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Antimicrobial Activity against Some Saprophytic and Pathogenic Microorganisms of *Bacillus species* Strains Isolated from Natural Spring Waters in Bulgaria

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Authors' contributions

This work was carried out in collaboration between all authors. Author YT managed the literature searches, designed the study and wrote the first draft of the manuscript. Author NVZ isolated the Bacillus sp. strains from the thermal springs. Authors YT and IK performed the experiments and wrote the protocols. Author ZD supervised the work. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

It is well known that many members of genus *Bacillus* possess an antimicrobial activity against a variety of microorganisms. In this study we present a primary screening for antimicrobial activity against some saprophytic and pathogenic microorganisms of nineteen strains identified as *Bacillus cereus*, *Bacillus thuringiensis* and *Bacillus subtilis*, isolated from eight natural thermal springs in two districts - Haskovo and Stara Zagora, Bulgaria. The inhibitory activities of the *Bacillus* sp. strains were determined by agar-well diffusion method. Test microorganisms were preliminarily included into the agar medium, whereas the *Bacillus* sp. strains were added to the wells. After 48 hours of incubation, the antimicrobial effects were determined by measuring the diameter of zones of inhibition around the wells. Most of *Bacillus* sp. strains showed high antimicrobial activitiy against the molds *Penicillium* sp., *Aspergillus oryzae*, *Aspergillus niger*, *Aspergillus awamori*, *Fusarium moliniforme* and *Rhizopus* sp. Inhibitory activity against the mold *Mucor* sp. and

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bacterium *Enterococcus faecalis* ranged between low and moderate. Two of the strains (*Bacillus cereus* 52/GI1 and *Bacillus thuringiensis* 56/H3) possessed moderate antimicrobial activity against bacterium *Pseudomonas aeruginosa* and yeasts *Saccharomyces cerevisiae*. One strain (*Bacillus subtilis* 47/YA1) had high activity against the mold *Mucormucedo*; one strain (*Bacillus cereus* 36/GI6) showed moderate activity against yeasts *Candida utilis*, while bacterium *Escherichia coli* was not inhibited at all.

Keywords: Antimicrobial activity; bacillus; bacteriocins; thermal springs; Bulgaria.

1. INTRODUCTION

Bacteria belonging to the genus *Bacillus* are widely spread in nature and can be easily isolated from a variety of foods of plant and animal origin, soil and natural water sources.

Members of the bacterial genus *Bacillus* are aerobic or facultatively anaerobic Gram-positive or Gram-variable spore-forming rods. The vegetative cells range from 0.5 by 1.2 to 2.5 by 10 μm in diameter and can grow at optimal temperatures ranging from 25 to 37ºC, although thermophilic and psychrophilic members are capable of growth at temperatures as high as 75ºC or as low as 3ºC. Some species can flourish at extremes of acidity and alkalinity, ranging from pH 2 to 10. The extreme heterogeneity of the genus is reflected in the wide variety of ecological niches that the many species occupy and in the debate over their taxonomic status. The G+C content of the DNA of species within the genus can vary from 32 to 69%, and many species may subsequently be reclassified into different taxonomic groupings. Most strains are catalase positive, possess peritrichous flagella and sporulate in air, which differentiates them from the clostridia [1].

The genus *Bacillus* is divided into three broad groups, depending on the morphology of the spore and sporangium. *B. cereus*, *B. megatenium*, *B. anthracis*, *B. thuringiensis*, and *B. cereus var. mycoides* are all found in the large-cell subgroup of group 1, i.e., Gram-positive rods that produce central or terminal ellipsoid or cylindrical spores that do not distend the sporangia. Protoplasmic inclusions of poly-beta-hydroxybutyrate are found in the large-celled species but not in the small-celled subgroup comprising *Bacillus subtilis*, *Bacillus pumilus*, and *Bacillus lichenifornis*, which form a separate subgroup. Most of the clinically important *Bacillus* isolates are found in group 1. Group 2 species are Gram-variable and have swollen sporangia with central or terminal ellipsoid spores. This group contains mainly *Bacillus circulans*, *Bacillus macerans*, *Bacillus polymyxa*, *Bacillus popillae*, *Bacillus larvae*, *Bacillus lentimorbus*, *Bacillus alvei*, *Bacillus stearothermophilus* and *Bacillus brevis*. Group 3 is dominated by the heterogeneous and Gram-variable *Bacillus sphaericus* species. The sporangia are swollen, with spherical terminal or subterminal spores [2-4].

Members of the genus *Bacillus* are known to produce a wide arsenal of substances, including peptide and lipopeptide antibiotics, and bacteriocins, which often have an antimicrobial effect on closely related organisms. These compounds have been extensively studied because of their potential applications in the food industry as natural biopreservatives and in pharmaceuticals as antimicrobials [4-6].

Bulgaria is one of the richest countries in natural mineral springs in Europe and takes a second place after Iceland. Their total number is 225 and approximately 70% of them (148) are located in the southern part of the country, especially around the towns of Haskovo and

Stara Zagora. Thermal springs in these two districts have water temperature between 40ºC and 57ºC and they are famous as important balneotherapy and spa centers. Chemical composition and physical properties of these mineral waters are shown at Table 1A. However, a little is known about the microorganisms in these hot waters and their influence on human health and other microorganisms.

