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Selection of Lactobacilli with Probiotic Properties

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Abstract

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The aim of the present work was to select strains from genus Lactobacillus with properties, allowing their inclusion in probiotics and probiotic foods - survival in model conditions of digestion, high antimicrobial activity and resistance to some of the applied in medicinal treatment antibiotics.

The resistance of lactic acid bacterial strains against different concentrations of bile salts, low and neutral values of pH and presence of pepsin was studied. It was shown that they survive at low (pH 2.0) and neutral (pH 7.0) pH values and pepsin and overcome the bile barrier. The antimicrobial activity against conditional pathogens, toxicogenic and pathogenic bacteria and enterobacteria was investigated according to the agar diffusion method. The highest activity exhibited *Lactobacillus acidophilus* and *Lactobacillus delbrueckii ssp. bulgaricus*. The resistance of the lactobacilli against most of the antibiotics, used in medicine was investigated. It was shown that the antibiotic resistance is strain specific.

As a result of the conducted investigation were selected Lactobacillus strains, which conform to the requirements for probiotic cultures.

Key words: Lactobacilli, probiotics, artificial gastric juice, antibiotic resistance, antimicrobial activity

Introduction

Fuller (1989) defined as probiotics "a live microbial feed supplement, which beneficially affects the host animal by improving its intestinal microbial balance". According to the same author (1992), probiotics are biologically active preparations, containing live cells and metabolites of stabilized, autochthonous microorganisms, which improve the colonization and composition of the intestinal microflora in animals and humans, and have a stimulating effect on the digestive processes and the immune system of the host. Later Salminen et al. (1998) defined probiotics as food supplements with viable microorganisms with beneficial effect on human health.

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In the composition of probiotics are included not only lactic acid bacteria and bifidobacteria (*Lactobacillus, Streptococcus, Enterococcus, Lactococcus, Bifidobacterium*), but also representatives of genus Bacillus and fungi from genera *Saccharomyces* and *Aspergillus*. The main components of probiotics are the lactic acid bacteria and bifidobacteria (*Lactobacillus, Streptococcus, Enterococcus, Pediococcus, Lactococcus, Leuconostoc*), which are applied in the production of probiotic foods as well (Salminen et al., 1998, Salminen and von Wright, 1998; Gibson, 2004; Saarela and Mogensen, 2000; Wolfson, 1999).

Not all lactobacilli can be included in the composition of probiotics and probiotic foods, but only these, possessing the ability to adhere to the epithelial cells (Lehto and Salminen 1997, Ouwehand et al. 2001); to survive the conditions of the stomach and the intestines (Gasser 1994, Kashtan 1990); to reproduce in the digestive tract; to produce antimicrobial substances (organic acids and bacteriocins); they must have antimicrobial activity against conditional pathogens, toxicogenic and pathogenic microorganisms (Barefoot and Klaenhammer, 1983; Klaenhammer, 1993; Eswaranandam et al., 2004) and be safe for clinical and food applications (Salminen et al., 1998; Donohue et al., 1998; Saxelin and Salminen, 1996a; Saxelin et al., 1996b)

This necessitates the selection of lactic acid bacteria strains with probiotic properties.

Materials and Methods

In the study were used lactic acid bacteria strains from NCIMCC (National Bank for Industrial Microorganisms and Cell Cultures): *Lactobacillus delbrueckii subsp. bulgaricus* NCIMCC 144, *Lactobacillus delbrueckii subsp. bulgaricus* NCIMCC 26, *Lactobacillus delbrueckii subsp. bulgaricus* NCIMCC 144-1, *Lactobacillus delbrueckii subsp. bulgaricus* NCIMCC B 48, *Lactobacillus delbrueckii subsp. bulgaricus* NCIMCC GB, *Lactobacillus delbrueckii subsp. bulgaricus* NCIMCC LB-M, Lactobacillus acidophilus, *Lactobacillus acidophilus* NCIMCC 2, *Lactobacillus helveticus* NCIMCC H, *Lactobacillus casei* NCIMCC C, *Lactobacillus plantarum* NCIMCC 226-15 and from the collection of the Department of Microbiology and Organic Chemistry at UFT: *Lactobacillus delbrueckii subsp. bulgaricus* BB, *Lactobacillus delbrueckii subsp. bulgaricus* BG, *Lactobacillus acidophilus* A.

The antimicrobial activity of the investigated lactic acid bacteria strains was determined against the following pathogenic microorganisms - *Escherichia coli, Salmonella sp., Proteus vulgaris, Pseudomonas aeruginosa, Staphylococcus aureus subsp. aureus, Klebsiella pneumoniae subsp. pneumoniae, Enterococcus faecalis, Candida albicans* with human origin, which were granted by HMI-Plovdiv, HMI-Sofia, Bulgaria.

