IDENTIFICATION AND EXAMINATION OF SOME PROBIOTIC PROPERTIES OF A STRAIN FROM THE GENUS *LACTOBACILLUS,* ISOLATED FROM SPONTANEOUSLY FERMENTED YOGURT

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Abstract

The intake of probiotic microorganisms helps to restore and maintain the balance of the gastrointestinal microflora, which has a beneficial effect on the overall health of the individual. In order to be included in the composition of probiotic preparations or as starter cultures in the production of functional probiotic foods or beverages, newly isolated Lactobacillus strains must be identified and a number of probiotic properties must be demonstrated. Lactobacillus Pr2 isolated from spontaneously fermented yogurt was identified by physiological and biochemical (API 50 CHL) and molecular genetic (ARDRA-analysis and sequencing of the 16S rRNA gene) as a representative of the species Lactobacillus delbrueckii ssp. bulgaricus. The presence of some probiotic properties of Lactobacillus delbrueckii ssp. bulgaricus Pr2: resistance under model conditions of the gastrointestinal tract, antibiotic resistance profile, antimicrobial activity against pathogenic microorganisms and freeze-drying survival were examined. It was found that Lactobacillus delbrueckii ssp. bulgaricus Pr2 was highly sensitive to the effects of low pH values and the presence of enzymes (pepsin, pancreatin), to medium to high concentrations of bile salts in the medium (up to 1%), as well as to most of the antibiotics included in the study. However, the strain demonstrated high antimicrobial activity against pathogenic microorganisms and very good survival during the freeze-drying process. Lactobacillus delbrueckii ssp. bulgaricus Pr2 may be included in the composition of probiotics or as a starter culture for probiotic foods or beverages, but before that it is necessary to conduct additional research on the presence of other probiotic properties of the strain.

Keywords: *probiotic, Lactobacillus delbrueckii ssp. bulgaricus, ARDRA-analysis, sequencing, antimicrobial activity*

INTRODUCTION

Any disturbance in the balance of the gastrointestinal microflora caused by pathogenic microorganisms, mutagenic factors, various chemical agents leads to a number of diseases. *Lactobacillus* species from the gastrointestinal microflora can restore and maintain the intestinal homeostasis [1]. They are imported in the form of concentrates known as probiotic preparations.

Probiotics are living microorganisms that, when administered in adequate amounts, contribute to maintaining the balance of the intestinal microflora, which leads to the improvement of the overall health of the individuals. They contain significant amounts of living microorganisms, their metabolites and other compounds of microbial, plant or animal origin, which stimulate the growth of lactobacilli and bifidobacteria from the natural human microflora, which in turn improves the functioning of the digestive tract and related organs and systems.

Not all lactobacilli can be included in the composition of probiotics and probiotic foods, but only those that exhibit certain properties [2, 3]: 1) to be part of the natural microflora in humans and animals; 2) to have the ability to adhere to epithelial cells or cell lines; 3) to survive under the conditions of the stomach and intestines, i.e. to survive under acidic pH in the stomach and to be resistant to the action of bile juice [4]; 4) to be able to reproduce in the gastrointestinal tract since by assimilating the substrate predominantly, they suppress and expel pathogenic and toxigenic microorganisms from the biological niche; 5) to allow the conduction of industrial cultivation; 6) to demonstrate antimicrobial activity against opportunistic, carcinogenic and pathogenic microorganisms, which is associated with inactivation of their enzyme systems, overcoming their adhesion, inhibiting their growth and expulsion from the biological niche, resulting in normalization of the gastrointestinal tract microflora; 7) to produce antimicrobial substances [5]; 8) to modulate the immune response; 9) to be safe for clinical and nutritional use.

Finished preparations must contain high concentration of viable cells (no less than 10^9cfu/g), with retained activity after concentration and drying, and provide a long shelf life.

The isolation and examination of new lactobacilli strains from various sources allows the selection of strains with probiotic potential for use as components in the composition of probiotic preparation and probiotic fermented foods.

The aim of the present work was to identify and examine some probiotic properties of a strain of the genus *Lactobacillus* isolated from spontaneously fermented yogurt.

MATERIALS AND METHODS

1. Microorgamisms

The studies in the present work were performed with *Lactobacillus* Pr2 isolated from spontaneously fermented yogurt. The strain was identified by the application of physiological, biochemical and molecular genetic methods.

The following reference cultures were used in the molecular-genetic identification of *Lactobacillus* Pr2: *Lactobacillus delbrueckii* ssp. *bulgaricus* DSM 20081, *Lactobacillus delbrueckii* ssp. *lactis* DSM 20072, *Lactobacillus helveticus* DSM 20075, *Lactobacillus casei* ssp. *rhamnosus* LMG 6400.

The following test microorganisms were used in the examination of the antimicrobial activity of *Lactobacillus* Pr2: *Escherichia coli* ATCC 8739, *Staphylococcus aureus* ATCC 25923, *Listeria monocytogenes* NBIMCC 8632, *Salmonella enteritidis* ATCC 13076.

2. Media

2.1. MRS broth. Composition (g/dm^3): peptone from casein - 10; yeast extract - 4; meat extract - 8; glucose - 20; K_2HPO_4 - 2; sodium acetate - 5; diammonium citrate - 2; $MgSO_4$ - 0.2; $MnSO_4$ - 0.04; Tween 80 - 1 cm^3; pH = 6.5. The medium was autoclaved for 15 minutes at 118 °C.

2.2. Sterile skimmed milk with titratable acidity of 16 - 18°T. Composition (g/dm^3): skimmed milk powder (Scharlau). The medium was autoclaved for 15 minutes at 118 °C.

2.3. MRS agar. Composition (g/dm^3): MRS broth; agar - 20. The medium was autoclaved for 15 minutes at 118 °C.

2.4. Saline solution. Composition (g/dm^3): NaCl - 5. The medium was autoclaved for 15 minutes at 118 °C.

2.5. LAPTg10 broth. Composition (g/dm^3): peptone - 15; yeast extract - 10; trypton - 10; glucose - 10. pH was adjusted to 6.6 - 6.8 and Tween 80 - 1cm^3/dm^3 was added. The medium was autoclaved for 15 minutes at 118 °C.

