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Antagonistic Activity of *Bacillus* Bacteria against Food-Borne Pathogens

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Abstract

Bacillus bacteria have attracted the attention of scientists as promising probiotics because of their versatile antimicrobial activity and established health benefits on the host. In this study, seven *Bacillus* strains were identified and analyzed for antagonistic activity against broad spectrum of food borne pathogens. All strains were identified as *B. subtilis*, based on the results of morphological, biochemical characterization and 16S rDNA sequence analysis. *B. subtilis* strains demonstrated antagonistic activity against test-cultures of pathogens, including multiresistant strains. Reference *Bacillus* strains, derived from the commercial probiotics did not show antagonistic activity against tested strains of pathogens. Three the most active cultures were studied for production of biosurfactants. Crude biosurfactants were isolated and analyzed by oil spread test and inhibition activity against *Salmonella*, *Shigella* and *Staphylococcus* cultures. Biosurfactants from three tested *B. subtilis* strains gave positive oil spread test. Inhibition activity of biosurfactants was found only against *Staphylococcus* strains. Production of biosurfactants depended on the incubation conditions of *Bacillus* culture. Best results were obtained after cultivation of bacilli in starch broth at 30°C. The concentration of produced biosurfactant increased in time with growth of bacteria and reached the maximum at 30 hours of incubation.

Keywords: *Bacillus subtilis*; Antagonistic activity; Food borne pathogens; Biosurfactants

Abbreviations: MRSA: Methicillin Resistant *Staphylococcus aureus*; SA - Starch Agar; TBS - Trypticase Soy Agar; NA - Nutrient Agar; CFU – Colony Forming Unit; OD_{600} – Optical Density at a wavelength of 600 nm

Introduction

Foodborne pathogens are among the most significant problems in maintaining the health of the population. In 2011 the CDC estimates that each year roughly 1 in 6 Americans (or 48 million people) gets sick, 128,000 are hospitalized, and 3,000 die of foodborne diseases. The leading causes of foodborne illnesses in the United States are *Salmonella* and *Shigella* [1,2]. *Staphylococcus aureus* is among top five pathogens contributing to domestically acquired foodborne illnesses. Staphylococcal food poisoning is estimated to account for 241,148 foodborne illnesses per year in the United States, according to the CDC information (http://www.cdc.gov/foodborneburden/2011-foodborne-estimates.html). Foodborne illnesses are routinely treated with several classes of antibiotics. However, the use of these antibiotics has become problematic as over the years there have been numerous reports of cases of multi-antibiotic resistant food borne pathogens, worldwide [3-5]. In the United States, the proportion of methicillin-resistant *S. aureus* (MRSA) isolates from patients in intensive care units increased from 1992 to 2003 by 3% per year. Moreover, there is a great concern that the continued use of these drugs will result in the emergence of new resistant strains of these bacteria [6-9]. Colonization of the intestinal tract with MRSA may have important clinical implications, such as development of antibiotic-associated diarrhea, environmental dissemination, subsequent risk of infections and toxic shock syndrome [10-14]. Since foodborne infections have a dramatic impact on morbidity and mortality, particularly of infants and children, new approaches for cost effective and easy-to-deliver prophylaxis and treatment of these infections are highly desirable.

One of the growth areas in the control of foodborne infections is the use of probiotics [7]. Probiotic prophylaxes and therapies are gaining wider acceptance as more scientific data emerge regarding the interaction between pathogen and beneficial microbes in the human intestinal tract and molecular mechanisms of probiotics' action. Probiotic bacteria which confer beneficial effect for the host and have pronounced antagonistic activity against these pathogens are expected to present a clear alternative in the prevention and treatment of foodborne infections.

Bacteria of the *Bacillus* genus possess a great potential as probiotic cultures. *Bacillus* bacteria are among the most widespread microorganisms in nature. These bacteria are known to be producers of more than 200 antibiotics. *Bacillus* antibiotics differ in their structure, as well as spectrum of activity [15]. Some strains of *Bacillus* synthesize bacteriocines, which are only effective against bacteria of the same species, others produce antibiotics against Gram-negative bacteria and still other strains have a wide spectrum of antibiotic activity (including antifungal and antiprotozoan) [16]. Thus, it is possible to find strains with unique spectrum of activity among *Bacillus* bacteria. The aim of this work was to isolate and characterize *Bacillus* strains with pronounce activity against food borne pathogens.