In the present work were used the following nutrient media: MRS-agar (MERCK, Germany); LAPTg10 (19);

LBG (Luria-Bertani with 1% glucose) - for determination of the antimicrobial activity.

Analytical methods

A. Determination of the viable counts of lactobacilli:

The strains were cultivated in skimmed cow's milk for a fixed time at the optimal temperature for their development. The cultures were diluted tenfold according to the method of serial dilutions in saline solution (Macrae et al., 1993). After that, they were spread on Petri dishes with MRS and LAPTg10 media (Portillo et al., 1988), which were incubated for 3 days at 37 °C until single colonies were formed.

B. Method for determination of the gastric juice resistance of lactobacilli (Valdez and Taranto, 1990):

The cultures were incubated for 16 h in MRS-broth. The culture medium was then centrifuged at 5000 min^{-1} for 10 min. This was followed by two-act washing of the pellet with 0.1 M phosphate buffer, pH 7.0. The cells were resuspended to the original volume with the buffer. Buffer with pH 2.0, containing 2 $g.dm^{-3}$ NaCl and 3.2 g pepsin with activity 0.7 FIP $U.mg^{-1}$ and buffer with pH 7.0 and the same content were incubated with 2% of the bacterial suspension. They were thermostated at 37 °C and samples were taken on the 0, 2, 4 and 24 h of incubation. Proper dilutions were made according to the method of the serial tenfold dilutions in 0.1% peptone water and plated in MRS-agar. The plates were incubated at 37 °C until single colonies were formed. The colonies were count and the results were expressed as colony-forming units per cm^3 ($CFU.cm^{-3)}$.

C. Method for determination of the bile tolerance of lactobacilli (Valdez and Taranto, 1990; Amor et al., 2002):

The first steps, until the bacterial suspension was obtained, were the same as those in the method for determination of the gastric juice resistance of lactobacilli. MRS-broth (50 cm^3), containing different concentrations of bile salts (0; 0.05; 0.1; 0.15; 0.3; 1 %) was incubated with 0.5 cm^3 of the cell suspension. The lactic acid bacteria were cultivated at 37 °C and the viable cell counts were determined on the 0, 2, 4, 6, 8 and 24 h according to method A.

D. Method for determination of the antimicrobial activity. Agar diffusion method

From the test-microorganisms were prepared bacterial suspensions in saline solution. 100 cm^3 of melted and cooled LBG-agar was incubated with 1 cm^3 of the suspension and 20 cm^3 of this medium was pored into each Petri dish. After the medium solidified and was cooled wells with d=6 mm were made. In each of them were pipetted 60 µl from the Lactobacillus culture medium. The plates were incubated at 37 °C for 18-24 h. The diameter of the zones of clearance was determined. Zones with diameter grater than 6 mm were taken for inhibitory.

E. Determination of the antibiotic resistance of lactobacilli

The agar diffusion method was used,

but with paper disks with a definite antibiotic concentration. The lactobacilli were cultivated in their growth medium and centrifuged at 5000 min^{-1} for 10 min. The biomass was resuspended in saline solution to the original volume and the growth media were inoculated with 0.1 cm^3 of this suspension. The plates were left for 1-2 h at room temperature and after that the disks with d=6 mm were placed. The Petri dishes were incubated at 37 °C for 24-48 h and the zones of clearance were determined.

F. Method for determination of the components of the supernatant liquid

The components of the supernatant liquid were determined by HPLC with Perkin Elmer - Series 4, USA, column RP C 18.10 μm, 25cm Ø 4.6 mm; electrophotometric detector with λ = 206 nm, mobile phase - KH_2PO_4 (0.02 N) buffer with pH 2.25 and flow rate - 1$cm^3 \cdot min^{-1}$, injector - RHEODYNE - 7125-0.75, sample quantity - 6 μl.

Results

The lactic acid bacteria inhabit the digestive tract of humans and animal organisms. These beneficial for the health of the host microorganisms metabolize substrates with the production of lactic, acetic and other organic acids and bacteriocins, through which they change the conditions of the tract, suppress, and push out of the biological niche the pathogenic and toxigenic microbes. Because of this reason they are regulators of the gut microflora. Not all Lactobacillus strains can fulfill this role, but only those capable of surviving and reproducing in the diverse conditions of the digestive tract. This necessitates the selection of Lactobacillus strains with certain properties, known as probiotic properties.