2.6. LAPTg10 agar. Composition (g/dm^3): LAPTg10 broth; agar - 20. The medium was autoclaved for 15 minutes at 118 °C.

3. Physiological and biochemical methods

3.1. Determination of the biochemical profile

The API 50 CHL system (BioMerieux SA®, France) was used to identify *Lactobacillus* Pr2 based on its ability to utilize 49 different carbon sources. Fresh 24-hour culture of *Lactobacillus* Pr2 was centrifuged for 15 minutes at 5000xg. The resulting biomass pellet was washed twice with PBS-buffer and resuspended in API 50 CHL medium, part of the kit (dark blue colour). The API strips were placed in the incubation boxes, the microtubules were inoculated with the cell suspension thus prepared and were sealed with sterile liquid paraffin. The results were reported at the 24th hour and the 48th hour of incubation at 37±1°C. The reading was performed according to the colour change in the microtubules in comparison with the control (microtube 0). The colour changing from dark blue to green or yellow was reported as a positive result (consumption of the respective substrate). The obtained results were processed with apiweb® identification software.

3.2. Determination of tolerance to bile salts (method modified by Denkova Z., 2005 [6]*)*

Fresh 24-hour culture of *Lactobacillus* Pr2 was centrifuged for 15 minutes at 5000xg. The biomass pellet obtained was washed twice with PBS-buffer and resuspended to the original volume in PBS-buffer. 0.2 cm^3 of the cell suspension was incubated with 5 cm^3 of MRS broth with bile salt concentration of 0%, 0.15%, 0.3%, 0.6% or 1%. Cultivation was then carried out for 24 hours 37±1°C, taking samples at the 0th, 2nd, 4th , 6th, 8th and 24th hour to determine the concentration of viable lactobacilli cells (cfu/cm^3).

3.3. Determination of survival at low pH in the presence of pepsin and at alkaline pH in the presence of pancreatin (Denkova Z., 2005 [6]*)*

Fresh 24-hour culture of *Lactobacillus* Pr2 was centrifuged for 15 minutes at 5000xg. The resulting biomass precipitate was washed twice with PBS buffer and resuspended to the original volume in PBS buffer. 0.2 cm^3 of the cell suspension was incubated with 5 cm^3 of pH = 2 buffer solution containing 0.5% NaCl and pepsin (3.2 g/dm^3) (Sigma, 2,500 - 3,500 U/mg protein), buffer solution with pH=4.5 and pancreatin; and buffer solution with pH=7 and pancreatin at 37±1°C for 24 hours. Aliquots to determine the total number of viable cells (cfu/cm^3) were taken at 0th, 2nd, 4th and 24th hour.

3.4. Determination of the antibiotic susceptibility profile

Laboratory susceptibility testing of fast-growing microorganisms was performed using the agar-diffusion method with paper discs by Bauer, Kirby et al., 1966 [7]. Fresh 24-hour culture of *Lactobacillus* Pr2 was used to inoculate by spread plating plates with MRS-agar. Standard disks soaked in different antibiotics were placed in the plates. The plates were incubated for 48 hours at 37±1°C. The diameter (in mm) of the inhibition zones formed around each of the antibiotic discs was recorded. The following designations were used for the interpretation of the results: R - resistant (the inhibition zone being smaller than 8 mm), SR - intermediately sensitive (the inhibition zone being between 8 mm and 16 mm), S - sensitive (the inhibition zone being larger than 16 mm).

3.5. Determination of the antimicrobial activity – agar-diffusion method with wells

Culture fluid (CF), biomass (B) and neutralized acellular supernatant (NASN) (pH 6.5) obtained from a 24-hour culture of *Lactobacillus* Pr2 were used to determine the antimicrobial activity of *Lactobacillus* Pr2. The antimicrobial activity was tested against the following test microorganisms: *Escherichia coli* ATCC 8739, *Staphylococcus aureus* ATCC 25923, *Listeria monocytogenes* NBIMCC 8632, *Salmonella enteritidis* ATCC 13076.

Each of the test pathogenic microorganisms (suspension concentration of 10^7cfu/cm^3) was inoculated by spread plating into agar plates and wells (7 mm in diameter) were prepared after agar solidification. 0.1 cm^3 of CF, B and NASN were pipetted into the wells and allowed to diffuse into the agar for 1 hour at 4±2°C. The Petri dishes were then incubated at 37±1°C for 24 h, after which the inhibition zones around the wells in mm were recorded.

4. Molecular-gentic methods

4.1. Isolation of total DNA

DNA isolation was performed using E.Z.N.A.® DNA isolation kit according to the manufacturer's instructions.

4.2. 16S rDNA amplification and ARDRA-analysis

16S rDNA amplification. All PCR reactions were performed using a PCR kit - PCR VWR, 25 µl in Progene cycler (Techne, UK) according to the manufacturer's instructions, using 50 ng total DNA of the test strain and 10 pmol primers in each reaction. DNA from the studied strain was amplified using universal primers for 16S rDNA - 27f (5'AGAGTTTGATCMTGGCTCAG3 ') [8] and 1492r (5'ACCTTGTTACGACTT3') [8]. The amplification program included: denaturation - 95°C for 3 min, 35 cycles - 93°C for 30 s, 55°C for 30 s, 72°C for 1 min, final elongation - 72°C for 7 min.

ARDRA-analysis. The PCR product obtained by the amplification of the 16S rDNA of the studied strain was subjected to restriction for 1 hour with the fast-digest (fD - fast digest) endonucleases *Eco* RI, *Hae* III and *Alu* I (Boehringer Mannhem GmbH, Germany) with a concentration of 10 units/µl.

The resulting products were visualized on a 2% agarose gel stained with ethidium bromide solution (0.5 µg / cm^3) using the UVP Documentation System (U.K.).

4.3. Purification of the product of the PCR reaction - 16S rDNA - from TAE-agarose gel

The purification of the 16S rDNA was performed with a DNA purification kit (GFX MicrospinTM) according to the manufacturer's instructions.

4.4. Sequencing of the 16S rRNA gene

The sequencing of the 16S rRNA gene was performed by the Sanger method by Macrogen Europe Laboratory, the Netherlands. The entire 16S rRNA gene sequence was obtained using *CLC Sequence Viewer* software and the resulting entire sequence was compared to the sequences in the online database using the BLASTn algorithm, identifying the strain to a species level with the respective percentage of confidence.

5. Freeze-drying

The freeze-dried concentrate of *Lactobacillus* Pr2 was obtained by a combined method for technological treatment - immobilization and freeze-drying, in the following stages of the process - primary processing, freezing and freeze-drying. Primary processing involved dilution of the *Lactobacillus* Pr2 cultural suspension, equilibration, dosing, immobilization of the cell suspension by retention (inclusion) in the respective polymer matrix, that served as a cryoprotective medium as well.

