D-fruccose	-	-	-	-
L-fruccose	-	-	-	-
D-arabitol	-	-	-	-
L-arabitol	-	-	-	-
Gluconate	-	-	-	-
2-keto-gluconate	-	-	-	-
5-keto-gluconate	-	-	-	-
Identification	Bacillus subtilis/ amyloliquefaciens	Bacillus cereus S74	Bacillus thuringiensis B62	Bacillus subtilis 0-2
Reliability, %	99.8	99	99	99

Table 5(60): Species affiliation of the strains isolated from waters in Haskovo region.

Strain	Species affiliation (Reference strain)	Reliability, %
N. Valcheva P1	Bacillus methylotrophicus PY	99.9
N. Valcheva P2	Bacillus cereus S74	99.9
N. Valcheva Y1	Bacillus thuringiensis B62	99.9
N. Valcheva Y2	Bacillus subtilis 0 - 2	99.9

Table 6(63): Species affiliation of the strains isolated from waters in the Stara Zagora region.

Strain	Species affiliation (Reference strain)	Reliability, %
N. Valcheva BTh	Bacillus thuringiensis B62	99.9
N. Valcheva BV 2-1-2	Bacillus vallismortis	99.9
N. Valcheva BA 2-1-3	Bacillus amyloliquefaciens	99.9
N. Valcheva BL 3-1	Bacillus licheniformis	100
N. Valcheva BAP 4-1	Bacillus altitudinis/pumilus	99.9
N. Valcheva BS 2-1-1	Bacillus subtilis	99.9
N. Valcheva BV 2-1-2	Bacillus vallismortis	99.9
N. Valcheva BS 2-1-1	Bacillus subtilis	99.9
N. Valcheva BL 3-1	Bacillus vallismortis	99.9
N. Valcheva BA 2-1-3	Bacillus amyloliquefaciens	99.9
N. Valcheva BTh	Bacillus thuringiensis B62	99.9
N. Valcheva BV 2-1-2	Bacillus vallismortis	99.9
N. Valcheva BA 2-1-3	Bacillus amyloliquefaciens	99.9
N. Valcheva BCG 1-1	Bacillus cereus group	99.9
N. Valcheva BAP 4-1	Bacillus altitudinis/pumilus	99.9
N. Valcheva BCG 1-1	Bacillus cereus group	99.9

Table 7(67): Species affiliation of strains isolated from waters in Yambol region.

Strain	Species affiliation (Reference strain)	Reliability, %
N. Valcheva BTh	Bacillus thuringiensis B62	99.9
N. Valcheva BCG 25-1	Bacillus cereus group	99.9
N. Valcheva BA 22-1	Bacillus amyloliquefaciens	99.9
N. Valcheva BCG 12-1	Bacillus cereus group	100

Table 8(71): Species affiliation of the strains isolated from waters in Sliven region.

Strain	Species affiliation (Reference strain)	Reliability, %
N. Valcheva BS 17-1	Bacillus subtilis	99.9
N. Valcheva BV 17-1	Bacillus vallismortis	99.9
N. Valcheva BA 17-1	Bacillus amyloliquefaciens	99.9
N. Valcheva BL 15-1	Bacillus licheniformis	100
N. Valcheva BL 15-	Bacillus licheniformis	99.9

Table 9(75): Species affiliation of the strains isolated from waters in the Burgas region.

Strain	Species affiliation (Reference strain)	Reliability, %
N. Valcheva BL 14-1	Bacillus licheniformis	99.9
N. Valcheva BCG 12-1	Bacillus cereus group	99.9
N. Valcheva BrB 24-1	Brevibacillus spp.	99.9
N. Valcheva PsA 10-1	Pseudomonas aeruginosa	100
N. Valcheva BL 14-1	Bacillus amyloliquefaciens	99.9
N. Valcheva BCG 12-1	Bacillus subtilis	99.9

Table 10(79): Species affiliation of the strains isolated from waters in the Varna region.

Strain	Species affiliation (Reference strain)	Reliability, %
N. Valcheva BL 7-1	Bacillus licheniformis	99
N. Valcheva BS 5-1-1	Bacillus subtilis	99
N. Valcheva BV 5-1-2	Bacillus vallismortis	98
N. Valcheva BA 5-1-3	Bacillus amyloliquefaciens	98

Table 10(83): Species affiliation of the strains isolated from waters in Plovdiv region.

Strain	Species affiliation (Reference strain)	Reliability, %
N. Valcheva BS 2-1-1	Bacillus subtilis	99
N. Valcheva BC 16-1	Bacillus cereus group	99
N. Valcheva BAP 13-1	Bacillus altitudinis/pumilus	99
N. Valcheva BL 3-1	Bacillus licheniformis	100
N. Valcheva BS 2-1-1	Bacillus subtilis	99
N. Valcheva BV 2-1-2	Bacillus vallismortis	98
N. Valcheva BA 2-1-3	Bacillus amyloliquefaciens	93
N. Valcheva BA 2-1-3	Bacillus amyloliquefacien	98
N. Valcheva BIAS hs – 1	Aeromonas sobria hs – 1	97
N. Valcheva BIKO LF – 1	Klebsiella oxytoca LF – 1	99
N. Valcheva BIBASP SDF004	Bacillus amyloliquefaciens subsp.plantarum SDF004	99
N. Valcheva SIBT KU4	Bacillus thuringiensis KU4	100
N. Valcheva BVBC S72	Bacillus cereus S72	99

***Plovdiv Region**

Thirteen microbial strains isolated from the following sources were identified:

-Thermal healing spring “KEI Momina Banya” in Hisarya

-Thermal healing spring “KEI Momina Salza” in Hisarya

-Hypothermal healing spring “KEI Stublata” in Hisarya

-Thermal healing spring “KEI Central catchment” in Banya

-Thermal healing spring “Driller 1-Kokalche” in Banya

-Thermal healing spring “Driller-Bulgarian Rose” in Banya

-Isothermal healing spring “KEI Men’s Bath” in Banya

-Isothermal healing spring “KEI Women’s Bath” in Banya

-Hypothermal healing non-thermal spring “Banski Izvor” in the village of Narechenski

Bani

-Hypothermal healing non-thermal spring “Banski Izvor” in the village of Narechenski

Bani

-Hypothermal healing spring “Salt Spring” in the village of Narechenski Bani

-Healing spring “Ochno Izvorche” in the village of Narechenski Bani

-”Bajova Voda” spring in the village of Bachkovo

The results showed that the strains had comparable indicators: three were Gramnegative, the remaining ten were Gram-positive, five were facultative anaerobes and the remaining eight were aerobes.

The species affiliation of the strains is presented in Table 10. The data show a very high level of reliability in their identification.