In this article we present a primary screening for antimicrobial activity against some saprophytic and pathogenic microorganisms of nineteen *Bacillus* sp. strains, isolated during 2012 - 2013 from eight natural thermal springs, located in two of the most important Bulgarian balneotherapy districts - Haskovo and Stara Zagora. The inhibitory activities of *Bacillus* sp. strains were determined by agar-well diffusion assay. Antimicrobial effects were determined by measuring the diameter ofzones of inhibitionaround the wells.

2. MATERIALS AND METHODS

2.1 *Bacillus* sp. Isolates

We tested a total of nineteen strains, isolated during 2012 – 2013 from eight natural thermal springs in two districts - Haskovo and Stara Zagora, Bulgaria. The strains were sequenced at Macrogen Europe Laboratory, Netherlands and identified as *Bacillus cereus* (n=11), *Bacillus thuringiensis* (n=6) and *Bacillus subtilis* (n=2). The strains were named of the springs they derived as presented at Table 1B.

2.2 Test Microorganisms

In the present study following microorganisms, came from collection of the Department of Microbiology at University of Food Technologies, Plovdiv, Bulgaria, were used:

Bacteria :*Escherichia coli* ATCC 25922; *Enterococcus faecalis* NBIMCC 3360; *Pseudomonas aeruginosa* ATCC 9027;
Yeasts :*Candida utilis*; *Saccharomyces cerevisiae*;
Molds :*Aspergillus awamori*; *Aspergillus niger*; *Aspergillus oryzae*; *Fusarium moliniforme*; *Mucormucedo*; *Mucor* sp.; *Penicillium* sp.; *Rhizopus* sp.

2.3 Culture Media

Bacillus sp. strains were propagated in modified LB medium (LBG) containing 10g tryptone, 5g yeast extract, 10g NaCl and 10g glucose dissolved in 1L of deionized water. The pH of the medium was adjusted to 7.0 – 7.5 by using of 1N NaOH and autoclaved for 20 min. at 121℃ and 15 psi. For agar medium was used LBG medi um (prepared as above), with addition of 15g/L agar agar before autoclaving.

2.4 Agar-Well Diffusion Assay

The antimicrobial activity of the *Bacillus* sp. strains was determined by agar-well diffusion assay [7]. We performed it in two consecutive stages.

Table 1A. Chemical composition and physical properties of thermal spring waters in Haskovo and stara Zagora districts, Bulgaria

Parameter	Measure unit	MPV	District							
			Haskovo						Stara Zagora	
			H1	H2	GI	YA	D	R	PB	SMB
pH	pH units	6.5÷9.5	6.8	6.9	6.7	7.4	6.8	6.9	7.9	6.9
Oxidation	mgO_2/dm^3	5.0	0.7	0.6	1.7	0.6	1.3	0.7	0.3	0.6
Chlorides (Cl^-)	mg/dm^3	250.0	46.0	49.0	94.0	23.0	20.0	26.0	17.0	26.0
Nitrates (NO_3^-)	mg/dm^3	50.0	6.0	10.0	171.0	6.0	17.0	84.0	3.0	7.0
Nitrites (NO_2^-)	mg/dm^3	0.5	0.04	0.03	0.02	0.01	0.01	0.02	-	-
Ammonium ions (NH^{4+})	mg/dm^3	0.5	0.13	0.12	0.18	0.04	0.05	0.08	-	-
Sulfates (SO_4^{2-})	mg/dm^3	250.0	723.0	72.0	173.0	165.0	97.0	272.0	22.0	14.0
Calcium (Ca)	mg/dm^3	150.0	158.0	164.0	194.0	30.0	102.0	144.0	12.0	72.0
Magnesium (Mg)	mg/dm^3	80.0	20.0	22.0	44.0	21.0	74.0	46.0	-	30.0
Phosphates (PO_4^{3-})	mg/dm^3	0.5	0.1	0.1	0.5	0.1	0.4	-	-	-
Manganese (Mn)	mg/dm^3	50.0	1024.0	844.0	34.0	8.0	8.0	35.0	3.0	-
Iron (Fe)	$\mu g/dm^3$	200.0	845.0	533.0	24.0	16.0	30.0	20.0	30.0	16.0
Fluorides (F^-)	mg/dm^3	1.5	2.5	2.9	0.7	1.3	0.1	1.0	2.3	-
Conductivity	$\mu S/dm^3$	2000.0	1966.0	1962.0	1524.0	841.0	970.0	1194.0	670.0	750.0

Legend: MPV - Maximal permissible value; H1 – Haskovo mineral spa 1; H2 – Haskovo mineral spa 2; GI – Gorskiizvor; YA – Yabalkovo; D – Dobrich; R – Radievo; PB – Pavel banya; SMB – Stara Zagora mineral spa