Materials and Methods

Bacterial strains

Seven bacterial strains (16k, M1-1, 11-89, M2-3, 101, BSB, 105), isolated from environment, were used in this study. Morphological characterization of the cultures was done with high resolution CitoViva microscope [17,18]. Gram reaction and catalase activity were analyzed

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in tested strains. Bacterial cultures were identified using API 50 CHB tests (bioMerieux, Marcy-l'Etoile, France). For further characterization, the 16S rRNA gene was PCR amplified using universal 16S primers that correspond to positions 0005F and 0531R. Products of sequencing reactions were analyzed with an ABI 3100- AVANT Genetic Analyzer in MIDI Labs (Newark, DE). Sequence analysis was performed using BLAST and Sherlock® DNA microbial analysis software and database

Probiotic strains

Bacillus cultures from commercial probiotics were studied as the reference strains (Table 1).

Test-cultures

Test-cultures of *Salmonella*, *Shigella* and *Staphylococcus* were obtained from the culture collection of Auburn University (Auburn, AL). Stock cultures were maintained at -20°C in NZY medium, supplemented with 25% (v/v) glycerol.

Antagonistic activity of *Bacillus* strains

Activity of *Bacillus* strains against pathogens were studied by the method of delayed antagonism in solid nutrient medium [19]. Briefly, *Bacillus* strains were inoculated as a line on the surface of a nutrient media. After 72 h of growth at 30 or 37°C overnight test-cultures were inoculated as a perpendicular line to the *Bacillus* culture. The plates were incubated for 24 h at 37°C. The antagonistic activity was detected as a zone of pathogens' growth inhibition. Different media were tested to assess the antagonistic activity of *Bacillus* strains–NZY, starch agar (SA), trypticase soy agar (TBS) and nutrient agar (NA). Starch agar composed of starch (10 g/L), peptone (5 g/L), NaCl (0.5 g/L), agar (15 g/L) was used previously for cultivation of *Bacillus* probiotic strain [20]. Test-cultures of pathogens were grown overnight in NZY medium at 37°C.

Biosurfactant evaluation

For preparation of the inoculum, *Bacillus* strains were grown in NZY medium overnight at 37°C on shaker-incubator (200 rpm). Seed cultures were inoculated into nutrition media (1 mL of overnight culture into 250-mL flack with 50 mL of tested medium). Two media were used for cultures cultivation: SA and a fermentation media for biosurfactant production by *B. subtilis* natto, composed of 5.0 g/L sucrose, 20.0 g/L peptone, 0.5 g/L yeast extract, 0.02 g/L $MgSO_4 \bullet 7H_2O$, 1.4 g/L $Na_2HPO_4 \bullet 12H_2O$, 0.4g/L KH_2PO_4 [21]. *Bacillus* strains were incubated 24 hours at 30° and 37°C. After incubation the bacterial cells were precipitated by centrifugation at 10,000 x g at 4°C for 10 min. Cell free supernatant was acidified to pH 2.0 with 1N HCl and left overnight at 4°C to precipitate. The resulting precipitate was collected by centrifugation at 10,000 x g for 20 min. The supernatant was discarded and the remaining pellet was resuspended in 5 mL of methanol and left for extraction for 4 hours at room temperature. Methanol extract was centrifuged at 10,000 x g for 30 min and supernatant was transferred into a preweighed 50 mL glass and evaporated overnight under the vaccum in an exicator with silicagel. The glass was weighed again to determine the net weight of the crude biosurfactant. Obtained samples were diluted in deionized sterile water (pH 8.0) for further testing.

Bacillus strain	Probiotic	Producer
B. cereus IP 5832	Bactisubtil	Cassenne Marion, Paris, France
B. cereus DM-423	Cereobiogen	Keda Drugs Trade Co Ltd under Dalian university of Medical Sciences, China
B. clausii	Enterogermina	Sanofi -Synthelabo, Milan, Italy

Table 1: Characterization of *Bacillus* probiotic strains.

Biosurfactant production during *Bacillus* cultivation

Bacillus strain was cultivated in 250 mL flasks with 50 mL of starch medium at 30°C for 32 hours. At different time intervals, the fermentation medium was sampled for determination of biomass and biosurfactant concentration.