The immobilization matrix used was hydrocolloid and contained a 4% solution of highly esterified apple pectin and a 1.2% solution of sodium alginate in a ratio of 1:1. The cellular connection to the carrier was carried out by mixing the cell suspension with the carrier (hydrocolloid matrix) in the following sequence of technological steps:

1. Preparation of the cell suspension by culturing *Lactobacillus* Pr2 in MRS-broth medium for 24 hours at 37±1°C.

2. Mixing the cell suspension with the polymer solution at a temperature of 40 - 45°C and homogenizing for 1 hour and 30 minutes in a bioreactor with a stirring rate of 500 rpm.

The material thus prepared was subjected to freeze-drying, which combines two methods of preservation - freezing and drying under high vacuum. The samples were pre-frozen in chambers with forced air convection at a temperature of -30°C to -35°C for 12-15 hours.

Parameters	**Unit**	**Parameter value**
I. Object of research	-	Lactobacilli cellular suspensions with cryoprotective medium - pectin and sodium alginate
II. Layer thickness	mm	11
III. **Freezing**		
1.Freezing temperature	°C	-30°C to – 35°C
2. Freezing rate	°C/sec	Slow method - 0.05 - 0.06
3. Eutectic temperature	°C	– 37.00
4. Curing temperature	°C	– 40.00
IV. Freeze-drying		
1. Load on the bearing surface	kg/m^2	10.60
2. Drying temperature	°C	– 30.0
3. Temperature in the desublimator	°C	from - 55.0 to - 60.0 – before drying from - 65.0 to - 70.0 – in final drying
4. Partial pressure	Pa	from 30.0 to 34.0
5. Chamber pressure	Pa	from 20.0 to 27.0
6. Final drying temperature	°C	+ 30
7. Maximum product temperature	°C	30
8. Residual moisture content	%	from 1.37 to 3.09 /according to BSS – no more than 6.0%/
9. Process duration	h	from 12 to 14
10. Storage conditions		In envelopes of three-layer aluminum foil, closed under vacuum; stored at relative humidity no more than 35% at 20-22°C

Table 1. Parameters of the freeze-drying process of the cell suspensions

Freeze-drying was carried out under vacuum conditions in a sublimation installation "Hochvakuum-TG - 16.50" with contact heating of the plates in ICFT - Institute of Cryobiology and Food Technology – Sofia. The process parameters were as described in Table 1.

6. Microbiological studies of the freeze-dried concentrates

1. Microbiological status of native and freeze-dried samples – according to BSS 1670-82 and Ordinance № 5 of the Ministry of Health - SG 39/84, BSS EN ISO 4833:2004.

Indicators:

- lactic acid bacteria, CFU/g;
- total number of mesophilic aerobic and facultative anaerobic bacteria - CFU/g;
- *Escherichia coli* in 0.1 g of the product (ISO 16649-2:2001);
- pathogenic microorganisms, including *Salmonella* sp. (BDS EN ISO 6579:2003) in 25.0 g of the product;

- coagulase-positive staphylococci in 1.0 g of the product (BDS EN ISO 6888-1:2005 + A1:2005);
- sulfite-reducing clostridia in 0.1 g of the product (ISO 15213:2003);
- spores of microscopic molds, CFU/g (BDS ISO 6611:2006);
- yeast, CFU/g (BDS ISO 6611:2006);

7. Processing of the results

Data from triplicate experiments were processed using Microsoft Office Excel 2013 software, using statistical functions to determine the standard deviation and maximum estimation error at significance levels of $\alpha < 0.05$.

RESULTS AND DISCUSSION

1. Characteristics of Lactobacillus Pr2

1.1. Phenotypic characteristics of Lactobacillus Pr2

The characterization of newly isolated strains begins with assessment of the culture purity and microscopic morphological control. The micro- and macromorphological characteristics of *Lactobacillus* Pr2 are presented in Table 2.

When cultured on LAPTg10-agar medium, the isolated strain grew in the form of bulging whitish colonies, 2-3 mm in size, irregular in shape, with jagged edges. The cells were arranged singly and in chains and were long rods with rounded edges.

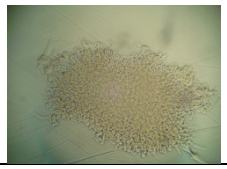
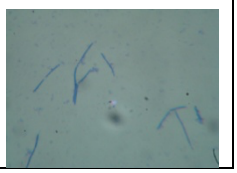
Strain	Colonial characteristics		Cellular morphology	
	Description of the colonies	Visualization	Description of the cells	Visualization
Pr2	Star-shaped colonies, with strongly serrated edges, bulging, whitish, 2-3 mm in size.		Long thick rods with rounded edges, arranged singly, in pairs and in chains.	

Table 2. Colonial characteristics and cellular morphology of *Lactobacillus* Pr2

1.2. Physiological and biochemical characteristics of Lactobacillus Pr2

After characterization according to the main morphological criteria (Table 2), the studied strain was subjected to determination of its biochemical profile with the API 50 CHL rapid identification system (Biomerieux, France). According to its ability to utilize 49 carbon sources (Table 3), the species affiliation of the strain with the corresponding percentage of reliability (87,1%) to the species *Lactobacillus delbrueckii* ssp. *bulgaricus* was determined.

Analyzes using API 50 CHL give rapid and reproducible identification of some lactobacilli species, but sometimes with a low percentage of discrimination. Using only the classical phenotypic and physiological-biochemical characteristics does not always allow for a reliable differentiation of individual species of lactobacilli, especially considering that lactobacilli also have phenotypic variability [9]. Therefore, according to modern notions of taxonomic determination of lactobacilli, molecular genetic methods are applied, especially for cases with a relatively low percentage of discrimination by classical methods [9].