Table 1B. *Bacillus* sp. strains investigated in present study

Isolate (spring)	District	Reference strain	Identity, %
Haskovo mineral spa 4 (32/H4)	Haskovo	*Bacillus cereus XA5-11*	100
Gorskiizvor 6 (36/GI6)	Haskovo	*Bacillus cereus JN267*	100
Pavel banya (37/PBGK)	Stara Zagora	*Bacillus thuringiensis B62*	99
Haskovo mineral spa 2 (40/H2)	Haskovo	*Bacillus cereus S74*	99
Gorskiizvor 4 (41/GI4)	Haskovo	*Bacillus cereus S74*	99
Pavel banya (42/BGFRA)	Stara Zagora	*Bacillus cereus A7-5*	97
Stara Zagora mineral spa (43/SMB)	Stara Zagora	*Bacillus thuringiensis B62*	99
Haskovo mineral spa 6 (44/H6)	Haskovo	*Bacillus cereus JN267*	98
Dobrich 1 (45/D1)	Haskovo	*Bacillus thuringiensis B62*	99
Haskovo mineral spa 1 (46/H1)	Haskovo	*Bacillus subtilis 0-2*	99
Yabalkovo 1 (47/YA1)	Haskovo	*Bacillus subtilis A2*	99
Haskovo mineral spa 5 (49/H5)	Haskovo	*Bacillus cereus JN267*	99
Gorskiizvor 5 (50/GI5)	Haskovo	*Bacillus thuringiensis B62*	99
Radievo 2 (51/R2)	Haskovo	*Bacillus thuringiensis B62*	99
Gorskiizvor 1 (52/GI1)	Haskovo	*Bacillus cereus KH2*	93
Radievo 1 (53/R1)	Haskovo	*Bacillus cereus LH1*	99
Gorskiizvor 3 (54/GI3)	Haskovo	*Bacillus cereus S433Ba-98*	97
Gorskiizvor 2 (55/GI2)	Haskovo	*Bacillus cereus WIF15*	98
Haskovo mineral spa 3 (56/H3)	Haskovo	*Bacillus thuringiensis B62*	99

2.5 Preparation of Test Microorganism Suspensions and Agar Medium

Test bacteria and yeasts were cultured on LBG agar medium for 24 hours at 30ºC, excepting *Escherichia coli* and *Enterococcus faecalis* which were cultured at 37ºC. The mold fungi were grown on malt extract agar (MEA) at 30ºC for 7 days or until sporulation. Then the test microorganisms were stored at 4ºC. Suspensions were prepared after vigorously shaking with sterile 0.5% NaCl. The concentrations of test microorganisms cells were determined using a Thoma's haemocytometer. The yeast and bacterial suspensions were prepared with a titer of 1.0 x 10^9cfu ml^{-1}. The suspensions of mold fungi contained spores and/or fractions of mycelium - at a concentration of 1.0 x 10^5cfu ml^{-1} [8]. Test microorganisms were inoculated in a preliminarily melted and tempered to 45 - 48ºC LBG agar medium. Inoculated LBG agar medium was transferred in quantity of 20ml in sterilized Petri dishes (d=10 cm) and allowed to solidify. After this, six wells (d=6 mm) per plate were cut.

2.6 *Bacillus* sp. Samples Preparation and Implementation of the Experiment

Each strain of *Bacillus* sp. was propagated in parallel in two tubes containing liquid LBG medium for 24 hours at 30ºC. Then one of the tubes was stored at 4ºC and another one was centrifuged at 3000 g^{-1} for 5 min. and cell-free supernatant was collected. Then the cells (biomass) were washed and resuspended in 0.5% NaCl. The supernatant and cells were also stored at 4ºC.

Two replicates of each sample (supernatant, cells and whole culture) were added in quantity of 60 μλ to the agar wells. After 48 hours of incubation at relevant conditions (37ºC for *Escherichia coli* and *Enterococcus faecalis*and 30ºC for the rest of indicator microorganisms), the antimicrobial effects were determined by measuring the diameter

ofzones of inhibitionaround the wells.

Microorganisms with inhibition zones in size 18 mm or more were considered as sensitive; moderately sensitive were those in which the zones were from 12 to 18 mm; resistant were those where the inhibition zones were up to 12 mm or completely missing [8].

3. RESULTS AND DISCUSSION

3.1 *Bacillus cereus* Strains

As seen at Table 2A, almost all *Bacillus cereus* strains (excepting *B. cereus* 52/GI1 and *B. cereus* 55/GI2), showed strong antifungal activity against the mold *Penicillium* sp. Four isolates (*B. cereus* 36/GI6, *B. cereus* 40/H2, *B. cereus* 53/R1and *B. cereus* 54/GI3) possessed high activity against the mold *Rhizopus* sp. In regard to the antagonistic activity to the mold *Mucor* sp., strains *B. cereus* 32/H4, *B. cereus* 44/H6 and *B. cereus* 54/GI3 showed insignificant inhibitory effect. The cells and whole culture of the strains*B. cereus* 40/H2, *B. cereus* 41/GI4 and *B. cereus* 49/H5 showed similar inhibitory effect. The supernatant of these three strains had no activity against *Mucor* sp., probably due to the association of antibacterial compounds to the cell walls and they do not release into the culture medium. None of the strains possessed activity against *M. mucedo*.

Regarding the antifungal properties of *B. cereus*strains towards representatives from genus *Aspergillus*, all of them showed pronounced activity against the plant pathogens *Aspergillus oryzae* and *Aspergillus niger* (with exception of *B. cereus* 44/H6). The strains *B. cereus* 36/GI6, *B. cereus* 44/H6, *B. cereus* 49/H5, *B. cereus* 53/R1, *B.cereus* 54/GI3 and *B. cereus* 55/GI2 had strong antagonistic effect towards *Aspergillus awamori*. High antifungal activity of all strains against the mold *Fusarium moliniforme* was observed (Table 2B).