Survival of lactobacilli in conditions, close to the conditions in the digestive tract

While choosing lactic acid bacteria, which can be included as components of probiotics and probiotic foods, it is required, that they are resistant to the acid conditions of the stomach, as well as to the bile acids in the small intestines. The bacteria, which enter the digestive tract, pass through an active acidity range from pH 2.0 to pH 8.0. Different microorganisms vary in their ability to survive in such extreme conditions. Some of them perish entering the stomach, and others survive long enough and preserve their viability and reproductive capacity. In the concentrated form of probiotics or with probiotic foods, the lactobacilli reach the stomach in the conditions of low pH values and the presence of pepsin. After that, together with the digested food, they pass though the small intestines, where the concentration of bile acids is up to 0.3% and reach the large intestine, which has a neutral pH value.

We investigated the ability of *Lactobacillus delbrueckii subsp. bulgaricus* strains from the Bulgarian gene fund to develop in model conditions of digestion - enzymes and low and neutral pH values, the presence of bile salts. Their antimicrobial activity towards pathogens and conditional pathogens was defined and their antibiotic resistance was studied.

At low pH values and in the presence

Table 1

Reduction of the viable cell counts of strains *Lactobacillus delbrueckii subsp. bulgaricus* at acid (pH 2.0) and neutral pH values (pH 7.0) and in the presence of pepsin

Strain	Viable cell counts, cfu cm^{-3}							
	0 h		2 h		4 h		24 h	
	pH 2.0 + pepsin	pH 7.0 + pepsin	pH 2.0 + pepsin	pH 7.0 + pepsin	pH 2.0 + pepsin	pH 7.0 + pepsin	pH 2.0 + pepsin	pH 7.0 + pepsin
Lactobacillus bulgaricus 26	$6.3 . 10^6$	$2.2 . 10^7$	$1.3 . 10^6$	$7.9 . 10^6$	$3 . 10^2$	$2.1 . 10^6$	10	$5 . 10^4$
Lactobacillus bulgaricus 144	$9.4 . 10^5$	$5 . 10^5$	$3 . 10^4$	$2 . 10^5$	$5 . 10^3$	$2.9 . 10^5$	10	10
Lactobacillus bulgaricus 144-1	$9 . 10^4$	$1 . 10^5$	$1 . 10^4$	$1 . 10^4$	$2 . 10^3$	$3 . 10^4$	80	$3 . 10^2$
Lactobacillus bulgaricus B48	$1 . 10^7$	$2.2 . 10^7$	$3 . 10^4$	$6.3 . 10^6$	$3 . 10^3$	$9.8 . 10^6$	$2 . 10^2$	$3 . 10^6$
Lactobacillus bulgaricus LB-M	$1.5 . 10^5$	$6 . 10^4$	$1 . 10^4$	$2 . 10^4$	$1 . 10^3$	$6.5 . 10^6$	10	10
Lactobacillus bulgaricus BB	$1.5 . 10^6$	$4.3 . 10^6$	$2.5 . 10^5$	$4.1 . 10^6$	$1.3 . 10^5$	$4 . 10^4$	$2 . 10^2$	$2.3 . 10^4$
Lactobacillus bulgaricus BG	$1 . 10^6$	$1.3 . 10^6$	$2 . 10^4$	$2 . 10^4$	$1 . 10^3$	$3.2 . 10^6$	10	$2 . 10^2$
Lactobacillus bulgaricus GB	$9.8 . 10^6$	$7.9 . 10^6$	$1 . 10^3$	$2 . 10^6$	$1 . 10^3$	$1 . 10^3$	30	$7 . 10^3$
Lactobacillus bulgaricus J	$3 . 10^5$	$2 . 10^5$	$4 . 10^4$	$2 . 10^4$	$2 . 10^4$	$6 . 10^3$	20	$1.8 . 10^3$

of the enzyme pepsin most of the *Lactobacillus delbrueckii subsp. bulgaricus* strains showed a three-order reduction in viable cell counts for the time of exposure to these conditions in the organism (about 4 hours) (Table 1.). All of the studied representatives of this species remained viable and their cell counts reach up to 10^3 cfu.cm^{-3}. More resistant to these conditions were the cells of *Lactobacillus delbrueckii subsp. bulgaricus* 144, *Lactobacillus delbrueckii subsp. bulgaricus BB* and *Lactobacillus delbrueckii subsp. bulgaricus J*, which exhibited a one-order decrease of the viable cell counts (Table 1).

All of the investigated *Lactobacillus delbrueckii subsp. bulgaricus* strains from the Bulgarian collection distinguished themselves by high resistance towards low pH values and pepsin, as well as neutral values of pH in the presence of pepsin. The viable cell counts were reduced and the degree of reduction was associated with the individual features of the strains.

The three *Lactobacillus acidophilus* strains, which were studied, were influenced by the low and neutral values of pH in the presence of pepsin in a different manner. Their viable cell counts remained high for the time of stay in the stomach and the degree of reduction was also associated with the individual features of the strains (Table 2).