#	Carbon source	*Lactobacillus* Pr2
1	**Glycerol**	-
2	**Erythriol**	-
3	**D-arabinose**	-
4	**L-arabinose**	-
5	**Ribose**	-
6	**D-xylose**	-
7	**L-xylose**	-
8	**Adonitol**	-
9	**β-metil-D-xyloside**	-
10	**Galactose**	-
11	**D-glucose**	+ (90 – 100 %)
12	**D-fructose**	+ (90 – 100 %)
13	**D-mannose**	+ (90 – 100 %)
14	**L-sorbose**	-
15	**Rhamnose**	-
16	**Dulcitol**	-
17	**Inositol**	-
18	**Manitol**	-
19	**Sorbitol**	-
20	**α-methyl-D-mannoside**	-
21	**α-methyl-D-glucoside**	-
22	**N-acetyl-glucosamine**	+ (90 – 100 %)
23	**Amigdalin**	-
24	**Arbutin**	-
25	**Esculin**	-
26	**Salicin**	-
27	**Cellobiose**	-
28	**Maltose**	-
29	**Lactose**	+ (90 – 100 %)
30	**Melibiose**	-
31	**Saccharose**	-
32	**Trehalose**	+ (90 – 100 %)
33	**Inulin**	-
34	**Melezitose**	-
35	**D-raffinose**	-
36	**Amidon**	-
37	**Glycogen**	-
38	**Xylitol**	-
39	**β-gentiobiose**	-

40	**D-turanose**	-
41	**D-lyxose**	-
42	**D-tagarose**	-
43	**D-fuccose**	-
44	**L-fuccose**	-
45	**D-arabitol**	-
46	**L-arabitol**	-
47	**Gluconate**	-
48	**2-keto-gluconate**	-
49	**5-keto-gluconate**	-

Table 3. Ability of *Lactobacillus* Pr2 to utilize 49 carbon sources included in the API 50 CHL identification system

1.3. Molecular-genetic characterization

Lactobacilli are of interest for use as both starter cultures and probiotic microorganisms and this requires their accurate identification and complete taxonomic characterization [10]. Many lactobacilli species have a high degree of similarity in terms of phenotypic and physiological characteristics and this leads to difficulties in their accurate taxonomic characterization [9].

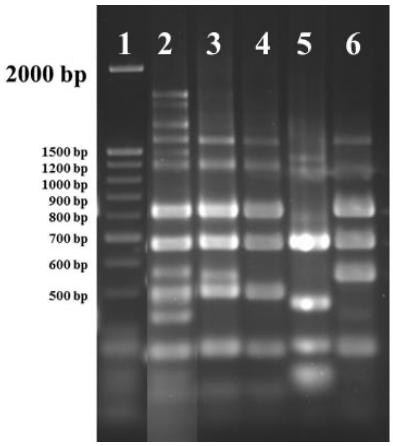
Therefore, according to modern polyphasic taxonomy, a combination of phenotypic and molecular genetic methods for typing and subtyping bacteria and in particular lactobacilli is used.

1.3.1. Genotypic characteristics of Lactobacillus Pr2

Lactobacilli are similar in phenotypic and physiological characteristics. Therefore, differentiating them is a difficult process. This requires the use of additional methods for their reliable identification and characterization. With the advancement of molecular biology, a set of molecular DNA techniques is replacing phenotypic methods of identification. Unlike phenotypic methods, molecular genetic methods are much more logical, faster, more reliable, and more reproducible, and can distinguish between subspecies and types within a species that are otherwise indistinguishable with phenotypic identification methods.

The ARDRA (Amplified Ribosomal DNA Restriction Analysis) method involves enzymatic amplification of the gene encoding the 16S rRNA using primers complementary to the conserved regions at both ends of the 16S rRNA gene, followed by restriction with restriction enzymes. The resulting profile is strictly specific to the particular species studied.

The amplified 16S rDNA of *Lactobacillus* Pr2 was subjected to ARDRA-analysis using the restriction enzymes *Hae* III (Fig. 1), *Alu* I (Fig. 2) and *Eco* RI (Fig. 3) in order to confirm the results of the species identification obtained by the biochemical identification method.



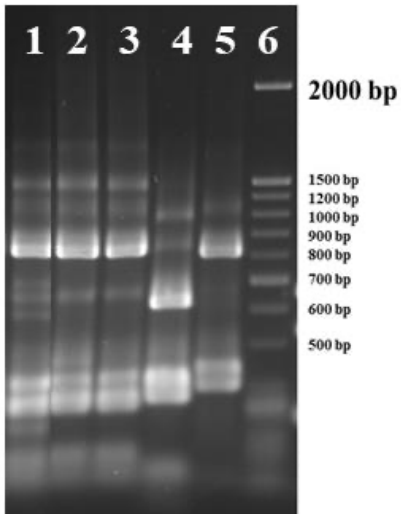
1. 100 bp Plus DNA Ladder
2. *Lactobacillus* Pr2
3. *L.d.* ssp. *bulgaricus* DSM 20081
4. *L.d.* ssp. *lactis* DSM 20072
5. *L. helveticus* DSM 20075
6. *L. casei* ssp.*rhamnosus* LMG 6400

Fig. 1. Restriction profile with *Hae* III

The restriction profile of *Lactobacillus* Pr2 with the enzyme *Hae* III was similar to that of *Lactobacillus delbruecki* ssp. *bulgaricus* DSM 20081 and *Lactobacillus delbruecki* ssp. *lactis* DSM 20072 (Fig. 1), which required the conduction of ARDRA-analysis using other enzymes - *Alu* I and *Eco* RI in order to confirm the species affiliation of the strain.

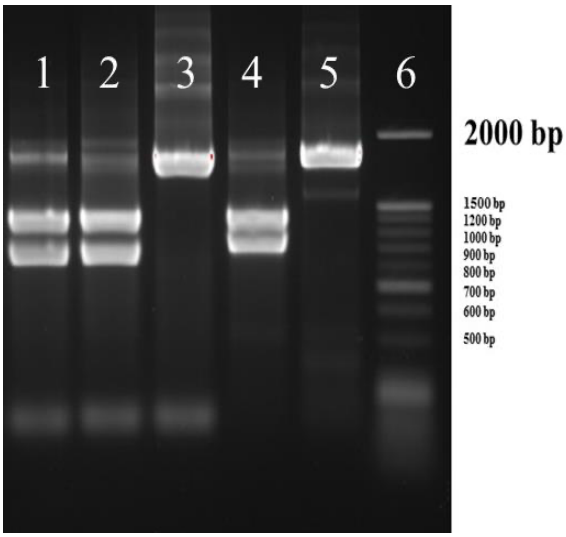
The restriction profile of *Lactobacillus* Pr2 with the restriction endonuclease *Alu* I was again similar to that of *Lactobacillus delbruecki* ssp. *bulgaricus* DSM 20081 and *Lactobacillus delbruecki* ssp. *lactis* DSM 20072 (Fig. 2). The profile of *Lactobacillus* Pr2 with the restriction enzyme *Eco* RI (Fig. 3) categorically confirmed the species affiliation of *Lactobacillus* Pr2 to the species *Lactobacillus delbruecki* ssp. *bulgaricus*.