***Pazardzhik Region**

Thirteen microbial strains isolated from the following sources were identified: - Hyperthermal healing spring “Driller No 3-Varvara” in the village of Varvara

-Hyperthermal healing spring “Driller No 5-Varvara” in the village of Varvara

-Hyperthermal healing spring “Driller No 6-Varvara” in the village of Varvara

-Thermal healing spring KEI No 2 “Vetren dol” in the village of Varvara

-Isothermal healing spring KEI No 1 “Bancheto” in the village of Banya - Thermal healing spring Driller No 2 “Naklonen” in the village of Banya

-Subthermal spring in the town of Oborishte

-Subthermal spring in Oborishte – Panagyurishte

-Hypothermal spring Panagyurishte – Buta

-Thermal healing spring “Strelcha”

-Hyperthermal spring Driller No 5 “Syarna Banya-Velingrad”

-Hyperthermal healing spring Driller No 3 “Mizinka-Velingrad”

-Thermal healing spring Driller No 7KG “Velova Banya-Velingrad”

The results show that the strains had comparable indicators as 12 were Gram-positive and one was Gram-negative; seven were facultative anaerobes and six - aerobes. The species affiliation of the strains is presented in Table 11. The data show a very high level of reliability in their identification.

***Sofia Region**

Three microbial strains isolated from the following sources were identified:

-Isothermal healing spring “Driller No 3-Gorna Banya”

-Hyperthermal healing spring KEI “Pchelinski Bani”

-Hyperthermal healing spring KEI “Sofia-Center”

The results show that the strains had comparable indicators as all were Gram-positive; one of them was a facultative anaerobe, and two were aerobes. The species affiliation of the strains is presented in Table 12. The data show a very high level of reliability in their identification.

***Lovech Region**

Three microbial strains isolated from the following sources were identified:

-Thermal healing spring “Driller No P-1hg-Krushuna”

-Isothermal healing spring “Driller No L-2-Shipkovo”

-Thermal healing spring “Driller No P-1hg-Chiflik”

The results show that the strains had comparable indicators as all were Gram-positive; one of them was a facultative anaerobe, and the other two were aerobes. The species affiliation of the strains is presented in Table 13. The data show a very high level of reliability in their identification.

***Blagoevgrad Region**

Sixteen microbial strains isolated from the following sources were identified:

-Hyperthermal healing spring “Rupi 1” – nine strains

-Hyperthermal healing spring “Rupi-2” – seven strains

The results show that the strains had comparable indicators: four were Gram-negative, and the remaining 12 were Gram-positive; two were facultative anaerobes, two were obligate aerobes, and the other 12 were aerobes.

The species affiliation of the strains is presented in Table 14. The data show a very high level of reliability in their identification. The comparative analysis showed that 21 different types of microorganisms were identified, summarized in the Table 15. The species distribution of the identified microorganisms by region is presented in the Table 16.

Examining the different representatives of the genus Bacillus in the water sources in our study did not contradict the data from the literature since their resistance to higher water temperatures was known. As a pattern, it was established that in all investigated hyperthermal springs of Bulgaria, the mesophilic transforming bacteria Bacillus subtilis, Bacillus amyloliquefaciens, and Bacillus vallismortis always live together. They probably mutually support their development, reproduction, and adaptation by releasing secondary metabolites outside the cell in the struggle for survival against thermal and hyperthermal organisms. The strains of Bacillus subtilis, Bacillus thuringiensis, Bacillus cereus and Bacillus methylotrophicus were most common in the water sources in this work.

Bacillus cereus is a Gram-positive bacterium, facultative anaerobe and can produce protective endospores like other members of the genus. Its virulence factors include cerolysin and phospholipase C. Bacillus thuringiensis was also identified in large part of the studied spring waters. It is a Gram-positive, soil-dwelling bacterium. Furthermore, many extracellular compounds produced by B. thuringiensis species, including phospholipases, chitinases, proteases, δexotoxins, were used as insecticidal proteins and antibiotic compounds with antifungal activity. The other identified microorganism of the genus is Bacillus subtilis. It is a Grampositive, catalase-positive, aerobic, rod-shaped bacterium with the ability to form an endospore, allowing it to survive in extreme environmental conditions. The species is generally considered safe, although some strains are closely related to pathogenic B. cereus and B. thuringiensis. Bacillus methylotrophicus is a Gram-positive bacterium that occurs in soil and other sources. It has proven antimicrobial, cytotoxic and insecticidal properties, which can be explained by the synthesized lipopeptides and other biologically active substances.

***Conclusion:** The number of different microorganisms doesn’t depend on the number of studied springs. Still, it depends on temperature, pH, different types of dissolved salts (cations and anions), and dissolved gases in the studied spring water.

-The most widespread are Bacillus subtilis and Bacillus amyloliquefaciens, identified in 10 out of 11 investigated regions, followed by Bacillus licheniformis in nine areas and Bacillus vallismortis in six areas. Bacillus thuringiensis and Geobacillus stearothermophilus strains were in three areas. All types of microorganisms were in a different combination in a region, and there was no region with the same bacterial variety. The synthesis of biologically active substances depends on the type of microorganism and does not depend on the area of isolation of the strain.

-The identified single species of microorganisms are distributed in three regions - Burgas (Brevibacillus spp., Pseudomonas aeruginosa), Plovdiv - (Bacillus amyloliquefaciens subs. Plantarum, Klebsiella oxytoca, Aeromonas sobria) and Blagoevgrad (Bacillus megaterium, Bacillus simplex, Pseudomonas fluorescens, Stenotrophomonas maltofila).

-All microorganisms have specific requirements for a nutrient medium, temperature range and optimal temperature, pH range, and optimal pH of development and biosynthesis. Therefore their type and distribution in areas with different physicochemical compositions, temperature, and pH of the springs are strictly specific.

-The quantity of the facultative thermophile Bacillus licheniformis – 15 is the largest, followed by the mesophiles Bacillus subtilis – 12, Bacillus amyloliquefaciens – 11, Bacillus

vallismortis, and Bacillus cereus group – 9. The reason for the distribution could be due to their wide temperature and pH range and unpretentiousness regarding nutrients.

-It is considered a phenomenon that strains of Bacillus subtilis, Bacillus amyloliquefaciens, and Bacillus vallismortis always live together in hyperthermal waters. It is most likely due to the purpose of protection from adverse conditions and their ability to sporulate. The other strains are two, three, four, or single, which indicates their high specificity and requirements regarding conditions and nutritional environment of development.

5.3. Determination of Biological Properties of Identified Strains

5.3.1. Enzyme activity

The summary of the data showed that the following isolated strains of bacteria exhibited the highest enzyme activity (amylolytic, proteolytic, and lipolytic activity):

1. Bacillus thuringiensis - N. Valcheva BTh B62 – amylolytic activity 25 mm, proteolytic activity 26.5 mm;

2. Bacillus methylotrophicus - N. Valcheva BMTh PY4 – amylolytic activity 24 mm, proteolytic activity 20 mm;

3. Bacillus subtilis - N. Valcheva BS 17 -1, N. Valcheva BS 2-1-1, N. Valcheva BS 2-2-2 – amylolytic activity 26 mm, proteolytic activity 27 mm;

4. Bacillus amyloliquefaciens - N. Valcheva BA 22-1 – amylolytic activity 26 mm, proteolytic activity 27 mm, lipolytic activity 22 mm;

5. Bacillus licheniformis - N. Valcheva BL 3–1, N. Valcheva BL 30-1, N. Valcheva BL 7-1 – amylolytic activity 26 mm, proteolytic activity 27 mm, lipolytic activity 24 mm;

6. Geobacillus stearothermophilus - N. Valcheva GbSt region of Pazardzhik, Sofia and

Blagoevgrad – amylolytic activity 25 mm, proteolytic activity 27 mm, lipolytic activity 24 mm;

7. Bacillus cereus group - N. Valcheva BCG 25-1 – amylolytic activity 24 mm, proteolytic activity 26 mm, lipolytic activity 23 mm.