Antibacterial activity of *B. cereus*strains was low (Table 2C). Ten of eleven strains (excepting *B. cereus* 55/GI2) showed weak to moderate activity towards the Gram-positive bacterium *Enterococcus faecalis*, but had no effect on Gram-negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa*, as well as on the yeasts *Saccharomyces cerevisiae* and *Candida utilis* (excepting *B. cereus* 52/GI1 which had weak effect against *Pseudomonas aeruginosa* and *Saccharomyces cerevisiae*, and *B. cereus* 36/GI6 which possessed moderate activity against *Candida utilis*).

It is assumed that antimicrobial activity of *Bacillus cereus* is in a result of proteins with bactericidal effect, called cereins, produced at the beginning of the stationary phase. According to some investigations, the mode of action of cereins is to interfere with cell membranes and the cell wall [4,5].

Table 2A. Antifungal activity of *B. cereus* strains against the molds *Penicillium* sp., *Rhizopus* sp., *Mucor* sp. and *Mucor mucedo*

Isolate		Inhibition zones, mm			
		Test microorganism (1.0 x 10^5cfu ml^{-1})			
		***Penicillium* sp.**	***Rhizopus* sp.**	***Mucor* sp.**	***Mucor mucedo***
Bacillus cereus 32/H4	Supernatant	22.0	-	10.5	-
	Cells	25.0	-	12.0	-
	Culture	23.0	-	12.0	-
Bacillus cereus 36/GI6	Supernatant	20.0	20.5	-	-
	Cells	21.0	20.0	-	-
	Culture	21.5	20.0	-	-
Bacillus cereus 40/H2	Supernatant	23.5	20.0	-	-
	Cells	29.0	21.5	11.0	-
	Culture	29.0	23.5	12.0	-
Bacillus cereus 41/GI4	Supernatant	23.0	-	-	-
	Cells	22.0	-	10.5	-
	Culture	25.0	-	11.0	-
Bacillus cereus 42/BGFRA	Supernatant	17.5	-	-	-
	Cells	17.0	-	-	-
	Culture	18.0	-	-	-
Bacillus cereus 44/H6	Supernatant	19.0	-	11.0	-
	Cells	21.5	-	10.0	-
	Culture	21.0	-	11.5	-
Bacillus cereus 49/H5	Supernatant	11.0	-	-	-
	Cells	13.0	-	10.0	-
	Culture	13.0	-	10.5	-
*Bacillus cereus*52/GI1	Supernatant	-	-	-	-
	Cells	-	-	-	-
	Culture	-	-	-	-
Bacillus cereus 53/R1	Supernatant	17.0	23.0	-	-
	Cells	20.0	22.0	-	-
	Culture	21.0	27.0	-	-
Bacillus cereus 54/GI3	Supernatant	20.0	26.5	10.0	-
	Cells	22.0	18.5	10.0	-
	Culture	19.0	22.0	10.0	-
Bacillus cereus 55/GI2	Supernatant	-	-	-	-
	Cells	-	-	-	-
	Culture	-	-	-	-

Table 2B. Antifungal activity of *B. cereus*strains against the molds from genus *Aspergillus* and *Fusarium moliniforme*

Isolate		Inhibition zones, mm			
		Test microorganism (1.0 x 10^5cfu ml^{-1})			
		Aspergillus oryzae	***Aspergillus awamori***	***Aspergillus niger***	***Fus. moliniforme***
Bacillus cereus 32/H4	Supernatant	20.0	-	22.5	17.0
	Cells	21.0	-	27.0	19.0
	Culture	20.0	-	29.5	22.0
Bacillus cereus 36/GI6	Supernatant	18.0	23.5	25.5	26.5
	Cells	21.0	23.0	26.5	29.0
	Culture	18.0	25.0	21.5	27.0
Bacillus cereus 40/H2	Supernatant	22.0	-	22.0	20.0
	Cells	21.0	-	23.0	20.0
	Culture	20.0	-	27.0	19.0
Bacillus cereus 41/GI4	Supernatant	20.0	-	27.0	18.0
	Cells	23.0	-	20.5	20.0
	Culture	22.0	-	17.5	22.0
Bacillus cereus 42/BGFRA	Supernatant	21.0	-	25.5	23.5
	Cells	22.0	-	27.5	22.5
	Culture	22.5	-	21.0	23.0
*Bacillus cereus*44/H6	Supernatant	17.0	16.0	-	13.0
	Cells	19.0	16.0	-	18.0
	Culture	18.5	18.0	-	19.0
Bacillus cereus 49/H5	Supernatant	20.0	29.0	22.5	25.0
	Cells	15.5	19.0	25.5	26.5
	Culture	18.0	16.0	22.0	24.0
*Bacillus cereus*52/GI1	Supernatant	19.0	-	25.5	16.5
	Cells	20.0	-	27.0	16.0
	Culture	19.0	-	26.0	10.0
*Bacillus cereus*53/R1	Supernatant	17.0	25.0	34.5	22.0
	Cells	19.0	19.0	30.0	21.0
	Culture	22.0	20.0	32.0	24.0
Bacillus cereus 54/GI3	Supernatant	15.5	23.5	26.5	21.0
	Cells	10.0	19.5	23.5	16.0
	Culture	14.0	21.0	23.5	23.5
Bacillus cereus 55/GI2	Supernatant	18.0	20.0	25.0	30.0
	Cells	15.0	18.0	23.0	15.0
	Culture	18.0	24.0	25.5	28.0

Table 2C. Antibacterial activity of *B. cereus*strains against *Escherichia coli*, *Enterococcus faecalis*, *Pseudomonas aeruginosa* and yeasts *Saccharomyces cerevisiae* and *Candida utilis*