As a result of the conducted studies, the species identification of *Lactobacillus* Pr2 was confirmed. For its complete identification another molecular genetic method for genotyping was used - sequencing of the gene for the 16S rRNA. The 16S rRNA gene was partially sequenced by Macrogen Europe Laboratory, the Netherlands, using 27F and 1492R universal primers; the whole 16S rDNA was obtained using *CLC Sequence Viewer* software and the resulting sequences were processed with the online BLASTn software. The results of the sequential analysis of the 16S rDNA demonstrated that *Lactobacillus* Pr2 belonged to the species *Lactobacillus delbruecki* ssp. *bulgaricus* with a percentage of similarity between the 16S rDNA sequence of *Lactobacillus* Pr2 and the partial 16S rDNA sequence of *Lactobacillus delbruecki* ssp. *bulgaricus* ATCC 11842 of 99% (Fig. 4).



1. *Lactobacillus* Pr2
2. *L.d.* ssp. *bulgaricus* DSM 20081
3. *L.d.* ssp. *lactis* DSM 20072
4. *L. helveticus* DSM 20075
5. *L. casei* ssp. *rhamnosus* LMG 6400
6. 100 bp Plus DNA Ladder

Fig. 2. Restriction profile with *Alu* I



1. *Lactobacillus* Pr2
2. *L.d.* ssp. *bulgaricus* DSM 20081
3. *L.d.* ssp. *lactis* DSM 20072
4. *L. helveticus* DSM 20075
5. *L. casei* ssp. *rhamnosus* LMG 6400
6. 100 bp Plus DNA Ladder

Fig. 3. Restriction profile with *Eco* RI

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Query  13    ATACATGCA-GTCGAGCGAGCTGAATTCAAAGATCCCTTCGGGGTGATTTGTTGGACGCT  71
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Sbjct  45    ATACATGCAAGTCGAGCGAGCTGAATTCAAAGATTCCTTCGGGATGATTTGTTGGACGCT  104

Query  72    AGCGGCGGATGGGTGAGTAACACGTGGGCAATCTGCCCTAAAGACTGGGATACCACTTGG  131
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Query  192   TAAGCTGTCACTTTAGGATGAGCCCGCGGCGCATTAGCTAGTTGGTGGGGTAAAGGCCTA  251
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Query  552   TAAAGCGAGCGCAGGCGGAATGATAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAA  611
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Query  792   CGTAAACGATGAGCGCTAGGTGTTGGGGACTTTCCGGTCCTCAGTGCCGCAGCAAACGCA  851
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Query  852   TTAAGCGCTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGG  911
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Query  972   CTTGACATCCTGTGCTACACCTAGAGATAGGTGGTTCCCTTCGGGGACGCAGAGACAGGT  1031
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Query  1032  GGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGC  1091
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Query  1451  AGACAG  1456
            || |||
Sbjct  1485  AGGCAG  1490
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Fig. 4. Comparison between the nucleotide sequence of the 16S rDNA of *Lactobacillus* Pr2 and the 16S rDNA sequence of *Lactobacillus delbruecki* ssp. *bulgaricus* ATCC 11842

2. Examination of some probiotic properties of Lactobacillus delbrueckii ssp. bulgaricus Pr2

A series of studies have been conducted to determine the probiotic potential of *Lactobacillus delbrueckii* ssp. *bulgaricus* Pr2.

2.1. Antimicrobial activity of Lactobacillus delbrueckii ssp. bulgaricus Pr2

A number of criteria apply to the selection of probiotic strains, one of the most important being their ability to increase the host's natural defenses against enteropathogens by producing antimicrobial substances or by competitively inhibiting and eliminating these pathogens [11].

In this regard, the antimicrobial activity of *Lactobacillus delbrueckii* ssp. *bulgaricus* Pr2 by the agar-diffusion method with wells was examined (Table 4).

$d_{inhibition\ zone}$, [mm] / Strain (Sample)		*Staphylococcus aureus* ATCC 25923	*Escherichia coli* ATCC 8739	*Listeria monocytogenes* NBIMCC 8632	*Salmonella enteritidis* ATCC 13076
Pr2	Culture liquid (CL)	11,00±1,00	10,00±0,00	14,50±0,50	8,00±0,00
	Biomass (B)	8,00±0,00	-	10,00±0,50	8,00±0,00
	Neutralized Acellular Supernatant (NASN)	-	-	9,00±0,00	-

Table 4. Antimicrobial activity of *Lactobacillus delbrueckii* ssp. *bulgaricus* Pr2 against pathogenic microorganisms. The values are given in mm.

Lactobacillus delbrueckii ssp. *bulgaricus* Pr2 demonstrated antimicrobial activity against *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 8739, *Listeria monocytogenes* NBIMCC 8632, *Salmonella enteritidis* ATCC 13076, as the culture liquid (CL) had higher antimicrobial activity compared to the biomass sample (B).

The neutralized acellular supernatanat did not show any antimicrobial activity against *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 8739 and *Salmonella enteritidis* ATCC 13076.

All three samples of *Lactobacillus delbrueckii* ssp. *bulgaricus* Pr2 had antimicrobial activity against *Listeria monocytogenes* NBIMCC 8632.

The results obtained support the understanding of the mechanisms of the antagonistic action in probiotic strains. Probiotics are able to affect intestinal pathogens by producing antibacterial substances, including lactic acid, acetic acid and antibiotic-like substances (bacteriocins), by competing for nutrients and/or attachment sites, by affecting enzyme activity or by increasing the activity of macrophages and specific antibody levels [12]. A number of authors have investigated the activity of selected lactobacilli strains against various pathogens [13]. Most often, lactobacilli strains exhibit antimicrobial effect against bacterial pathogens by producing metabolites such as lactic acid and acetic acid and subsequently lowering the pH [12].

2.2 Antibiotic resistance

Antibiotics are substances with antimicrobial action that affect Gram-positive and Gram-negative bacteria. They inhibit the growth of microbial cells or destroy them. For this to happen, any antimicrobial substance must penetrate the cell, form a conjugate with a specific cellular structure that is involved in life processes (DNA replication and cell division) or suppress them completely. The effect of antibiotics on microbial cells is associated with inhibition of cell wall synthesis (inhibition of peptidoglycan synthesis or addition of other components), transcription and replication and the formation of the polypeptide chains. When applied, they destroy not only pathogenic microorganisms, but also beneficial microflora [14]. Knowledge of the antibiotic resistance profile of lactobacilli with

probiotic potential is essential and one of the conditions for selection of potential probiotic strains. This is an important criterion in the selection of probiotic cultures due to the possibility of conducting combination therapy with antibiotic and probiotic in order to restore the normal microflora of the gastrointestinal tract and/or urogenital tract [15]. Unfortunately, normally occurring bacteria, including lactobacilli, can serve as a source of antibiotic resistance genes, transferring them to various pathogenic microorganisms. The presence of acquired factors for antibiotic resistance is considered highly undesirable in Europe [15]. Precise analysis of the data on the spectrum of antibiotic resistance in the selection of probiotic strains is needed due to the possibility of transfer of genetic elements that determine resistance [15]. Rules and criteria for the evaluation of transferable resistance genes in starter cultures and probiotics for use in animals and humans have been developed [16].