The studies carried out to determine the enzyme activity of the isolated strains showed that each culture is characterized by a specific enzyme set (Fig. 4).

Bacillus cereus (P1), Bacillus thuringiensis (P2), Bacillus methylotrophicus (X1), and

Bacillus subtilis (X2) strains were characterized by alkaline phosphatase, lipase (C4), lipase (C8), acid phosphatase and α-glucosidase activity. Unlike the other four studied strains, Bacillus thuringiensis (P2) possessed leucine arylamidase and naphthol – AS – BI – phosphohydrolase activity.

The data on the amylase activity of Bacillus licheniformis BL 3-1, BL 30-1, and BL 71– did not contradict the data from the literature. The data on enzyme activity of the investigated strain Geobacillus stearothermophilus - N. Valcheva GbSt from water sources in the Varna, Burgas, and Blagoevgrad regions did not contradict the data from the literature.

5.3.2. Antimicrobial Activity

The test microorganisms used in the study are some of the most common pathogenic microorganisms. Molds (mainly Penicillium sp.) and yeasts are known as organisms responsible for food spoilage. Some molds (Fusarium sp., Aspergillus niger, Aspergillus oryzae) are important plant pathogens, and the mycotoxins released by them cause poisoning in humans and domestic animals. The results with the most pronounced inhibitory effect against pathogenic toxigenic microorganisms.

***Bacillus cereus**

The results of the antimicrobial activity of N. Valcheva BCG 25-1 /Yambol region/ strain showed no inhibitory activity against mold fungi of the genus Mucor. The strain exhibited a pronounced inhibitory effect against Aspergillus oryzae and A. niger. It had high antimicrobial activity against A. awamori, Fusarium moliniforme, and Penicillium sp. and a well-expressed antimicrobial effect against Rhizopus sp. N. Valcheva BCG 25-1 /Yambol region/ strain showed weak inhibitory action against Pseudomonas aeruginosa ATCC 9027 and Enterococcus faecalis.

***Bacillus Thuringiensis**

The antimicrobial activity results of N. Valcheva BTh B62 /Stara Zagora region/ strain exhibited a pronounced inhibitory effect against the molds Penicillium sp., Fusarium moliniforme, and genus Aspergillus. The studied strain had weak activity against Mucor sp., Pseudomonas aeruginosa ATCC 9027, and Enterococcus faecalis. The strain is resistant to Rhizopus sp. and the test culture Escherichia coli. N. Valcheva BTh B62 /Stara Zagora region/ strain had a weak inhibitory action against the tested yeast Saccharomyces cerevisiae.

***Bacillus Subtilis**

The antimicrobial activity of N. Valcheva BS 17-1, N. Valcheva BS 18, N. Valcheva BS 22-1 /Sliven, Sofia, and Blagoevgrad regions/ showed that all three strains exhibited a pronounced inhibitory effect against the mold fungi Penicillium sp . and Fusarium moliniforme. The strains did not show an antimicrobial effect against the test cultures of the genus Mucor, except for the cell suspension and the culture medium against Mucor sp. The strains showed a highly pronounced inhibitory effect against the Aspergillus genus's representatives and weak antimicrobial activity against Enterococcus faecalis. The test cultures Escherichia coli ATCC 25922 and Pseudomonas aeruginosa ATCC 9027 were resistant to the action of the studied samples/ No inhibitory action was found against studied yeast.

***Bacillus Methylotrophicus**

The antimicrobial activity of strain N. Valcheva BMTh PY5 / Haskovo region/shows a pronounced inhibitory effect against the molds Penicillium sp., Fusarium moliniforme, Mucor mucedo. The observed antimicrobial effect against Rhizopus sp. was weaker; test cultures were resistant against studied bacteria, and growth stimulation was established against studied yeast strains.

5.3.3. The Adhesive Capacity to Human Epithelial Cells

In the adhesion studies on the human carcinoma epithelial cell line HT-29, 12 bacteria of the genus Bacillus and genus Geobacillus, identified in ordinary, mountain, and mineral waters from different regions of the country and development conditions, were selected. The analysis results for the adhesion of bacteria of the genus Bacillus and the genus Geobacillus are presented in Table 17 and images 1-14.

The results show that the most strongly adherent strain of the studied bacteria from the genus Bacillus and genus Geobacillus is strain N. Valcheva Bacillus subtilis 0-2, identified from the thermal healing spring "Haskovo mineral baths" with a water temperature of 57.4°C, region Haskovo, showed moderate adhesion with 10 adherent bacteria on 10 epithelial cells. The adhesion ability of microorganisms does not depend on the isolation location; that could be due to the different fineness of the adhesion sites of bacteria on epithelial cells. Moderate induction of transforming growth factor beta TGF-beta is favorable for antiinflammatory effect. The immunomodulatory properties of the studied strains are presented in Table 18. The data show that all 12 strains increased the synthesis of transforming growth factor beta TGF-beta relative to the control, which was from a cell line without the presence of bacteria. The strains with the most significant induction of transforming growth factor beta TGFbeta were: B. thuringiensis B62, the combination BS + BV + BA (Bacillus subtilis + Bacillus vallismortis + Bacillus amyloliquefaciens) 8-1, Bacillus subtilis 0-2. The degree of induction by these strains is not as substantial as with specially selected intestinal probiotic strains. However, the increase in the synthesis of transforming growth factor beta TGF-beta by the two strains mentioned above and the combination shows a moderate anti-inflammatory effect. None of the 12 tested strains increased its concentration compared to the control (cell line without bacteria) regarding the cytokine IL-8. The two strains with the strongest inhibition of the production of the cytokine IL-8 were: Bacillus subtilis 0-2 and Bacillus thuringiensis B62. In addition to suppressing the synthesis of the cytokine IL-8, both strains induced the synthesis of transforming growth factor beta TGF-beta. They have the most significant anti-inflammatory and immunomodulatory properties. The strength of the antiinflammatory effects of the above-mentioned two strains cannot be compared with more significant anti-inflammatory effects of selected probiotics such as specific strains of Lactobacillus acidophilus, Lactobacillus bulgaricus, etc. In the case of the two strains Bacillus subtilis 0-2 and Bacillus thuringiensis B62, a moderate (not strong) anti-inflammatory, the

immunomodulatory effect can be claimed.

Conclusion: The studied strains have the following biological properties - amylolytic, proteolytic, and lipolytic enzyme activity.