Surface activity of biosurfactants was measured by an oil spreading test [22,23]. Briefly, 20 µL of crude oil was added to a Petri dish (90 mm diameter) with 50 mL of distilled water to form a thin membrane. Ten microliters of sample was put onto the center of the oil membrane. The diameter of the oil-displaced circle area was measured. Each sample was tested in triplicate.

Antimicrobial activity of biosurfactants was evaluated by an agar well diffusion method [20]. Prepared suspensions of test-cultures in PBS (10^8 CFU/mL) were inoculated onto the surface of agar medium (100 µL of suspension on each plate). Wells (6 mm diameter) were made with a sterile cork borer. 50 µL of the test solutions were added to each well. Plates were incubated for 24 hours at 37°C. Zones of test-cultures growth inhibition were measured.

Statistics

Statistical analyses (t- Test and ANOVA) were performed using Microcal™ Origin® version 6.0 (Northhampton, MA) to validate the signification of the results. The data are presented as means (± SD) of at least three replicates.

Results

Identification of *Bacillus* strains

The microscopic study of bacterial cultures showed these strains to be Gram-positive rods, less than 1 µm in diameter. All strains sporulated aerobically without swelling of the cell and produced catalase. These data indicated that tested strains belong to *Bacillus* genus. Additional testing with API 50CHB kit resulted in identification of all cultures as *B. subtilis*. Partial sequence of 16S rRNA gene confirmed the obtained results of biochemical identification

Antagonistic activity

Antagonistic activity of *B. subtilis* cultures was tested on different nutrient media at two temperatures: 30° and 37°C. All cultures showed prominent growth on selected media at both temperature, but no antagonistic activity was indicated at 37°C. *Bacillus* cultures inhibited the growth of pathogenic bacteria only after growth on SA at 30°C (Table 2).

Strains BSB, 16K and 105, showed the highest antagonistic activities, were used in further experiments with broad spectrum of *Salmonella* and *Staphylococcus* strains, including clinical isolates. *Bacillus* cultures from commercial probiotics were tested as reference strains. Antagonistic activity of bacilli was studied after growth on SA at 30°C. *B. subtilis* strains were highly effective against all tested strains of *Salmonella* and *Staphylococcus* (Table 3, Figures 1 and 2). Commercial *Bacillus* strains showed no antagonistic activity against test-cultures.

Biosurfactant production

Biosurfactant production was tested in two media–starch broth and fermentation medium, used for surfactant production by *B. subtilis* natto [21]. *B. subtilis* strains were incubated in two medium at 30°C and 37°C. Production of biosurfactant was assessed by the oil spreading technique and by inhibition of test-cultures growth. The

Bacillus subtilis strain/ Medium*	Zone of test-cultures growth inhibition, mm					
	Staphylococcus aureus ATCC12600	*Salmonella typhimurium* ATCC 13311	*S. dublin* SA 2424	*S. enteritidis*	*Shigella sonnei*	*Shigella flexneri*
BSB3						
NZY; NA; TSA	0	0	0	0	0	0
SA	28.3 ± 0.3	29.6 ± 0.6	25.6 ± 0.3	20.3 ± 0.8	23.3 ± 0.8	21.3 ± 0.3
M1-1						
NZY; NA; TSA	0	0	0	0	0	0
SA	14.6 ± 1.4	6.7 ± 1.7	5.0 ± 0	7.0 ± 0	11.7 ± 0.3	11.0 ± 0.6
16k						
NZY; NA; TSA	0	0	0	0	0	0
SA	29.3 ± 0.3	25.6 ± 0.3	16.0 ± 0.6	22.0 ± 0.6	21.6 ± 0.3	19.6 ± 0.8
11-89						
NZY; NA; TSA	0	0	0	0	0	0
SA	20.3 ± 0.6	17.7 ± 0.7	15.3 ± 0.3	13.6 ± 0.6	15.7 ± 0.7	14.6 ± 0.3
M2-3						
NZY; NA; TSA	0	0	0	0	0	0
SA	19.7 ± 0.9	23.7 ± 0.8	15.7 ± 1.2	21.7 ± 0.9	20.3 ± 0.3	21.0 ± 0.6
101						
NZY; NA; TSA	0	0	0	0	0	0
SA	17.3 ± 0.7	6.2 ± 0.6	4.3 ± 0.3	18.6 ± 0.9	19.7 ± 0.3	15.3 ± 0.6
105						
NZY; NA; TSA	0	0	0	0	0	0
SA	34.1 ± 1.2	25.7 ± 0.8	16.2 ± 0.3	23.6 ± 0.6	24.3 ± 0.3	21.2 ± 0.3

*NZY: NZY agar; NA: Nutrient Agar; TSA: Trypticase Soy Agar; SA: Starch Agar

Table 2: Antagonistic activity of *B. subtilis* strains on different media.