20 antibiotics with different mechanism of action from the main groups used in medical practice were selected and the susceptibility of *Lactobacillus delbrueckii* ssp. *bulgaricus* Pr2 to each one of them was tested (Table 5). *Lactobacillus delbrueckii* ssp. *bulgaricus* Pr2 was resistant to penicillin, cefamandole, tetracycline and ciprofloxacin.

#	Mechanism of action	Antibiotic		Concentration	**Pr2**
1	Inhibition of cell wall synthesis	Penicillin	P	10 E/disc	R
2		Azlocillin	Az	75 µg/disc	S
3		Piperacillin	P	100 µg/disc	S
4		Ampicillin	A	10 µg/disc	S
5		Oxacillin	O	1 µg/disc	S
6		Amoxicillin	Ax	25 µg/disc	S
7		Vancomycin	V	30 µg/disc	S
8		Cefamandole	Cm	30 µg/disc	R
9	Inhibition of protein synthesis	Tetracycline	T	30 µg/disc	R
10		Doxycycline	D	30 µg/disc	S
11		Gentamicin	G	10 µg/disc	S
12		Kanamycin	K	30 µg/disc	S
13		Tobramycin	Tb	10 µg/disc	S
14		Amikacin	Am	30 µg/disc	S
15		Lincomycin	L	15 µg/disc	S
16		Chloramphenicol	C	30 µg/disc	S
17		Erythromycin	E	15 µg/disc	S
18	Inhibition of DNA synthesis and/or cell division	Rifampin	R	5 µg/disc	S
19		Nalidixic acid	Nx	30 µg/disc	S
20		Ciprofloxacin	Cp	5 µg/disc	R

d < 8 mm – **R**; 8 < d < 16 mm – **SR**; d > 16 mm – **S**.

Table 5. Antibiotic resistance profile of *Lactobacillus delbrueckii* ssp. *bulgaricus* Pr2

Screening for potentially transferable resistance to chloramphenicol, clindamycin, erythromycin, gentamicin, rifampin, tetracycline in lactobacilli is recommended [15]. *Lactobacillus delbrueckii* ssp. *bulgaricus* Pr2 should be tested for potentially transferable resistance to tetracycline (Table 5).

2.3. In vitro determination of the ability of Lactobacillus delbrueckii ssp. bulgaricus Pr2 to survive under conditions simulating different parts of the gastrointestinal tract

Digestive juices in the gastrointestinal tract of mammals are a serious barrier to the entry of microorganisms. The choice of probiotic bacteria to be included as components of probiotics and functional foods requires that they be resistant to effects in different parts of the gastrointestinal tract [17]. Bacteria that enter there with food first enter the stomach under conditions of low pH values and in the presence of pepsin. Then, together with the digested food, they go to the small intestine, where the concentration of bile acids reaches 0.3%, and pass to the large intestine, where the pH values are neutral to slightly alkaline. Those who survive extreme conditions in the stomach are then subjected to stress in the small intestine caused by bile and pancreatic juices, and only survivors reach the colon and colonize mucosal surfaces [17].

2.3.1. Resistance of Lactobacillus delbrieckii ssp. bulgaricus Pr2 to different pH values and enzymes

One of the main criteria to consider when selecting potential probiotic strains in order to ensure their viability and functionality when taken orally is to be tolerant to the acidic stomach pH, which can reach pH=1.5, and the concentration of bile salts in the duodenum [16]. Pepsin has a pH optimum of action at pH = 1 - 2. It can attack peptide components in the cell wall. This affects the cells in the logarithmic growth phase. The cells in the stationary growth phase are resistant to its action, retain their viability and, upon removal of the factor, form colonies on the surface of the solid nutrient medium LAPTg10-agar. At neutral pH values in both the buffer and the culture medium, pepsin action is suspended and in the presence of nutrients, microbial cells grow and multiply, resulting in an increase in their concentration in the medium.

The resistance of *Lactobacillus delbrueckii* ssp. *bulgaricus* Pr2 to the model conditions of the gastrointestinal tract: survival at pH = 2 + pepsin, at pH = 4.5 + pancreatin and at pH = 7 + pancreatin was examined. *Lactobacillus delbrueckii* ssp. *bulgaricus* Pr2 was most sensitive to pH = 2 + pepsin.

At pH = 2 + pepsin the concentration of viable cells was reduced from the beginning of the process and by the 24th hour the concentration of active cells was 2x10^{1}cfu/cm^{3}. At pH = 4.5 + pancreatin at the 24th hour the number of active cells reached 3x10^{3}cfu/cm^{3}. At pH = 7 + pancreatin – at the 24th hour it reached 6x10^{3}cfu/cm^{3}.

During the retention of the food in the stomach - 1.5 to 3 hours - in an acidic environment (pH = 2 + pepsin) the number of viable cells of *Lactobacillus delbrueckii* ssp. *bulgaricus* Pr2 was reduced by 3logN. At pH = 4.5 + pancreatin the decrease in the number of viable cells was by 3logN by the 24th hour, and at pH = 7 + pancreatin the reduction was by 2.8logN by the 24th hour (Fig. 5).

2.3.2. Survival of Lactobacillus delbrueckii ssp. bulgaricus Pr2 at different concentrations of bile salts

Bile salts are composed of the salts of 4 types of bile acids (cholic, deoxycholic, chenodeoxycholic and lithocholic acids), each of which can bind to glycine or form more complex salts. Bile salts or acids can be synthesized from cholesterol or extracted from the blood. After eating, bile is poured into the duodenum. It consists of 97% water, 0.7% bile salts, 0.2% bilirubin, 0.51% lipids (cholesterol, fatty acids and lecithin) and 200 meq/l inorganic salts [18].

Bile salts are an important factor influencing probiotic strain survival in the intestinal tract. It is known that about three hours after eating the concentration of bile salts in the small intestine reaches about 0.3%, and the retention of food in the small intestine is about 4 hours [18]. This requires a study of the influence of different concentrations of bile salts on the growth of the studied strain in liquid nutrient medium (Fig. 6).