- Antimicrobial activity against various bacteria, yeasts, and molds.
- The adhesive ability to human epithelial cells.
- Anti-inflammatory, immunomodulating effect.

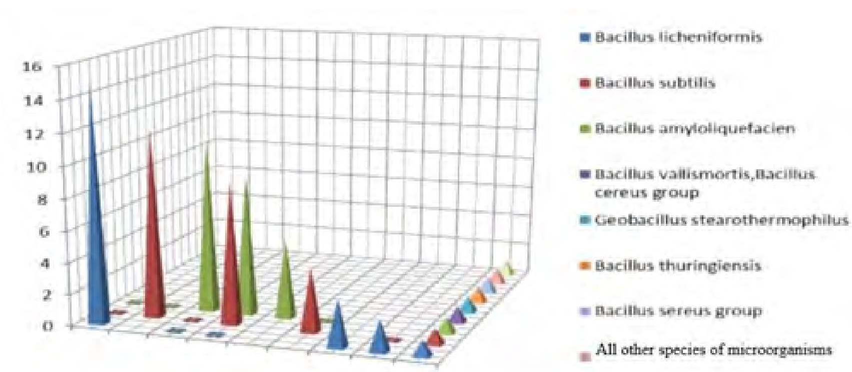

Figure 3: The largest number of microorganisms by species in the investigated waters in Bulgaria.

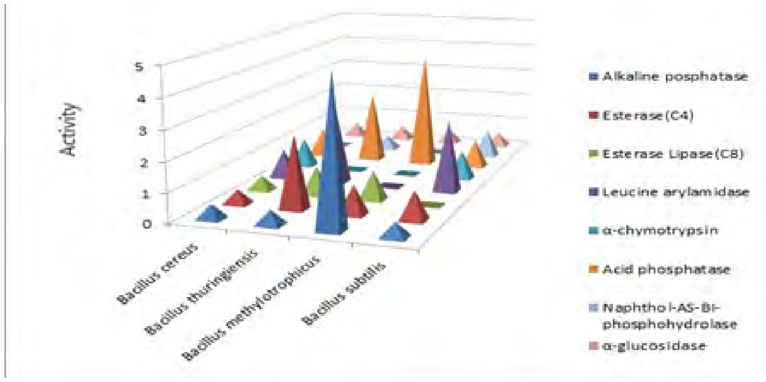

Figure 4: Enzyme profile of strains isolated from investigated waters in Haskovo region – Bacillus cereus (P1), Bacillus thuringiensis (P2), Bacillus methylotrophicus (Y1), Bacillus subtilis (Y2).
* enzyme activity is determined on a color scale from 0 (absence of enzyme activity) to 5 (maximum enzyme activity)

Table 11(87): Species affiliation of the strains isolated from waters in the Pazardzhik region.

Strain	Species affiliation (Reference strain)	Reliability, %
N. Valcheva BS 2-1-2	Bacillus subtilis	99.9
N. Valcheva BS 8-1-1	Bacillus subtilis	99.9
N. Valcheva BL 7-1	Bacillus licheniformis	99.9
N. Valcheva BL 7-1	Bacillus licheniformis	100
N. Valcheva BA 8-1-3	Bacillus amyloliquefaciens	99.9
N. Valcheva BV 8-1-2	Bacillus vallismortis	98.9
N. Valcheva BCG 10-1	Bacillus cereus group	99.9
N. Valcheva StX 14-2	Staphylococcus xylosus	99.9
N. Valcheva StX 14-2	Staphylococcus xylosus	99.9
N. Valcheva BL 7-1	Bacillus licheniformis	99.9
N. Valcheva GBSTh	Geobacillus stearothermophilus	99.9
	Table 11 (87) continued.	
N. Valcheva GBSTh	Geobacillus stearothermophilus	100
N. Valcheva BL 7-1	Bacillus licheniformis	99.9

Table 12(91): Species affiliation of the strains isolated from waters in the Sofia region.

Strain	Species affiliation (Reference strain)	Reliability, %
N. Valcheva BL 30-1	Bacillus licheniformis	99.9
N. Valcheva GbSt	Geobacillus stearothermophilus	99.9
N. Valcheva BS 2-1-1	Bacillus subtilis	99.9

Table 13(95): Species affiliation of the strains isolated from waters in Lovech region.

Strain	Species affiliation (Reference strain)	Reliability, %
N. Valcheva BL 8- 1	Bacillus licheniformis	99.9
N. Valcheva BA 2-1-3	Bacillus amyloliquefaciens	99.9
N. Valcheva BS 6-1-1	Bacillus subtilis	99.9

Table 14(99): Species affiliation of the strains isolated from waters in the Blagoevgrad region.

Strain	Species affiliation (Reference strain)	Reliability, %
N. Valcheva BCG 25-1	Bacillus cereus group	99.9
N. Valcheva BV 3-2-1	Bacillus vallismortis	99.9
N. Valcheva BS 2-2-2	Bacillus licheniformis	99.9
N. Valcheva BA 3-2-3	Bacillus amyloliquefaciens	99.9
N. Valcheva BM 2-3	Bacillus megaterium	99.9
N. Valcheva BsX 2-6	Bacillus simplex	99.9
N. Valcheva PsFl 3-8	Pseudomonas fluorescens	99.9
N. Valcheva GBSTt	Geobacillus stearothermophilus	99.9
N. Valcheva BL 6-1	Bacillus licheniformis	99.9
Ignatov § Valcheva GBSSt	Geobacillus stearothermophilus	99.9
Ignatov § Valcheva StMalt 1-1	Stenotrophomonas maltophilia a	99.9
Ignatov § Valcheva BV 3-2-1	Bacillus vallismortis	99.9
Ignatov § Valcheva BS 2-2-2	Bacillus subtilis	99.9
Ignatov § Valcheva BA 3-2-3	Bacillus amyloliquefaciens	99.9
Ignatov § Valcheva BM 2-3	Bacillus megaterium	99.9
Ignatov § Valcheva BsX 2-6	Bacillus simplex	99.9
Ignatov § Valcheva PsF 3-8	Pseudomonas fluorescens	99.9

Table 15(100): Identified microorganisms from water samples collected from different regions of the country.