#	Test -cultures	Zone of test-cultures inhibition (mm) by *B. subtilis* strains		
		105	BSB3	16 k
1	*S. typhimurium* Health 9491	0	23.8 ± 0.7	22.7 ± 0.3
2	*S. typhimurium* DT 104 Dairy	25.6 ± 0.3	25.8 ± 0.8	24.2 ± 0.6
3	*S. diarisonae*	25.3 ± 0.8	27.1 ± 0.9	26.3 ± 0.6
4	*S. panama* SA 3583	22.6 ± 0.3	25.3 ± 0.3	23.1 ± 0.7
5	*S. indica* SA 4401	23.1 ± 0.2	25.6 ± 0.7	25.2 ± 0.3
6	*S. derby* SARB 10	30.2 ± 1.3	31.4 ± 0.8	27.3 ± 0.6
7	*S. typhimurium* LT2	30.1 ± 0.6	32.3 ± 0.7	28.6 ± 0.3
8	*S. mission*	26.6 ± 0.3	25.8 ± 0.6	24.3 ± 0.7
9	*S. montevideo*	25.0 ± 0.0	25.3 ± 0.7	23.0 ± 0.6
10	*S. typhimurium* 6787	22.3 ± 0.3	24.3 ± 0.7	19.7 ± 0.8
11	*S. typhimurium* Heath 1390	21.7 ± 0.3	22.0 ± 0.6	21.7 ± 0.3
12	*S. bongori* SA 4910	21.7 ± 0.9	25.0 ± 0.6	23.7 ± 0.3
13	*S. typhimurium* Nal 1x fecal	19.7 ± 0.8	20.7 ± 0.3	18.0 ± 1.1
14	*S. minnesota*	30.7 ± 0.3	31.0 ± 0.7	27.7 ± 0.9
15	*S. salamae* SA 41106	10.0 ± 0.7	23.6 ± 0.3	18.7 ± 0.9
16	*S. typhimurium* 520-96	22.3 ± 0.6	24.0 ± 0.6	21.6 ± 0.7
17	*S.Thompson* 265-4	25.1 ± 0.3	26.3 ± 0.9	23.3 ± 0.6
18	*S. infantis* SARR 27	31.1 ± 0.9	30.3 ± 0.6	27.6 ± 0.3
19	*S. paratyphimurium*	30.2 ± 0.3	30.3 ± 0.6	29.6 ± 0.7
20	*S. typhimurium* DT 104 Swine	21.1 ± 0.2	24.6 ± 0.3	22.6 ± 0,3
21	*S. typhimurium* 9693	22.3 ± 0.3	25.7 ± 0.6	23.3 ± 0.1

Table 3: Anti-*Salmonella* activity of *Bacillus* strains.

best conditions for biosurfactant production for all *Bacillus* cultures were cultivation in starch broth at 30°C. Results in Figure 3A indicate that oil spread test for biosurfactants, produced by *B. subtilis* BSB3 and 16k at 30°C gave higher results than for *Bacillus* cultures, grown at 37°C. Study of inhibition activity of these biosurfactants against test-cultures, showed that activity of biosurfactant from *B. subtilis* BSB3, cultivated at 30°C was more pronounced, than at 37°C (Table 4; Figure 3B). Biosurfactants from *B. subtilis* 16k, incubated at different temperatures, demonstrated more consistent results. *B. subtilis* 105 produced biosurfactant, as it was confirmed by the oil spreading test, but this biosurfactant had lack of inhibition activity. Biosurfactants, produced by *Bacillus* strains demonstrated inhibition activity only against *Staphylococcus* cultures. Tested cultures of *Salmonella* and *Shigella* were resistant to these biosurfactants.