The cell is an open dynamic system and conditions for the entry of bile salts into the cytoplasm are probably created. The cytoplasmic membrane is composed of phospholipids containing fatty acids, which upon interaction with bile salts form compounds with a crystal lattice, that release the included

compound only under certain conditions. This in turn impairs the permeability of the cytoplasmic membrane and the transport of nutrients from the outside inwards and from the inside to the medium. Young growing cells are particularly sensitive. Cells in the stationary growth phase survive this stress and form colonies on the surface of the solid medium. Lower concentrations of bile salts in the medium allowed surviving cells to grow, and higher concentrations (0.6%, 1%) inhibited their growth (Fig. 6). The strain was affected by the presence of bile salts in the culture medium, and the degree of reduction in the concentration of viable cells depended on the concentration of bile salts in the medium.

The concentration of viable cells began to decrease from the first hours of culturing of the cells of *Lactobacillus delbrueckii* ssp. *bulgaricus* Pr2 in the presence of different concentrations of bile salts. The degree of reduction was directly proportional to the concentration of bile salts in the medium.

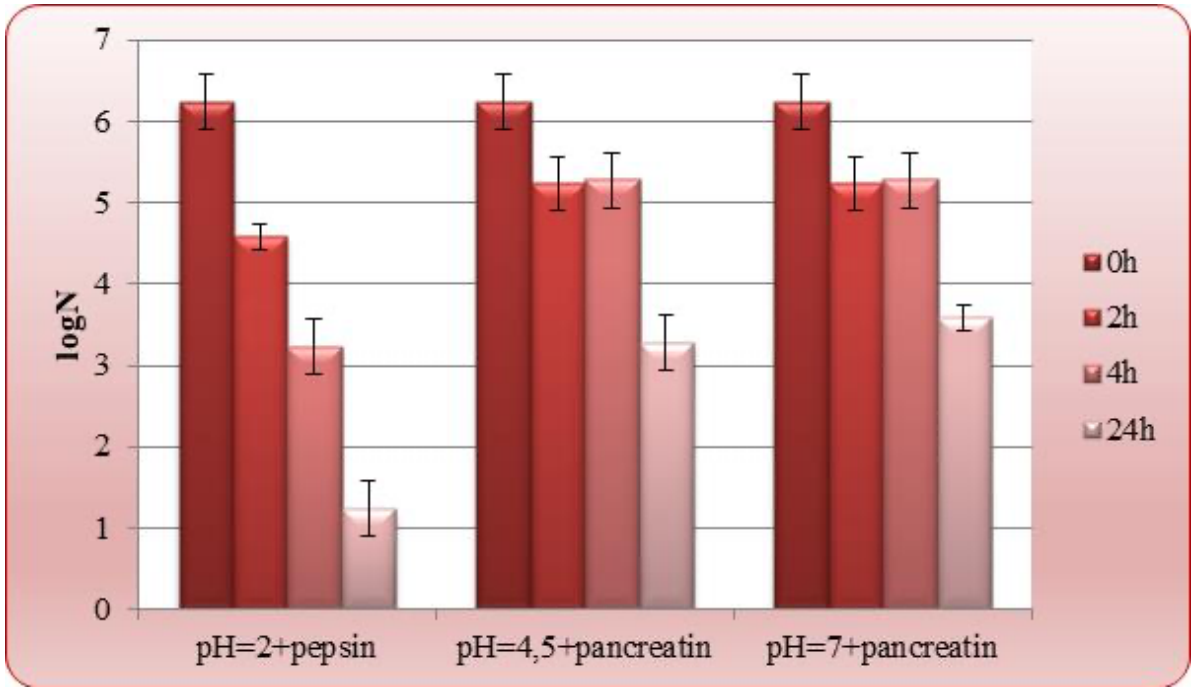


Fig. 5. Survival of *Lactobacillus delbrueckii* ssp. *bulgaricus* Pr2 cells at pH=2 + pepsin, pH=4.5 + pancreatin and pH=7 + pancreatin

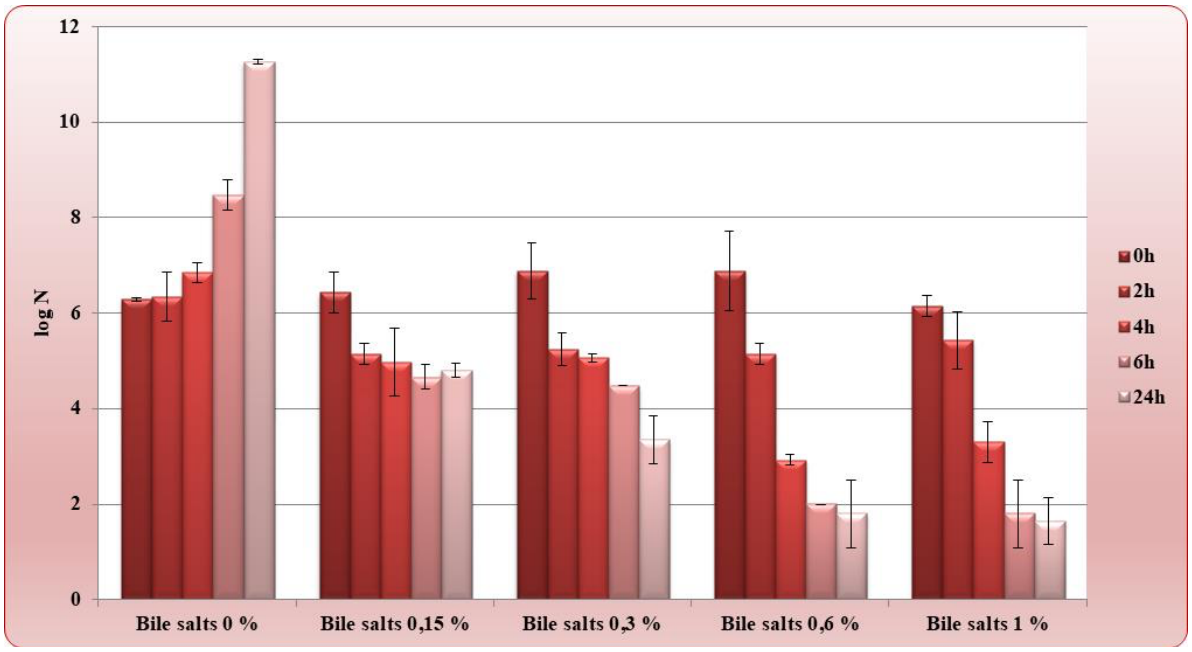


Fig. 6. Survival of *Lactobacillus delbrueckii* ssp. *bulgaricus* Pr.2 at different concentrations of bile salts in the medium

The reduction for 24 hours was 2logN in 0.15% bile salts, 4logN in 0.3% bile salts, 5logN in 0.6% bile salts and 1% bile salts (Fig. 6).