Region	Species affiliation (Reference strain)	Number of different types	Number of springs
Haskovo	Bacillus methylotrophicus	4	3
	Bacillus cereus		
	Bacillus thuringiensis Bacillus subtilis		
Stara Zagora	Bacillus cereus group	7	16
	Bacillus thuringiensis		
	Bacillus vallismortis		
	Bacillus amyloliquefaciens Bacillus licheniformis		
	Bacillus altitudinis/pumilus Bacillus subtilis		
Yambol	Bacillus thuringiensis	3	3
	Bacillus cereus group		
	Bacillus amyloliquefaciens		
Sliven	Bacillus subtilis	4	5
	Bacillus vallismortis		
	Bacillus amyloliquefaciens Bacillus licheniformis		
Burgas	Bacillus licheniformis	5	6
	Bacillus cereus group Brevibacillus spp.		
	Bacillus amyloliquefaciens Bacillus subtilis		
Varna	Bacillus licheniformis	4	5
	Bacillus subtilis		
	Bacillus vallismortis		
	Bacillus amyloliquefaciens		

Plovdiv	Bacillus subtilis Bacillus cereus group Bacillus altitudinis/pumilus Bacillus licheniformis Bacillus subtilis Bacillus vallismortis Bacillus amyloliquefaciens Aeromonas sobria	8	30
Pazardzhik	Bacillus subtilis Bacillus licheniformis Bacillus amyloliquefaciens Bacillus vallismortis Bacillus cereus group Staphylococcus xylosus Geobacillus stearothermophilus	7	12
Sofia	Bacillus licheniformis Geobacillus stearothermophilus Bacillus subtilis	3	4
Lovech	Bacillus licheniformis Bacillus amyloliquefaciens Bacillus subtilis	3	3
Blagoevgrad	Bacillus subtilis Bacillus cereus group Bacillus vallismortis Bacillus licheniformis Bacillus amyloliquefaciens Bacillus megaterium Bacillus simplex Pseudomonas fluorescens Geobacillus stearothermophilus Stenotrophomonas maltophilia	10	2

Table 16(101): Distribution of identified microorganisms by region.

Type of microorganism	Region	Number areas
Bacillus methylotrophicus	Haskovo	1
Bacillus cereus	Haskovo	1
Bacillus cerereus group	Stara Zagora, Yambol, Burgas, Plovdiv, Pazardzhik, Blagoevgrad	6
Bacillus thuringiensis	Haskovo, Stara Zagora, Yambol	3
Bacillus subtilis	Haskovo, Stara Zagora, Sliven, Burgas, Varna, Plovdiv, Pazardzhik, Sofia, Lovech, Blagoevgrad	10
Bacillus vallismortis	Stara Zagora, Sliven, Varna, Plovdiv, Pazardzhik, Blagoevgrad	6
Bacillus amyloliquefaciens	Stara Zagora, Yambol, Sliven, Burgas, Varna, Plovdiv, Pazardzhik, Sofia, Lovech, Blagoevgrad	10
Bacillus licheniformis	Stara Zagora, Sliven, Burgas, Varna, Plovdiv, Pazardzhik, Sofia, Lovech, Blagoevgrad	9
Bacillus altitudinis/pumilus	Stara Zagora, Plovdiv	2
Brevibacillus spp.	Burgas	1
Staphylococcus xylolosus	Pazardzhik	1

Geobacillus stearothermophilus	Pazardzhik, Sofia, Blagoevgrad	3
Bacillus megaterium	Blagoevgard	1
Bacillus simplex	Blagoevgrad	1
Pseudomonas fluorescens	Blagoevgrad	1
Bacillus amyloliquefaciens subsp.	Plovdiv	1
plantarum		
Klebsiella oxytoca	Plovdiv	1
Aeromonas sobria	Plovdiv	1
Pseudomonas aeruginosa	Burgas	1
Stenotrophomonas maltofila	Blagoevgrad	1

Table 17(123): Adhesion of Bacillus and Geobacillus bacteria to the human epithelial cell line HT-29.

No	Strain	TGF-beta, pg/cm3	IL-8, pg/cm3
1	N. Valcheva Bacillus subtilis 0-2 from thermal healing spring "Haskovo mineral baths", Haskovo region	1546	110
2	N. Valcheva § I. Ignatov Bacillus subtilis 2-2-2 hyperthermal healing spring "Rupi 2", Blagoevgrad district	1187	134
3	N. Valcheva Bacillus amyloliquefaciens subsp. plantarum SDF004 from the hypothermal healing spring "Salt Spring" in the village of Narechenski Bani, Plovdiv region	1294	127
4	N. Valcheva Bacillus vallismortis 17-1 hypothermal healing spring KEI "Hadzhi Dimitar", "Toplata Voda" area, town of Shivachevo, Sliven region	1297	139
5	N. Valcheva BS + BV + BA 8-1 from hyperthermal healing spring "Driller No 5-Varvara" in Pazardzhik region	1716	119
6	N. Valcheva Bacillus thuringiensis B62 thermal healing spring "Driller No C3-7 Pavel banya, Stara Zagora region	1801	112
7	N. Valcheva Bacillus methylotrophicus PY5 hypothermal spring "Nanyo Nanev" Yablkovo, Haskovo region	1431	136
8	N. Valcheva Bacillus altitudimis/ pumilus 13-1 from hypothermal healing spring "KEI Stublata" Hisarya, Plovdiv region	1279	154
9	N. Valcheva Geobacillus stearothermophilus Varvara from hyperthermal healing spring Driller No 3 "Mizinka-Velingrad" with water temperature 87.7°C, Pazardzhik region	1469	138
10	N. Valcheva Geobacillus stearothermophilus from hyperthermal spring borehole No 5 "Syarna Banya- Velingrad", Pazarzhik region	1413	131
11	N. Valcheva Geobacillus stearothermophilus from hyperthermal healing spring "Rupi 1", Blagoevgrad region	1407	126
12	N. Valcheva Geobacillus stearothermophilus from a hyperthermal healing spring KEI "Pchelinski Bani", Sofia region	1410	136
13	Control strain	938	157

TGF-beta – transforming growth factor beta /proinflammatory cytokine/, pg/cm^3 IL-8 – interleukin 8/proinflammatory cytokine/, pg/cm^3.

Table 18(124): Immunomodulatory properties of the studied strains.

No	Cluster	Strain	Bacteria/10 epithelial cells	Level of adhesion
1	I-1	N. Valcheva Bacillus subtilis 0-2 from the thermal healing spring "Haskovo mineral baths" with a water temperature of 57.4°C, Haskovo region	10	moderate
2	I-2	N. Valcheva § I. Ignatov Bacillus subtilis 3-2-2 from the hyperthermal healing spring "Rupi 2" with a water temperature of 74.3°C, Blagoevgrad region	7	moderately weak
3	I-3	N. Valcheva Bacillus amyloliquefaciens subsp.plantarum SDF004 from the hypothermal healing spring "Salt Spring" in the village of Narechenski Bani with a water temperature of 21.5°C, Plovdiv region	0	zero
4	I-4	N. Valcheva Bacillus vallismortis 17-1 hypothermal healing spring KEI "Hadji Dimitar", locality "Toplata Voda", city of Shivachevo, with water temperature 22.5°C, Sliven region	0	zero
5	I-5	N. Valcheva BS + BV + BA (Bacillus subtilis + Bacillus vallismortis + Bacillus amyloliquefaciens) 8-1 from the hyperthermal healing spring "Driller No 5 - Varvara"	0	zero
		in the village of Varvara with a water temperature of 83.7°C, Pazardzhik region		
6	I-6	N. Valcheva Bacillus thuringiensis B62 from thermal healing spring "Driller No C3-7 Pavel banya with water temperature 48.50°C, Stara Zagora region	5	weak
7	II-1	N. Valcheva Bacillus methylotrophicus PY5 from hypothermal spring "Nanyo Nanev" Yablekovo, Haskovo region	2	very weak
8	II-2	N. Valcheva Bacillus altitudimis/V. pumilus 13-1 hypothermal healing spring "KEI Stublata" Hisarya with water temperature 31°C, Plovdiv region	1	very weak
9	II-3	N. Valcheva Geobacillus stearothermophilus Varvara from hyperthermal healing spring Driller No 3 "Mizinka - Velingrad" with water temperature 87.7°C, Pazardzhik region	2	very weak
10	II-4	N. Valcheva Geobacillus stearothermophilus from hyperthermal spring Driller No 5 "Syarna Banya-Velingrad" with a water temperature of 84.6°C, Pazarzhik region	1	very weak
11	II-5	N. Valcheva Geobacillus stearothermophilus from the hyperthermal healing spring "Rupi 2" with a water temperature of 74.3°C, Blagoevgrad region	4	weak
12	II-6	N. Valcheva Geobacillus stearothermophilus from a hyperthermal healing spring KEI "Pchelni Bani" with a water temperature of 79°C, Sofia region	3	Very weak