The investigated *Lactobacillus helveticus H* strain proved to be resistant towards low pH values in the presence of pepsin, as well as neutral pH values and pepsin (Table 2). The viable cell counts were above 10^6 cfu.cm^{-3} on the 24 h at pH 7.0 and above 10^5 cfu.cm^{-3} at pH 2.0 and the presence of enzyme.

The development of the representatives of the mesophilic lactobacilli *Lactobacillus plantarum* 226-15 and *Lactobacillus casei subsp.* casei C was also studied at low and neutral pH values and the presence of pepsin (Table 2). The viable cell counts of Lactobacillus plantarum 226-15 decreased with one order on the 4th hour at low pH values of the medium.

More resistant were the cells of *Lactobacillus casei subsp. casei C*. The viable cells were above 10^4 cfu.cm^{-3} in the medium with pH 7.0 (the initial concentration of viable cells in the population was 10^6 cfu.cm^{-3}) and above 10^5 cfu.cm^{-3} on 24 h at pH 2.0 and pepsin (Table 2).

On the third hour after the intake of food the concentration of bile salts in the small intestines reaches 0.3%. The variations in the survival of the different lactobacilli at different concentrations of bile salts in the nutrient medium (0.05%, 0.1%, 0.15%, 0.3%, 1%) were fallowed. The low concentrations 0.05% ÷ 0.15% had a small influence over the resistance of the cells of the different *Lactobacillus delbrueckii subsp. bulgaricus* strains (Table 3 and Figure 1), which was expressed by retention of the viable cell counts. Some of the strains showed a small decrease of the cell counts in the first two hours and after that they increased again. This was probably a result of the reproduction of the resistant towards bile salts section of the strain. On Figure 1 is shown the survival of the cells of the *Lactobacillus delbrueckii subsp.* bulgaricus strains after 24 h cultivation at different bile salts concentrations in the medium. When the concentration was equal to

Table 2

Reduction of the viable cell counts of *Lactobacillus strains* at acid (pH 2.0) and neutral pH values (pH 7.0) and in the presence of pepsin

Strain	Viable cell counts, cfu cm^{-3}							
	0 h		2 h		4 h		24 h	
	pH 2.0 + pepsin	pH 7.0 + pepsin	pH 2.0 + pepsin	pH 7.0 + pepsin	pH 2.0 + pepsin	pH 7.0 + pepsin	pH 2.00 + pepsin	pH 7.0 + pepsin
Lactobacillus acidophilus	$3.4 .10^{7}$	$2.6 . 10^{7}$	$1.3 . 10^{5}$	$4.9 . 10^{6}$	$1.2 . 10^{5}$	$4.8 . 10^{6}$	20	$3 . 10^{2}$
Lactobacillus acidophilus A	$1 . 10^{7}$	$5.1 . 10^{6}$	$1.2 . 10^{5}$	$9 . 10^{5}$	$3 . 10^{4}$	$1 . 10^{5}$	$1.1 . 10^{2}$	$2.1 . 10^{4}$
Lactobacillus acidophilus 2	$5.6 . 10^{7}$	$4.32 . 10^{7}$	$1.1 . 10^{7}$	$3.86 . 10^{7}$	$7 . 10^{3}$	$3.7 . 10^{7}$	70	$4 . 10^{4}$
Lactobacillus helveticus H	$9.6 . 10^{7}$	$1 . 10^{8}$	$6.9 . 10^{6}$	$7.51 . 10^{7}$	$2.2 . 10^{5}$	$6.5 . 10^{7}$	$3 . 10^{4}$	$4.6 . 10^{5}$
Lactobacillus casei C	$2 . 10^{5}$	$1.3 . 10^{6}$	$2 . 10^{5}$	$6 . 10^{5}$	$2 . 10^{3}$	$5 . 10^{4}$	$5 . 10^{2}$	$1 . 10^{4}$
Lactobacillus plantarum 226-15	$2 . 10^{4}$	$2 . 10^{5}$	$2 . 10^{4}$	$2.1 . 10^{5}$	$1 . 10^{4}$	$1 . 10^{4}$	10	10

Table 3

Variation of viable cell counts of *Lactobacillus delbrueckii subsp. bulgaricus* at different concentrations of bile salts in the medium