Production of biosurfactant during *B. subtilis* BSB3 cultivation

Production of biosurfactant by *B. subtilis* BSB3 increased in

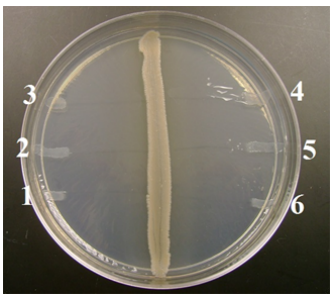

Figure 1: Anti-*Salmonella* activity of *Bacillus subtilis* BSB3: 1 – *S. paratyphimurium*, 2- *S. infantis* SARR 27, 3 – *S. thompson* 265-4, 4 – *S. minnesota*, 5 – *S. typhimurium* LT2, 6 – *S. derby* SARB 10.

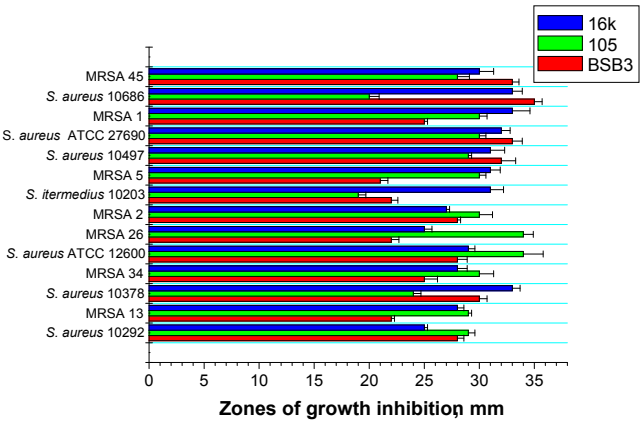

Figure 2: Antagonistic activity of *Bacillus subtilis* strains against *S. aureus* MRSA – methicillin resistant *S. aureus*.

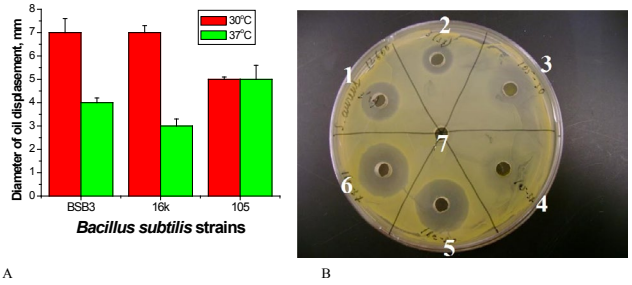

Figure 3: Activity of biosurfactants, produced by *B. subtilis* strains at different temperatures:
A- oil spread test; B- inhibition activity against *S. aureus* ATCC 12600;
Biosurfactants were isolated from *B. subtilis* strains, incubated at different temperatures: 1- strain BSB3 at 30°C; 2- strain BSB3 at 37°C; 3- strain 105 at 30°C; 4-strain 105 at 37°C; 5- strain 16k at 30°C; 6- strain 16 k at 37°C; 7- control (sterile water).

time and corresponded to the bacterial growth curve. The maximum production of biosurfactant was detected at 30 hours of bacteria cultivation (Figure 4).

Discussion

Bacillus bacteria are known to be effective antagonists of different pathogens [15,24]. In recent years bacilli were extensively studied as probiotics, due to their health benefits on the host [25,26]. A search for new *Bacillus* strains with pronounced antagonistic activity against food borne pathogens opens up promising expectations for treatment of these infections.

In the present study, newly isolated *Bacillus* strains were analyzed. All strains, identified as *B. subtilis*, were tested for their activity against *Salmonella*, *Shigella* and *Staphylococcus* strains. Antagonistic activity of *Bacillus* strains was detected only after cultivation on starch agar at 30°C. Incubation of bacilli on starch agar at 37°C, as well as on NZY, NA and TSA at 37°C and 30°C did not result in antagonistic effect. These outcomes are in accordance with our previous findings about conditions for production of antimicrobial compounds by *Bacillus* cultures [27]. Three *B. subtilis* strains, showed the highest activity against tested pathogens, were studied with broad spectrum of *Salmonella* and *Staphylococcus* cultures, including clinical multi-resistant strains. As reference strains, commercial *Bacillus* probiotic cultures from Bactisubtil, Cereobiogen and Enterogermina were used. None of the reference strains were active against tested pathogens. *B. subtilis* isolates demonstrated high activity of test-cultures' inhibition. Antagonistic activity was detected against all strains of *Salmonella* and *Staphylococcus*, including MRSA. Inhibition of MRSA by *Bacillus* cultures was shown by other authors [28-30], but no anti-*Salmonella* effect was found in the same strains.