Number of adhered bacteria per 10 epithelial cells: 0-3 – very weak; 4-6 – weak; 7-9 – moderately weak; 10-20 – moderate; 20-30 – moderately strong; 30-50 – strong; > 50 – very strong

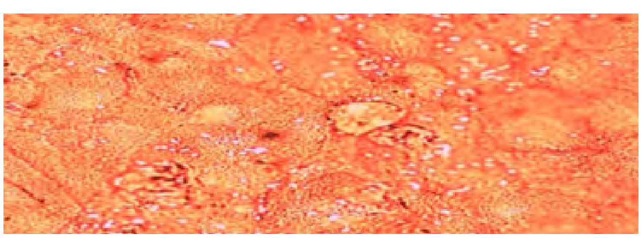

Image 1(174): Control image for lack of adhesion of bacteria on a human epithelial layer.

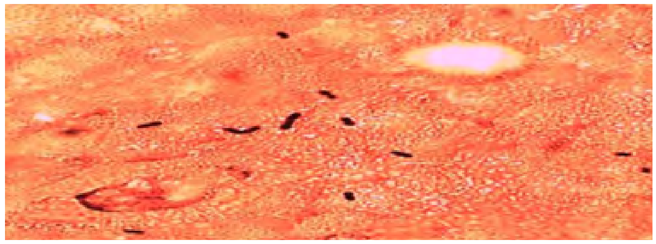

Image 2(175): Control image for moderate adhesion of bacteria on a human epithelial layer.

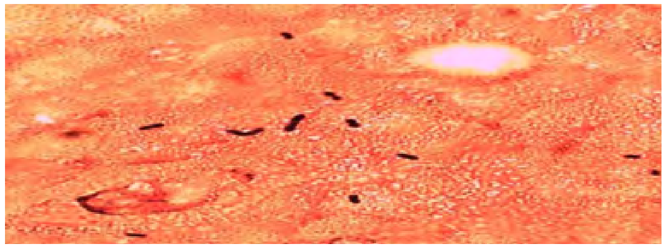

Image 3(176): Moderate adhesion of bacteria on human epithelial layer of N. Valcheva strain Bacillus subtilis 0-2 from thermal healing spring "Haskovo mineral baths" with water temperature 57.4°C, Haskovo region.

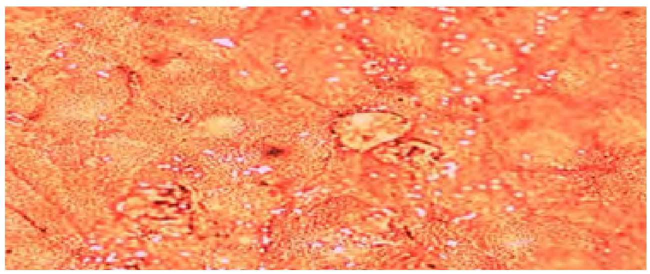

Image 4(177): Lack of adhesion of bacteria on human epithelial layer of N. Valcheva strain Bacillus amyloliquefaciens subsp. plantarum SDF004 from hypothermal healing spring "Salt spring" in Narechenski Bani village with water temperature 21.5°C, Plovdiv region. Figure 5 (8). Number of adherent bacteria per 10 epithelial cells.

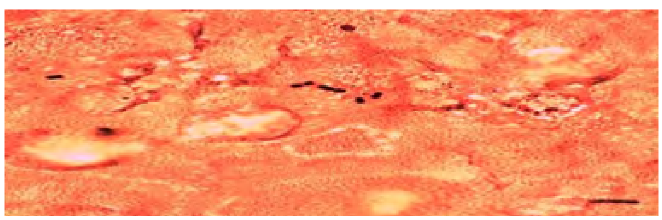

Image 5(178): Moderately weak adhesion of bacteria on human epithelial layer of Bacillus subtilis strain N. Valcheva § I. Ignatov Bacillus subtilis 3-2-2 from hyperthermal healing spring "Rupi 2" with water temperature 74.3°C, Blagoevgrad region.

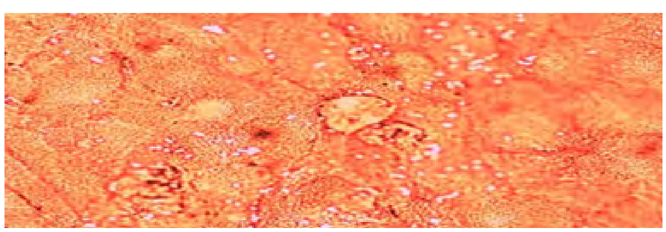

Image 6(179): Lack of adhesion of bacteria on human epithelial layer of N. Valcheva strain Bacillus vallismortis 17-1 from hypothermal healing spring KEI "Hadji Dimitar", locality "Toplata Voda" town of Shivachevo with water temperature of 22.5°C, Sliven region.

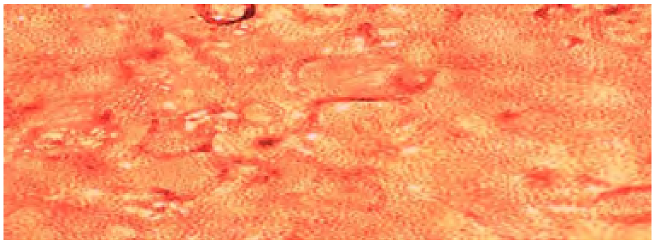

Image 7(180): Lack of adhesion of bacteria on human epithelial layer of strain N. Valcheva BS + BV + BA (Bacillus subtilis + Bacillus vallismortis + Bacillus amyloliquefaciens) 8-1 from hyperthermal healing spring "Driller No 5-Varvara" in the village of Varvara with temperature of water 83.7°C, Pazardzhik region.