Strain	Viable cell counts, cfu cm^{-3}											
	0 h			2 h			4 h			24 h		
	Concentration of bile salts, %											
	0	0.15	0.3	0	0.15	0.3	0	0.15	0.3	0	0.15	0.3
Lactobacillus bulgaricus BB	$2.1.10^6$	$2.0.10^6$	1.10^5	$3.4.10^6$	$4.2.10^6$	1.10^5	$3.5.10^6$	$4.2.10^6$	1.10^5	5.10^7	$6.7.10^5$	1.10^4
Lactobacillus bulgaricus 144	$7.5.10^6$	7.10^6	6.10^6	$4.2.10^7$	$4.7.10^6$	$1.6.10^6$	4.10^7	$9.2.10^6$	$3.9.10^6$	1.10^8	$3.7.10^7$	$3.3.10^6$
Lactobacillus bulgaricus 144-1	$1.6.10^6$	$7.4.10^5$	1.10^5	4.10^6	1.10^5	1.10^4	$4.6.10^6$	8.10^5	1.10^4	7.10^6	9.10^5	2.10^4
Lactobacillus bulgaricus B48	$2.9.10^6$	5.10^5	4.10^5	$4.6.10^6$	$1.4.10^6$	$1.9.10^5$	$4.1.10^6$	2.10^6	7.10^4	$1.1.10^7$	5.10^5	9.10^4
Lactobacillus bulgaricus LB-M	$2.7.10^6$	3.10^5	1.10^5	1.10^6	$3.2.10^6$	1.10^5	1.10^6	2.10^6	$1.6.10^5$	$1.2.10^8$	$2.9.10^6$	3.10^4
Lactobacillus bulgaricus 26	$3.8.10^6$	$3.1.10^6$	1.10^5	4.10^6	$3.7.10^6$	2.10^5	4.10^6	2.10^6	3.10^4	$2.2.10^7$	8.10^6	2.10^4
Lactobacillus bulgaricus BG	1.10^6	1.10^6	2.10^5	2.10^5	1.10^5	1.10^5	3.10^5	1.10^5	3.10^4	$1.2.10^8$	1.10^5	$3.8.10^5$
Lactobacillus bulgaricus GB	$3.6.10^6$	$4.1.10^5$	$2.6.10^5$	$3.7.10^6$	$2.8.10^6$	$1.3.10^5$	$1.2.10^6$	$3.8.10^6$	$3.3.10^4$	$1.7.10^7$	$1.5.10^6$	$5.5.10^4$
Lactobacillus bulgaricus j	$1.6.10^7$	9.10^5	1.10^5	$1.4.10^7$	7.10^5	1.10^3	$3.6.10^7$	$2.1.10^6$	1.10^2	2.10^7	8.10^5	10

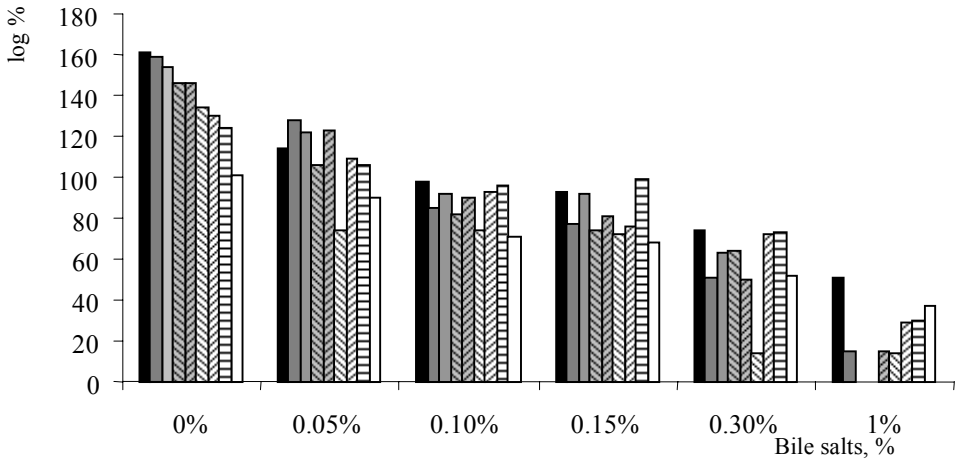

Fig. 1. Variation of viable cell counts of *Lactobacillus delbrueckii subsp. bulgaricus* after 24 h under the influence of different concentrations of bile salts in the medium.

(■) - *Lact. bulgaricus LBM;* (■) - *Lact. bulgaricus B48;* (■) - *Lact. bulgaricus BB;* (■) - *Lact. bulgaricus 144-1;* (■) - *Lact. bulgaricus GBL+;* (■) - *Lact. bulgaricus J;* (■) - *Lact. bulgaricus 144 D-;* (■) - *Lact. bulgaricus 26-12;* (□) - *Lact. bulgaricus BG*

that in the small intestines the viable cell counts of the studied strains were reduced in half. A high degree of resistance was demonstrated by the cell populations of *Lactobacillus delbrueckii subsp. bulgaricus LB-M, Lactobacillus delbrueckii subsp. bulgaricus GB and Lactobacillus delbrueckii subsp. bulgaricus 144.*

From the three investigated for bile tolerance acidophilic strains, *Lactobacillus acidophilus* and *Lactobacillus acidophilus 2* (Table 4 and Figure 2) after 8 h of incubation in a medium with 0.3 and 1% bile salts kept a high concentration of viable cells. These cultures showed a decrease in the viable cell counts on the 2 h of incubation, and after that it increased until the 6 h and then the growth was retained. An exception was *Lactobacillus acidophilus A* (Table 4), whose cells were more sensitive towards the salt content of the medium. At bile salts concentrations of 0.3 and 1% 2 h after the beginning of the fermentation process all of the cells lost their viability.