Bacteria of the *Bacillus* genus (predominantly, *B. subtilis*) produce various biosurfactants, which have a high potential for biotechnology and pharmacology [31]. These compounds vary in structure and spectrum of activity and usually are responsible for antimicrobial effects of *Bacillus* bacteria [21,32]. In our study *B. subtilis* strains produced biosurfactants after cultivation in starch broth at 30°C. Incubation of these cultures in fermentation medium, used for *B. subtilis* natto [21], resulted in lack of biosurfactants production. Presence of biosurfactant in cultivation medium was tested by the oil spread test and by inhibition activity against *Salmonella*, *Shigella* and *Staphylococcus* strains. It was shown elsewhere, that the oil spread test correlates with the biosurfactant production [23]. Biosurfactants from three tested *B. subtilis* strains gave positive oil spread test, showing the diameter of oil displacement from 3 to 7 mm. These results are in accordance with data for crude biosurfactants from *B. subtilis* natto [21] and from *B. subtilis* and *B. licheniformis* [33]. Inhibition activity of biosurfactants was found only against *Staphylococcus* strains and depended on the incubation temperature of *Bacillus* culture. Biosurfactant from *B. subtilis* BSB3, incubated at 30°C, demonstrated higher activity against

Test-cultures	Zone of test-cultures growth inhibition (mm) by biosurfactants from *B. subtilis*					
	BSB3 (30°C)*	BSB3 (37°C)**	16k (30°C)	16k (37°C)	105 (30°C)	105 (37°C)
S. aureus 10292	12	10	17	15	0	0
MRSA 13	10	9	13	11	0	0
S. aureus 10378	15	12	20	19	0	0
MRSA 34	11	8	0	0	0	0
S. aureus ATCC 12600	16	12	20	20	0	0
MRSA 26	15	10	13	13	0	0
MRSA 2	12	10	14	14	0	0
S. itermedius 10203	16	9	25	25	0	0
MRSA 5	12	9	15	14	0	0
S. aureus 10497	13	11	17	17	0	0
S. aureus ATCC 27690	13	11	15	15	0	0
MRSA 1	10	7	15	14	0	0
S. aureus 10686	15	12	18	18	0	0
MRSA 45	13	10	18	16	0	0

**Bacillus* strain was incubated at 30°C; ***Bacillus* strain was incubated at 37°C.

Table 4: Activity of biosurfactants against *Staphylococcus* test-cultures.

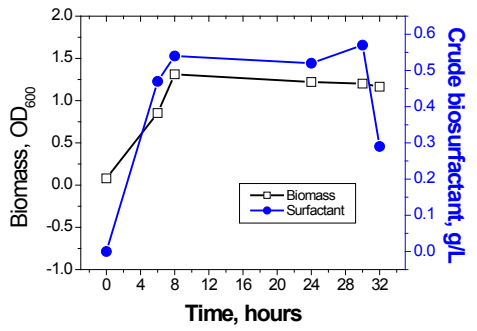

Figure 4: Kinetic of biosurfactant production during growth of *B. subtilis* BSB3.

Staphylococcus strains, as opposed to bacteria incubated at 37°C. Activity of biosurfactant, produced by *B. subtilis* 16 k did not changed with the change of incubation temperature. Biosurfactant from *B. subtilis* 105 showed no activity against tested pathogens. The inhibitory activity of *B. subtilis* may be the cumulative result of different antimicrobials, known for this bacteria [16]. Identified here biosurfactants play an important role in the anti-*Staphylococcus* activity for at least two *B. subtilis* strains – BSB3 and 16 k. Kinetic of biosurfactant production was similar to those reported for *Bacillus* cultures by other authors [33]. The concentration of biosurfactant increased in time with growth of bacteria and reached the maximum at 30 hours of incubation.

In the present study seven *Bacillus* strains were characterized for their activity against *Salmonella*, *Shigella* and *Staphylococcus* pathogens. Three strains showed pronounced antagonistic activity against broad spectrum of pathogenic cultures, including multi-resistant strains. Inhibitory effect on S. aures and MRSA strains was caused by production of biosurfactant, identified for two *B. subtilis* strains.

Further study of antimicrobial compounds, produced by *Bacillus* bacteria, will result in better understanding of the mechanisms of antagonistic activity of bacilli and selection of new strains, promising for biotechnology and pharmacology.

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