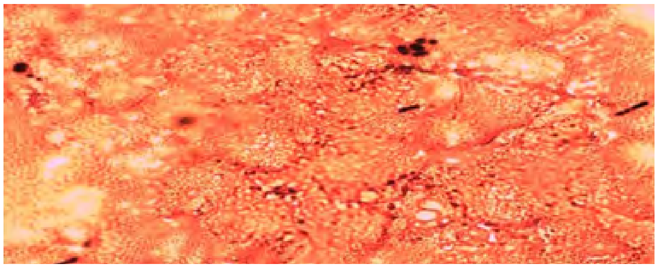

Image 8(181): Weak adhesion of bacteria on human epithelial layer of N. Valcheva strain Bacillus thuringiensis B62 from thermal healing spring "Driller No C3-7 Pavel banya with water temperature 48.50°C, Stara Zagora region.

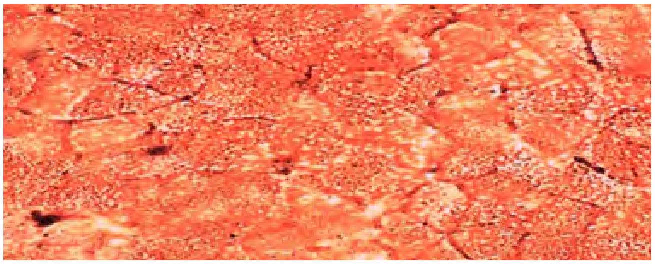

Image 9(182): Very weak adhesion on human epithelial layer of N. Valcheva Bacillus methylotrophicus PY5 strain from hypothermal spring "Nanyo Nanev" Yablkovo, Haskovo region.

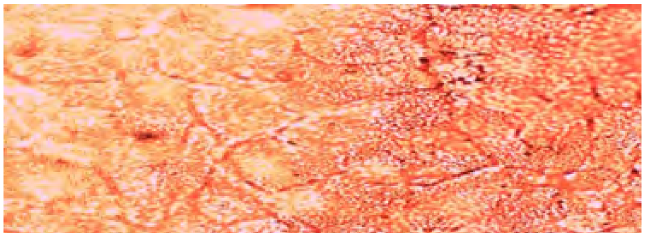

Image 10(183): Very weak adhesion on human epithelial layer of N. Valcheva strain Bacillus altitudimis/ pumilus 13-1 from hypothermal healing spring "KEI Stublata" Hisarya with water temperature 31°C, Plovdiv region.

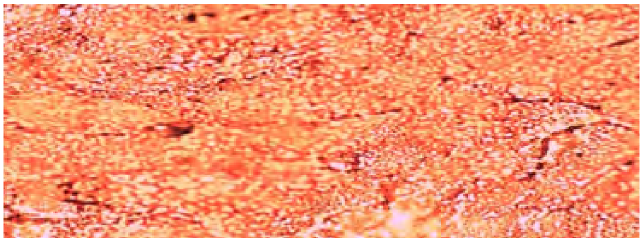

Image 11(184): Very weak adhesion on human epithelial layer of N. Valcheva strain Geobacillus stearothermophilus Varvara from hyperthermal healing spring Driller No 3 "Mizinka - Velingrad" with water temperature 87.7°C, Pazardzhik region.

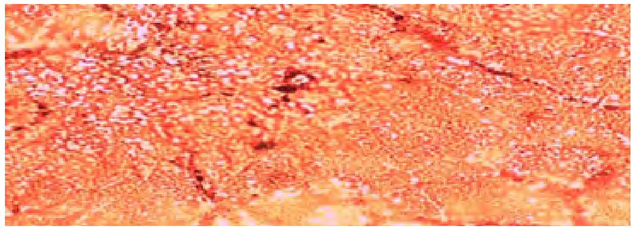

Image 12(185): Very weak adhesion on human epithelial layer of N. Valcheva strain
Geobacillus stearothermophilus Varvara, hyperthermal spring Driller No 5 "Syarna Banya-Velingrad" with water temperature 84.6°C, Pazarzhik region.

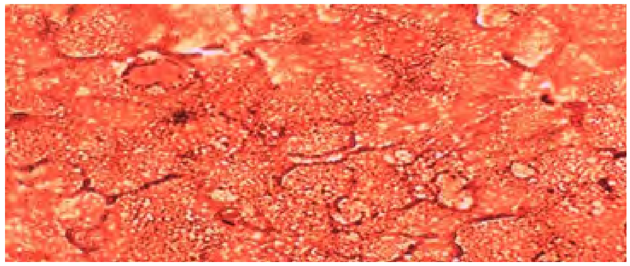

Image 13(186): Weak adhesion on human epithelial layer of strain N. Valcheva Geobacillus stearothermophilus Hyperthermal healing spring "Rupi 1" with water temperature 73.5°C, Blagoevgrad region.

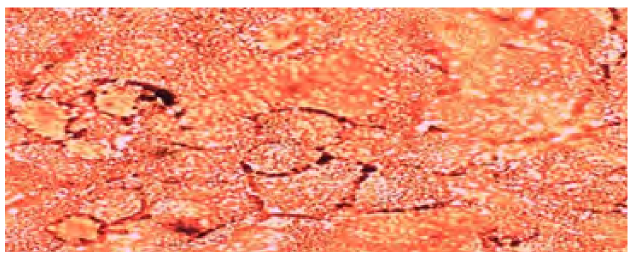

Image 14(187): Very weak adhesion on human epithelial layer of N. Valcheva strain
Geobacillus stearothermophilus from hyperthermal healing spring KEI "Pchelni Bani" with water temperature 79°C, Sofia region.

6. Conclusions

The obtained results in the dissertation thesis allow the following conclusions to be made:

1. Physical, chemical, and microbiological characterization of 90 mineral, mountain, and spring waters from 11 regions in the country – Haskovo, Stara Zagora, Yambol, Sliven, Burgas, Varna, Plovdiv, Pazardzhik, Sofia, Lovech, and Blagoevgrad – was carried out.

1.1. According to physical and chemical indicators, the water samples from only 24 water sources meet all the controlled parameters of Ordinance No 9/2001, SG No 30, and Decree No 178/ 23.07.2004 on water quality:

-Stara Zagora region: hypothermal spring in the village of Trakia with a water temperature of 20°C, hypothermal spring "St. Bogoroditsa" in the municipality of Maglizh with a water temperature of 21°C, hypothermal spring "Center" in the city of Maglizh with a water temperature of 21°C, a hypothermal spring in the village of Koprinka in the municipality of Maglizh with a water temperature of 21°C, a hypothermal spring in the city of Kazanlak in the municipality of Maglizh with a water temperature of 20°C, a hypothermal spring in Kren – Enina with a water temperature of 20°C, a hypothermal spring in the city of Enina with a water temperature of 20°C, hypothermal spring "Ayazmo" with a water temperature of 20°C, hypothermal spring "Trite Chuchura" with a water temperature of 20°C.

-Yambol region: hypothermal spring in the village of Okop with a water temperature of 20°C, hypothermal spring in the village of Karavelovo with a water temperature of 21°C.

-Sliven region: KEI "Hadzhi Dimitar" hypothermal healing spring, "Toplata Voda" locality, the town of Shivachevo with a water temperature of 22.5°C, "Gunchov Izvor" hypothermal healing spring with a water temperature of 21.5°C.