Isolate		Inhibition zones, mm				
		Test microorganism (1.0 x 10^9cfu ml^{-1})				
		E. coli	***Enteroc. faecalis***	***Ps. aeruginosa***	***Sacchar. cerevisiae***	***Candida utilis***
acillus cereus 32/H4	Supernatant	-	11.0	-	-	-
	Cells	-	13.0	-	-	-
	Culture	-	14.0	-	-	-
Bacillus cereus 36/GI6	Supernatant	-	14.0	-	-	13.0
	Cells	-	13.0	-	-	14.0
	Culture	-	15.0	-	-	14.0
*Bacillus cereus*40/H2	Supernatant	-	12.0	-	-	-
	Cells	-	15.0	-	-	-
	Culture	-	17.0	-	-	-
*Bacillus cereus*41/GI4	Supernatant	-	12.0	-	-	-
	Cells	-	12.0	-	-	-
	Culture	-	12.0	-	-	-
*Bacillus cereus*42/BGFRA	Supernatant	-	10.5	-	-	-
	Cells	-	13.0	-	-	-
	Culture	-	14.0	-	-	-
*Bacillus cereus*44/H6	Supernatant	-	14.0	-	-	-
	Cells	-	12.0	-	-	-
	Culture	-	10.0	-	-	-
Bacillus cereus 49/H5	Supernatant	-	12.0	-	-	-
	Cells	-	14.0	-	-	-
	Culture	-	14.5	-	-	-
Bacillus cereus 52/GI1	Supernatant	-	10.0	11.0	12.0	-
	Cells	-	12.0	10.0	12.0	-
	Culture	-	13.0	10.0	10.5	-
Bacillus cereus 53/R1	Supernatant	-	11.0	-	-	-
	Cells	-	13.0	-	-	-
	Culture	-	13.5	-	-	-
Bacillus cereus 54/GI3	Supernatant	-	10.0	-	-	-
	Cells	-	12.0	-	-	-
	Culture	-	15.5	-	-	-
Bacillus cereus 55/GI2	Supernatant	-	-	-	-	-
	Cells	-	-	-	-	-
	Culture	-	-	-	-	-

Legend: „-“ -no inhibition;d_{well} = 6 mm

According to other reports, bacteriocins are not usually associated to the cells and they are detectable in the culture supernatant [9,10]. As seen, the results in our study clearly showed that not only the supernatant, but also the cells (biomass) possessed activity against the test microorganisms. We suggested that this “phenomenon” was related to a presence of cell-associated substances with antimicrobial activity. Other possible reason for inhibitory activity of the biomass was after adding the cells to the wells, they started to grow again and continue to produce and release bacteriocins and other antibiotic substances, which diffused in the agar medium.

3.2 *Bacillus thuringiensis* Strains

As seen at Table 3A, all *Bacillus thuringiensis* strains demonstrated strong antagonistic effect on the mold *Penicillium* sp. In a contrast, none of the strains possessed activity against the mold *Rhizopus* sp. In general, antifungal activity of the strains against the mold *Mucor* sp. was considerably weaker and missing in the strain *B. thuringiensis*51/R2 and in supernatant of *B. thuringiensis*45/D1. The supernatant of *B. thuringiensis*45/D1 showed no activity, probably due to the association of compounds with antibacterial effect to the cells. The mold *Mucormucedo* remained unaffected.

Moderate to high antifungal activity of all *Bacillus thuringiensis* strains against *Aspergillus oryzae* was observed. In regard to the inhibitory activity against *Aspergillus niger*, four strains – *B. thuringiensis*37/PBGK, *B. thuringiensis*45/D1, *B. thuringiensis*50/GI5 and *B. thuringiensis*56/H3 showed significant effect. Only two of the strains - *B. thuringiensis* 37/PBGK and *B. thuringiensis*56/H3, possessed significant activity against *Aspergillus awamori*. Strong antifungal effect of all strains against the mold *Fusarium* sp. was ascertained (Table 3B).

As seen at Table 3C, *Bacillus thuringiensis* strains showed moderate activity against the Gram-positive bacterium *Enterococcus faecalis*. Only the strain *B. thuringiensis* 56/H3 was moderately active towards the Gram-negative bacterium *Pseudomonas aeruginosa* and yeast *Saccharomyces cerevisiae*. None of the strains inhibited *E. coli* and the yeasts *C. utilis*.

Many authors reported that *B. thuringiensis* produces bacteriocin-like inhibitory substances in the growth medium, and explain its antimicrobial activity with them. These antimicrobial peptides, called thuricins, exhibit a broad range of inhibitory activity against Gram-positive bacteria and several fungi [11,12,6].

3.3 *Bacillus subtilis* Strains

The results presented at Table 4A showed that *Bacillus subtilis*strains had high activity against the mold *Penicillium* sp. The strain *B. subtilis*46/H1 had no activity towards the mold *Rhizopus* sp., while the strain *B. subtilis*47/YA1 formed distinct inhibitory zones. Regarding the antifungal effect on *Mucor* sp., only the cells and culture of strain *B. subtilis*46/H1 showed low activity, while the second strain*B. subtilis*47/YA1 possessed significant effect only against the mold *Mucormucedo*.