Lactobacillus delbrueckii ssp. *bulgaricus* Pr2 had the ability to survive under the model conditions of the gastrointestinal tract, which is why it was defined as a potential probiotic culture. According to the criteria for *in vitro* evaluation of probiotic strains and studies, it can be concluded that *Lactobacillus delbrueckii* ssp. *bulgaricus* Pr2 combined useful properties that identify it as a promising strain for inclusion in the composition of probiotics and probiotic foods.

2.4. Freeze-dried concentrate

Lactobacillus delbrueckii ssp. *bulgaricus* Pr2 was cultured in a thermostat for 24 hours at 37±1°C. The obtained biomass was immobilized and freeze-dried in the presence of highly esterified apple pectin and sodium alginate. The microbiological status of the resulting freeze-dried concentrate after freeze-drying (Table 6) and the survival of the strain in the freeze-drying process (Fig. 8) were examined.

Parameter	**Norm according to BSS**	**Analysis**
1. Total number of mesophilic aerobic and facultative anaerobic bacteria, CFU/g	No more than 800	220 – 360
2. *Escherichia coli* in 0.1 g of the product	Not to be found	Not found
3. Sulfite-reducing clostridia in 0.1 g of the product	Not to be found	Not found
4. *Salmonella* sp. in 25.0 g of the product	Not to be found	Not found
5. Coagulase-positive staphylococci in 1.0 g of the product	Not to be found	Not found
6. Spores of microscopic molds, CFU/g	No more than 100	Not found
7. Yeast, CFU/g	No more than 100	Not found

Table 6. Microbiological status of the *Lactobacillus delbrueckii ssp. bulgaricus* Pr2 freeze-dried concentrate

The freeze-dried concentrate did not contain pathogenic microflora and met the standard requirements for microbial purity of food. The absence of insemination with pathogenic microorganisms proved that the entire technological process was carried out in accordance with sanitary norms and requirements.

The hydrocolloids used for mechanical immobilization and as cryoprotectants in the freeze-drying process were highly effective and the survival rate of the strain was high (Fig. 7).

The obtained results show the effectiveness of the combined hydrocolloid matrix as a carrier for immobilization and cryoprotective environment in the process of freezing and freeze-drying. The high survival of *Lactobacillus delbrueckii* ssp. *bulgaricus* Pr2 in the freeze-dried preparation was due to the optimal programming of the entire technological process - freezing conditions, appropriate cryoprotective medium, regime parameters of freeze-drying, properly defined cycle time, which provided low residual moisture content of the final product. Probably, in the form of hydrocolloid matrices for immobilization of cultures, they created an opportunity for: significant stabilization of the enzymatic activity of the immobilized cells; stabilization and overall increase of the activity of the immobilized probiotic system, which had a favorable effect on their survival after freeze-drying and during storage.

In addition, the very freeze-drying technology itself prevented the growth of pathogenic microflora. The greater the degree of dehydration, as in new organic products, is, the lower the survival of pathogenic microflora.

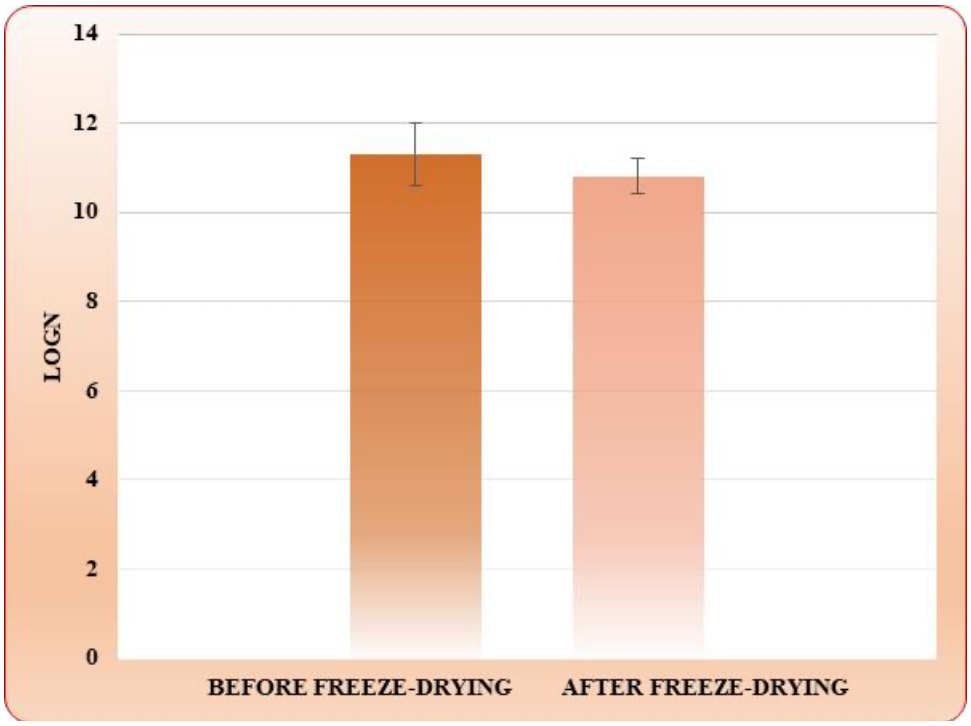


Fig. 7. Survival of *Lactobacillus delbrueckii* ssp. *bulgaricus* Pr2 in the process of freeze-drying

CONCLUSION

The newly isolated strain *Lactobacillus* Pr2 was identified by the application of cultural, microbiological, physiological and biochemical (API 50 CHL) and molecular genetic (ARDRA-analysis and sequencing of the gene for the 16S rRNA) methods as a representative of the species *Lactobacillus delbrueckii* ssp. *bulgaricus*. *Lactobacillus delbrueckii* ssp. *bulgaricus* Pr2 demonstrated good survival under the model conditions of the gastrointestinal tract (pH = 2 + pepsin, pH = 7 + pancreatin, high concentrations of bile salts) and high antimicrobial activity against pathogenic microorganisms. A freeze-dried preparation of *Lactobacillus delbrueckii* ssp. *bulgaricus* Pr2 with a

high content of viable cells after freeze-drying was obtained. After further investigation of other probiotic properties of *Lactobacillus delbrueckii* ssp. *bulgaricus* Pr2, the strain can be successfully applied in the composition of probiotic preparations and functional foods.

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