-Burgas region: hyperthermal healing spring in the village of Polyanovo with a water temperature of 51°C.

-Varna region: hyperthermal healing spring "Lekovita Voda" Goren Chiflik village, with water temperature 40°C, Varna region,

-Plovdiv region: hypothermal healing spring "Driller No 9" in the village of Dragoynovo with a water temperature of 20°C, hypothermal healing spring "Driller 1-Asenovgrad" in the town of Asenovgrad with a water temperature of 21°C, hypothermal spring "Bajova Voda " in the village of Bachkovo with a water temperature of 20 °C.

-Pazardzhik region: hyperthermal healing spring "Driller No 5-Varvara" in the village of Varvara with a water temperature of 83.7°C, a hypothermal spring in the town of Oborishte with a water temperature of 19°C, a hypothermal spring in Oborishte - Panagyurishte with a temperature of water 18°C, hypothermal spring Panagyurishte – Buta with water temperature 20°C.

-Sofia region: hypothermal healing spring "Driller TK No 1 "Ivanyane" town Bankya with water temperature 27°C.

1.2. According to microbiological indicators, the water samples from 15 water sources do not meet all the controlled microbiological parameters of Ordinance No 9/2001, SG No 30, and Decree No 178/ 23.07.2004 on the quality of drinking water.

-Stara Zagora region: spring water in the village of Trakia, spring water in "St. Nicholas Chudotvoretz" in the town of Maglizh, spring water in "St. Bogoroditsa" in Maglizh, spring water in the village of Koprinka, spring water in Enina.

-Yambol region: spring water in the village of Okop. - Sliven region: spring water in "Gunchov Izvor."

-Burgas region: spring water in the village of Sadievo.

-Varna region: spring water in "Lekovita voda" Goren Chiflik village.

-Plovdiv region: spring water in KEI "Mazhko banche" town of Banya.

-Panagyurishte region: spring water in the town of Oborishte, spring water in Oborishte - Panagyurishte, in spring water in Panagyurishte - Buta.

-Blagoevgrad district: spring water in "Rupi 1" and in spring water "Rupi 2".

1.3. A reduction in the water indexes has been established by treating it with metal ions/EVOdrop, electromagnetic waves or zeolite.

1.4. The relationship between biologically active substances – common duckweed extract (Lemna minuta Kunth.) extract, rose oil (Rosa damascena Mill.) and silivrea flower (Haberlea rhodopensis Friv.) to water and the formation of water clusters was determined.

2. Molecular genetic identification of 91 isolated microorganisms from 90 mineral, mountain, and spring waters from 11 regions of the country – Haskovo, Stara Zagora, Yambol, Sliven, Burgas, Varna, Plovdiv, Pazardzhik, Sofia, Lovech, and Blagoevgrad - was carried out for the first time. They belong to the following eight genera:

-genus Bacillus: B. subtilis, B. cereus group, B. altitudinis/pumilis, B. licheniformis, B.

vallismortis, B. amyloliquefaciens, B. methylotrophicus, B. thuringiensis, B. amyloliquefaciens subsp. plantarum, B. megaterium, B. simplex.

-genus Brevibacillus: Breviibacillus spp.

-genus Geobacillus: G. stearothermophilus.

-genus Aeromonas: A. hydrophila, A. sobria.

-genus Klebsiella: K. oxytoca.

-genus Pseudomonas: P. aeruginosa, P. fluorescens.

-genus Staphylococcus: S. xylosus.

-genus Stenotrophomonas: S. maltophilia.

3.Biological properties of the identified microorganisms were determined.

3.1. The strains with the highest enzyme activity were:

-Bacillus thuringiensis - N. Valcheva BTh B62 – amylolytic activity 25 mm, proteolytic activity 26.5 mm;

-Bacillus methylotrophicus - N. Valcheva BMTh PY4 – amylolytic activity 24 mm, proteolytic activity 20 mm;

-Bacillus subtilis - N. Valcheva BS 17-1, N. Valcheva BS 2-1-1, N. Valcheva BS 2-2-2 – amylolytic activity 26 mm, proteolytic activity 27 mm;

-Bacillus amyloliquefaciens - N. Valcheva BA 22-1 – amylolytic activity 26 mm, proteolytic activity 27 mm, lipolytic activity 22 mm;

-Bacillus licheniformis - N. Valcheva BL 3-1, N. Valcheva BL 30-1, N. Valcheva BL 7-1 – amylolytic activity 26 mm, proteolytic activity 27 mm, lipolytic activity 24 mm;

-Geobacillus stearothermophilus - N. Valcheva GbSt region of Pazardzhik, Sofia and

Blagoevgrad - amylolytic activity 25 mm, proteolytic activity 27 mm, lipolytic activity 24 mm;

-Bacillus cereus group - N. Valcheva BCG 25-1 – amylolytic activity 24 mm, proteolytic activity 26 mm, lipolytic activity 23 mm.

3.2. Antimicrobial activity of Bacillus sp. strains against saprophytic and pathogenic microorganisms: Penicillium sp., Fusarium moliniforme, Rhizopus sp., Aspergillus niger, Aspergillus oryzae, Aspergillus awamori, Mucor sp., and Enterococcus faecalis was determined. The most active strains were Bacillus methylotrophicus N. Valcheva BMTh /Haskovo region/, Bacillus cereus N. Valcheva BCG 25-1 /Yambol region/, Bacillus subtilis N. Valcheva BS 17-1 /Sliven region/, Bacillus subtilis N. Valcheva BS 18 /Sofia region/, Bacillus subtilis N. Valcheva BS 22-1 /Blagoevgrad region/ and Bacillus thuringiensis N. Valcheva BTh /Stara Zagora region/.

3.3. The adhesive ability to human epithelial cells was determined for the first time.

-A strain of N. Valcheva Bacillus subtilis 0-2, identified from a thermal healing spring "Haskovo mineral baths" with a water temperature of 57.4°C, is characterized by a moderate effect of 10 numbers of adhered bacteria on 10 epithelial cells.

-With moderately weak adhesion was strain N. Valcheva § I. Ignatov Bacillus subtilis

2-2-2 from hyperthermal healing spring “Rupi 2” with water temperature 74.3°C /Blagoevgrad region/ with seven adhered bacteria on 10 epithelial cells.

-With weak adhesion were strains N. Valcheva Bacillus thuringiensis B62 from the thermal healing spring “Driller No C3-7”, Pavel banya with water temperature 48.50 °C /Stara Zagora region/ and N. Valcheva Geobacillus stearothermophilus from hyperthermal healing spring “Rupi 1” with a water temperature of 73.5°C /Blagoevgrad region/, with respectively five and four adhered bacteria on 10 epithelial cells.

-All other strains had very weak or zero adhesion.

-With the most significant anti-inflammatory, immunomodulatory properties, i.e., with the most substantial inhibition of the production of the cytokine IL-8 (pg/cm3) and induction of the synthesis of transforming growth factor beta TGF-beta (pg/cm3) are strains Bacillus

subtilis 0-2 and B. thuringiensis B62.

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