Table 3A. Antifungal activity of *B. thuringiensis* strains against the molds *Penicillium*sp., *Rhizopus*sp., *Mucor* sp. and *Mucor mucedo*

Isolate		Inhibition zones, mm			
		Test microorganism (1.0 x 10^5cfu ml^{-1})			
		***Penicillium* sp.**	***Rhizopus* sp.**	***Mucor* sp.**	***Mucor mucedo***
Bacillus thuringiensis 37/PBGK	Supernatant	22.0	-	10.5	-
	Cells	22.5	-	11.0	-
	Culture	23.5	-	9.5	-
Bacillus thuringiensis 43/SMB	Supernatant	23.5	-	8.5	-
	Cells	22.0	-	11.0	-
	Culture	22.5	-	9.5	-
Bacillus thuringiensis 45/D1	Supernatant	24.5	-	-	-
	Cells	19.0	-	13.0	-
	Culture	19.0	-	11.5	-
Bacillus thuringiensis 50/GI5	Supernatant	20.0	-	10.5	-
	Cells	20.0	-	12.5	-
	Culture	25.0	-	11.5	-
Bacillus thuringiensis 51/R2	Supernatant	22.5	-	-	-
	Cells	22.5	-	-	-
	Culture	21.0	-	-	-
Bacillus thuringiensis 56/H3	Supernatant	22.5	-	9.5	-
	Cells	26.5	-	10.0	-
	Culture	32.5	-	11.5	-

Table 3B. Antifungal activity of *B. thuringiensis* strains against the molds from genus *Aspergillus* and *Fusarium moliniforme*

Isolate		Inhibition zones, mm			
		Test microorganism (1.0 x 10^5cfu ml^{-1})			
		Aspergillus oryzae	***Aspergillus awamori***	***Aspergillus niger***	***Fus. moliniforme***
Bacillus thuringiensis	Supernatant	17.0	24.0	24.5	20.0
37/PBGK	Cells	15.0	26.0	24.5	21.0
	Culture	14.0	28.5	13.5	22.0
Bacillus thuringiensis 43/SMB	Supernatant	18.0	-	-	18.0
	Cells	16.0	-	-	16.5
	Culture	17.0	-	-	19.0
Bacillus thuringiensis 45/D1	Supernatant	16.0	-	27.5	21.0
	Cells	18.0	-	20.0	19.0
	Culture	19.0	-	21.0	19.0
Bacillus thuringiensis 50/GI5	Supernatant	22.0	-	26.0	19.0
	Cells	24.0	-	21.0	20.0
	Culture	23.0	-	22.0	18.0
Bacillus thuringiensis 51/R2	Supernatant	24.0	-	-	22.0
	Cells	20.0	-	-	23.0
	Culture	21.0	-	-	24.0
Bacillus thuringiensis 56/H3	Supernatant	20.0	30.0	23.0	31.0
	Cells	18.0	16.0	26.0	27.0
	Culture	16.0	27.0	25.5	31.0

Table 3C. Antibacterial activity of *B. thuringiensis* strains against *Escherichia coli*, *Enterococcus faecalis*, *Pseudomonas aeruginosa* and yeasts *Saccharomyces cerevisiae* and *Candida utilis*

Isolate		Inhibition zones, mm				
		Test microorganism (1.0 x 10^9cfu ml^{-1})				
		E. coli	***Enteroc. faecalis***	***Ps. aeruginosa***	***Sacchar. cerevisiae***	***Candida utilis***
Bacillus thuringiensis 37/PBGK	Supernatant	-	12.0	-	-	-
	Cells	-	14.0	-	-	-
	Culture	-	15.5	-	-	-
Bacillus thuringiensis 43/SMB	Supernatant	-	12.0	-	-	-
	Cells	-	12.0	-	-	-
	Culture	-	12.0	-	-	-
Bacillus thuringiensis 45/D1	Supernatant	-	10.0	-	-	-
	Cells	-	13.0	-	-	-
	Culture	-	14.0	-	-	-
Bacillus thuringiensis 50/GI5	Supernatant	-	12.0	-	-	-
	Cells	-	14.0	-	-	-
	Culture	-	15.5	-	-	-
Bacillus thuringiensis 51/R2	Supernatant	-	10.0	-	-	-
	Cells	-	12.0	-	-	-
	Culture	-	15.0	-	-	-
Bacillus thuringiensis 56/H3	Supernatant	-	12.0	12.0	14.0	-
	Cells	-	13.0	10.0	15.5	-
	Culture	-	15.0	9.5	15.0	-

Legend: „-“ -no inhibition;d_{well} = 6 mm

As seen at Table 4B, the strain *B. subtilis* 47/YA1 possessed high activity against all of the tested representatives of genus *Aspergillus* and *Fusarium moliniforme*. The strain *B. subtilis*46/H1 was not active against *Aspergillus awamori*, but formed significant inhibition zones towards *Aspergillus oryzae, Aspergillus niger* and *Fusarium moliniforme*.

Antibacterial activity of *Bacillus subtilis* strains was very low (Table 4C). None of them inhibited the indicator bacteria and yeasts, excluding the strain *B. subtilis*46/H1, which affected insignificantly *Enterococcus faecalis*.