The cells of *Lactobacillus helveticus H*, like those of the acidophilic strains, were resistant to the presence of bile salts in a concentration, equivalent to that in the bile (Table 4). This strain demonstrated decrease of the viable cell counts at all salt concentrations during the first 2 h of the process, which was more obvious at the high bile salts concentrations 0.3 and 1%. This was followed by growth until the 8 h at the expense of the emerged resistant cells at the lower bile salts concentrations, and growth until the 4 h for the higher concentrations. After that the viable cell counts were reduced and reached 10^5 cfu/cm^3 on the 24 h at 0.3 and 1% bile salts (Figure 2).

Table 4

Reduction of the viable cell counts of *Lactobacillus* strains at different concentrations of bile salts in the medium

Strain	Viable cell counts, cfu cm^{-3}											
	0 h			2 h			4 h			24 h		
	Concentration of bile salts, %											
	0	0.15	0.3	0	0.15	0.3	0	0.15	0.3	0	0.15	0.3
Lactobacillus acidophilus	6.10^6	$2.1.10^6$	$3.5.10^5$	4.10^6	2.10^5	$3.5.10^5$	$1.9.10^6$	7.10^5	$3.8.10^5$	1.10^7	3.10^4	3.10^4
Lactobacillus acidophilus A	$4.3.10^6$	9.10^5	1.10^2	$2.9.10^6$	$1.8.10^6$	10	$2.8.10^6$	$2.7.10^6$	0	4.10^8	2.10^3	0
Lactobacillus acidophilus 2	2.10^6	$1.5.10^6$	$1.2.10^3$	2.10^6	$2.5.10^6$	$3.1.10^5$	2.10^6	6.10^6	7.10^5	$5.2.10^7$	3.10^4	1.10^4
Lactobacillus helveticus H	$1.5.10^7$	$1.8.10^7$	1.10^7	6.10^7	$1.6.10^7$	3.10^4	1.10^7	1.10^7	7.10^6	1.10^8	2.10^4	2.10^4
Lactobacillus casei C	$2.7.10^6$	$5.2.10^5$	2.10^5	$2.7.10^6$	$1.8.10^6$	3.10^5	2.10^6	7.10^5	1.10^5	$4.6.10^7$	3.10^4	2.10^4
Lactobacillus plantarum 226-15	$1.2.10^8$	$5.7.10^7$	$2.4.10^7$	$5.3.10^7$	$2.6.10^7$	$1.9.10^7$	$4.7.10^7$	$3.9.10^7$	$6.4.10^7$	$1.2.10^{10}$	$1.2.10^9$	$4.8.10^7$

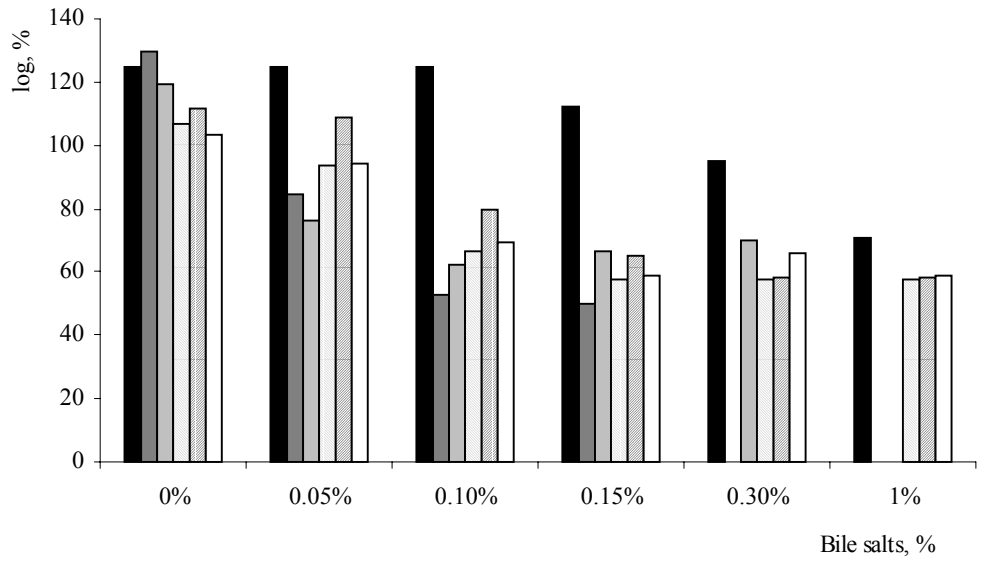

Fig. 2. Variation of viable cell counts of *Lactobacillus acidophilus, Lactobacillus helveticus H, Lactobacillus casei subsp. casei C* and *Lactobacillus plantarum 226-15* after 24 h under the influence of different concentrations of bile salts in the medium.