Table 4A. Antifungal activity of *B. subtilis* strains against the molds *Penicillium* sp., *Rhizopus* sp., *Mucor* sp. and *Mucor mucedo*

Isolate		Inhibition zones, mm			
		Test microorganism (1.0 x 10^5cfu ml^{-1})			
		***Penicillium* sp.**	***Rhizopus* sp.**	***Mucor* sp.**	***Mucor mucedo***
Bacillus subtilis	Supernatant	20.0	-	-	-
46/H1	Cells	19.0	-	10.0	-
	Culture	20.5	-	10.0	-
Bacillus subtilis	Supernatant	26.5	14.0	-	15.5
47/YA1	Cells	25.5	16.5	-	24.0
	Culture	24.0	18.5	-	26.0

Table 4B. Antifungal activity of *B. subtilis* strains against the molds from genus *Aspergillus* and *Fusarium moliniforme*

Isolate		Inhibition zones, mm			
		Test microorganism (1.0 x 10^5cfu ml^{-1})			
		Aspergilluso ryzae	***Aspergillusa wamori***	***Aspergillus niger***	***Fus. moliniforme***
Bacillus subtilis	Supernatant	16.0	-	20.5	18.0
46/H1	Cells	18.0	-	21.0	17.0
	Culture	20.0	-	22.5	23.0
Bacillus subtilis	Supernatant	26.0	25.0	28.0	25.0
47/YA1	Cells	10.0	22.5	27.5	22.0
	Culture	17.0	26.5	31.0	24.0

Table 4C. Antibacterial activity of *B. subtilis* strains against *Escherichia coli*, *Enterococcus faecalis*, *Pseudomonas aeruginosa* and yeasts *Saccharomyces cerevisiae* and *Candida utilis*

Isolate		Inhibition zones, mm				
		Test microorganism (1.0 x 10^9cfu ml^{-1})				
		E. coli	***Enteroc. faecalis***	***Ps. aeruginosa***	***Sacchar. cerevisiae***	***Candida utilis***
Bacillussubtilis	Supernatant	-	10.0	-	-	-
46/H1	Cells	-	14.0	-	-	-
	Culture	-	14.5	-	-	-
Bacillus subtilis	Supernatant	-	-	-	-	-
47/YA1	Cells	-	-	-	-	-
	Culture	-	-	-	-	-

Legend: „-“ -no inhibition;d_{well} = 6 mm

According to some reports, the inhibitory effect of *Bacillus subtilis* strains on other microorganisms, is due to the produced antimicrobial peptide, called subtilin and belonging to type-A lantibiotics. Besides subtilin, the different *B. subtilis* strains may produce other antimicrobial compounds as the broad-spectrum bacteriocin subtilosin A [13], lantibiotics, rhizocticin, surfactin, bacilysin, mycosubtilin, betacin, ericin, mersacidin etc., which are still not completely characterized [14,6].

Microorganisms, evaluated in this study as test microorganisms, are widely spread in the environment. Both molds (mainly *Penicillium* sp.) and yeasts are known as important spoilage organisms in different food products. Some mold fungi (*Fusarium* sp., *Aspergillus niger*, *Aspergillus oryzae*) are important pathogens of plants and produce mycotoxins which cause poisonings in humans and domestic animals. Bacteria used in this study are important pathogens causing food poisonings (*Escherichia coli*) or nosocomial infections (*Enterococcus faecalis*) in humans or can be causative agents of animal infections (*Pseudomonas aeruginosa*).

Generally, the results we obtained showed high antimicrobial activity of *Bacillus* sp. strains against majority of indicator microorganisms used – *Penicillium* sp., *Aspergillus oryzae*, *Aspergillus niger*, *Aspergillus awamori*, *Fusarium moliniforme* and *Rhizopus* sp., which makes these *Bacillus* strains perspective as producers of bacteriocins. Among the most active isolates are *B.cereus*36/GI6, *B. cereus*53/R1, *B.cereus* 54/GI3, *B. thuringiensis* 37/PBGK, *B. thuringiensis* 56/H3 and *Bacillus subtilis* 47/YA1.

Many *Bacillus* bacteriocins and lantibiotics with strong antibacterial and antifungal activity are interesting as means for therapy of human infections, caused by *L. monocytogenes*, *G. vaginalis*, *S. agalactiae* and others [15]. Some bacteriocin-producing *Bacillus* strains may be used as probiotics for treatment of long-term intestinal disorders due to their inhibitory activity against intestinal pathogens such as *C. perfringens*, *C. difficile*, etc. [16].

Bacteriocin-producing bacilli could be used as probiotics for livestock application, based on functional properties such as improvement of body weight of farm animals and poultry. Added to the feed, some bacteriocins improve the rumen fermentation (in ruminants) or reduce the poultry mortality by inhibition of pathogenic bacteria such as *C. jejuni*, *C. perfringens*, *Yersinia*, etc. [6].

Bacillus bacteriocins with strong inhibitory activity against staphylococci, could find practical applications in the control of mastitis in dairy cows. In recent studies, several bacteriocins from *B. thuringiensis* were tested against a collection of *S. aureus* isolates from dairy sources, which showed resistance to a variety of commercial antibiotics [17].

Consumer demands for minimally processed foods or “fresh foods” with no chemical preservatives have stimulated research interest in natural antimicrobial agents. Many studies present the effectiveness of application of bacteriocins as biopreservatives against spoilage of foods. It has been proved that some *Bacillus* sp. bacteriocins have a potential preservative application in different food substrates like in dairy products such as milk and cheeses. Some microbial peptides (BLIS) showed potential application in biopreservation of poultry meat, others - for preservation of seafood products, etc. [18,19].

Bacilli are naturally associated with soil and plants. For this reason, strains producing bacteriocins or BLIS with antibacterial or antifungal activity could be applied in the biological control of plant diseases. BLIS displaying antifungal activities, or their producer

strains, could be applied in the biocontrol of plant decay and postharvest control of fruits and vegetables [6].

4. CONCLUSION

The increasing microbial resistance to conventional antibiotics resulted in a growing interest to consider the bacteriocins synthesized by *Bacillus* sp. as alternative antimicrobials against a variety of microorganisms. Bacteriocin-producing *Bacillus* strains could be used for different applications towards saprophytic and pathogenic microorganisms in the fields of medicine, veterinary medicine, agriculture and food industry.

Based on the results obtained, the isolates with highest antimicrobial activity - *B. cereus* 36/GI6, *B. cereus* 53/R1, *B. cereus* 54/GI3, *B. thuringiensis* 37/PBGK, *B. thuringiensis* 56/H3 and *Bacillus subtilis* 47/YA1 are promising as bacteriocin-producing strains and their studying will continue in the future. Hopefully, the number of applications of bacteriocins will continue to increase, and the antimicrobial properties of members of *Bacillus* sp., which are widely disseminated in the nature, will be exploited in more rational ways.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Drobniewski FA. Bacillus cereus and related species. Clinical Microbiology Reviews. 1993;6(4):324–338.
2. Gordon RE, Haynes WC, Pang CHN. The genus Bacillus. United States Department of Agriculture Agricultural Handbook no. 427. U.S. Government Printing Office, Washington, D.C.; 1973.
3. Turnbull PCB, Kramer J, Melling J. Bacillus. In Topley and Wilson's principles of bacteriology, virology and immunity; 8-th ed. Edward Arnold, London. 1990;2:188 - 210.
4. Oscariz JC, Pisabarro AG. Characterization and mechanism of action of cerein 7, a bacteriocin produced by Bacillus cereus Bc7. Journal of Applied Microbiology. 2000;89:361–369.
5. Bizani D, Motta AS, Morrissy J, Terra R, Souto AA, Brandelli A. Antibacterial activity of cerein 8A, a bacteriocin-like peptide produced by Bacillus cereus. International Microbiology. 2005;8(2):125-131.
6. Abriouel H, Franz C, Ben Omar N, Galvez A. Diversity and applications of Bacillus bacteriocins (review). FEMS Microbiology Reviews. 2010;35:201–232.
7. Reeves DS. Antibiotic assays. In: Hawkey PM, Lewis DA editors. Medical Bacteriology, A Practical Approach. IRL Press, Oxford. 1989;195–221.
8. Todorova S, Kozhuharova L. Characteristics and antimicrobial activity of *Bacillus subtilis* strains isolated from soil. World J. Microbiol. Biotechnol. 2010;26:1207–1216.

9. Naclerio G, Ricca E, Sacco M, De Felice M. Antimicrobial Activity of a Newly Identified Bacteriocin of Bacillus cereus. Applied and Environmental Microbiology. 1993;59(12):4313–4316.
10. Risøen PA, Rønning P, Hegna IK, Kolstø AB. Characterization of a broad range antimicrobial substance from Bacillus cereus. Journal of Applied Microbiology. 2004;96:648–655.
11. Cherif A, Ouzari H, Daffonchio D, Cherif H, Slama KB, Hassen A, Jaoua S, Boudabous A. Thuricin 7: A novel bacteriocin produced by Bacillus thuringiensis BMG1.7, a new strain isolated from soil. Letters of Applied Microbiology. 2001;32:243–247.
12. Ahern M, Verschueren S, Van Sinderen D. Isolation and characterisation of a novel bacteriocin produced by *Bacillus thuringiensis* strain B439. FEMS Microbiology Letters. 2003;220:127–131.
13. Shelburne CE, An FY, Dholpe V, Ramamoorthy A, Lopatin DE, Lantz MS. The spectrum of antimicrobial activity of the bacteriocin subtilosin A. Journal of Antimicrobial Chemotherapy. 2007;9:297–300.
14. Stein T. Bacillus subtilis antibiotics: Structures, syntheses and specific functions. Molecular Microbiology. 2005;56(4):845–857.
15. Sutyak KE, Wirawan RE, Aroutcheva AA, Chikindas ML. Isolation of the Bacillus subtilis antimicrobial peptide subtilosin from the dairy product-derived Bacillus amyloliquefaciens. Journal of Applied Microbiology. 2008;104:1067–1074.
16. Lee KH, Jun KD, Kim WS, Paik HD. Partial characterization of polyfermenticin SCD, a newly identified bacteriocin of Bacillus polyfermenticus. Letters of Applied Microbiology. 2001;32:146–151.
17. Barboza-Corona JE, De la Fuente-Salcido N, Alva-Murillo N, Ochoa-Zarzosa A, L´opez-Meza JE. Activity of bacteriocins synthesized by *Bacillus thuringiensis* against Staphylococcus aureus isolates associated to bovine mastitis. Veterinary Microbiology. 2009;138:179–183.
18. Magnusson J, Schnurer J. Lactobacillus coryniformis subsp. coryniformis Strain Si3 Produces a Broad-Spectrum Proteinaceous Antifungal Compound. Applied and Environmental Microbiology. 2001;67(1):1–5.
19. Chen H, Hoover DG. Bacteriocins and their food applications. Comprehensive reviews in food science and food safety. 2003;2:82-100.

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