(■) - *Lactobacillus plantarum 226-15;* (▩) - *Lact. acidophilus A*; (▩) - *Lact. casei subsp. casei* C; (□) - *Lact. helveticus H;* (▨) - *Lact. acidophilus 2;* (□) - *Lact. acidophilus*

The cells of the mesophilic lactobacilli *Lactobacillus casei subsp. casei C* and *Lactobacillus plantarum 226-15* were the most resistant to the presence of bile salts in the medium. Not only so, but more than 0.3% of bile salts sustained the growth of both strains and they kept high viable cell counts of above 10^5 cfu/cm^3 for *Lactobacillus casei C* (Table 4), while *Lactobacillus plantarum 226-15* (Table 4) for the period 8-24 h at 1 % bile salts in the medium showed and increase of the viable cell counts (Figure 2). The high concentration of antimicrobial substances was a precondition for the emergence of resistant cells, which on their turn grew and reproduced and this was expressed by the increase of viable cells in the microbial population.

There is always a certain amount of nutrients in the stomach of the animal organisms, therefore the variation of the viable cell counts of *Lactobacillus acidophilus 2* and *Lactobacillus delbrueckii subsp. bulgaricus GB* in buffers with pH 2.0 and pH 7.0, as well as in the presence of pepsin and in nutrient medium with pH 2.0 and pH 7.0 and the presence of enzyme was followed.

Pepsin has a pH-optimum at pH 1-2. It can attack the peptide components of the cell wall. This influences the cells in the logarithmic phase of growth. The cells in the stationary phase are resistant to its action, they preserve their viabil-

Table 5

Antimicrobial properties of the lactobacilli

Strain	Inhibition zone, mm								
	Salmonella sp., $1.2.10^{12}$ cfu cm^{-3}*	*Candida albicans*, 5.10^{8} cfu cm^{-3}	*Proteus vulgaris*, 5.10^{11} cfu cm^{-3}	*Enterococcus faecalis*, $2.2.10^{11}$ cfu cm^{-3}	*Staphylococcus aureus*, $1.0.10^{11}$ cfu cm^{-3}	*Pseudomonas aeruginosa*, 7.10^{10} cfu cm^{-3}	*Klebsiella pneumoniae*, $1.0.10^{11}$ cfu cm^{-3}	*Escherichia coli*, $1.5.10^{10}$ cfu cm^{-3}	TA, °T
Lact. bulgaricus 26	10	10	9-10	14	8	10-12	20	15	180
Lact. bulgaricus BG	10	9	11	15-16	8	11-12	18-20	15	208
Lact. bulgaricus GB	10	9	9-10	14	7	10	20	13-14	196
Lact..helveticus H	11-12	10	10	12	8	11	17-18	12	148
Lact. acidophilus	11	9	10	15	8-10	12	18-21	15	192
Lact..bulgaricus LB-M	8	10	10	11	8	10-12	22	12-14	152
Lact. bulgaricus BB	11-12	10	10	13	9	9	21	10	152
Lact. plantarum 226-15	9-10	10	-	9	8	8	18	8	56
Lact. casei subsp. casei C	9	10	9	12	18	10-11	21	13	164
Lact..bulgaricus B 48	12	10	11	16	16	15	18	17	216
Lact. bulgaricus J	14-15	10-11	-	11-12	13-14	12	21	10	152
Lact. bulgaricus 144	10	9	10	10	8	12	18	10	124
Lact.bulgaricus 144-1	-	9	-	10	-	-	20	10	132
Lact. acidophilus 2	11	9-10	11	14	12	15-16	20-21	15	88
Lact. acidophilus A	11-12	10	11	15	10	16	20	14-15	200

* concentration of the cells of the test-microorganism in the agar medium.

ity and when the stress factor is removed, they form colonies on the surface of the LAPTg10-agar. At neutral pH values, both in the buffer and the nutrient medium, the action of the enzyme is suspended and in the presence of nutrients the microbial cells grow and reproduce.

Of interest were the interactions between the selected group of lactobacilli and pathogens, representatives of *Enterobacteriaceae*, causing agents of toxicoinfections and toxicoses, as well as fungal pathogens.

As test-microorganisms were used pathogenic microbes of human origin - *Salmonella sp., Candida albicans, Proteus vulgaris, Enterococcus faecalis, Staphylococcus aureus subsp. aureus, Pseudomonas aeruginosa, Klebsiella pneumoniae subsp. pneumoniae, Escherichia coli* with viable cell counts of the suspensions above 10^{10} cfu.cm^{-3}. The investigations were conducted according to agar diffusion method. The results from these experiments are presented in Table 5.

All of the studied *Lactobacillus* strains inhibited the growth of the enteropathogenic strains and to a greater extend that of *Klebsiella pneumoniae subsp. pneumoniae, Escherichia coli, Salmonella sp., Enterococcus faecalis*, and *Pseudomonas aeruginosa.* Weaker was their action against *Candida albicans* and *Proteus vulgaris.*

High antimicrobial activity against the pathogens showed the acidophilic strains. They inhibited the growth and development of all the investgated pathogens and *Lactobacillus acidophilus 2* had the strongest influence with relatively low titratable acidity of the culture medium (Table 5). A lower effect on the test-microorganisms was demonstrated by *Lactobacillus casei subsp. casei C* and *Lactobacillus plantarum 226-15* in comparison with the other lactobacilli. Significantly higher inhibitory activity was observed in the case of *Lactobacillus helveticus H*. Having in mind that the study was completed with the supernatant after centrifugation of the culture medium, it can be concluded that the inhibition was a result of the action of the metabolites produced and secreted by the microorganisms in the medium. Investigations were conducted to define the active components of the supernatant as organic acids through HPLC. The content of the main ingredients of the supernatant is

Table 6

Organic acid content in the culture medium of latic acid bacteria starins

Strain	Components of the culture medium, mg cm^{-3}			
	Lactic acid	Citric acid	Acetic acid	Tartic acid
Lact. bulgaricus GB	45.89	-	-	5.25
Lact. helveticus H	15.66	7.78	-	1.44
Lact. bulgaricus LB-M	13.31	6.55	-	1.15
Lact. plantarum 226-15	16.69	5.85	-	-
Lact. casei subsp. casei C	23.11	7.75	-	2.18
Lact .bulgaricus 144	12.88	6.25	-	1.18
Lact. acidophilus 2	33.47	7.54	-	2.92

Table 7

Influence of the different antibiotics on the growth of lactobacilli

No	Antibiotic	Concentration, μg /disc	*Lactobacillus bulgaricus 144*	*Lactobacillus bulgaricus GB*	*Lactobacillus acidophilus*	*Lactobacillus acidophilus A*	*Lactobacillus acidophilus 2*	*Lactobacillus helveticus*	*Lactobacillus plantarum*	*Lactobacillus casei C*	*Lactobacillus bulgaricus LB-M*
1	penicillin	10*	+++	-	-	-	-	+++	-	++	+
2	ampicyline	10	+++	+++	-	-	+++	+++	+++	+++	+++
3	cefamndole	30	+	+++	-	-	++	+++	+++	-	++
4	nalidixic acid	30	+++	+	+	-	+++	+++	+++	+++	++
5	cyprofloxacin	5	+++	+++	+++	+	+++	+++	+++	+++	++
6	amoxicyline	25	+++	++	-	-	+	++	-	+++	+
7	tetracycline	30	+++	+++	-	-	-	+++	+++	+++	-
8	oxacyllin	1	+++	+++	+	-	+++	+++	+++	+++	-
9	doxicycline	30	+++	-	-	-	+	+++	-	-	-
10	gentamycine	10	-	+++	-	-	-	++	+++	++	-
11	canamycine	30	-	-	-	+	+++	+++	+++	+++	+++
12	lyncomycin	15	+++	+++	++	-	-	+++	++	-	-
13	tobramycin	10	+++	+++	++	++	+	+++	+++	+++	++
14	amycacin	30	+	-	+++	-	-	+++	+++	+++	+
15	vancomycin	30	+++	-	-	-	-	+++	+++	+	-
16	azlocylin	75	+++	-	+	-	-	+++	+++	-	++
17	piperacyline	100	++	-	-	-	+	++	+-	-	++
18	erythromycin	15	-	++	-	-	-	+++	+-	++	++
19	rifampin	5	+++	+++	+	-	+++	+++	+++	-	-
20	chloramphenicol	30	+++	+	-	-	+++	+++	+++	+++	+
21	streptomycin	30	+++	-	-	+	+++	+++	+++	+++	+
22	clindamycin	2	+++	+	-	-	+++	+++	+	+++	-

* The concentration is expressed in U/disc; - - no growth; + - single colonies in the clearance zone; ++ - weak growth; +++ - dense growth

shown in Table 6.

The acids, which are predominant in the culture medium of the lactobacilli, are the lactic, citric and tartic acids. *Lact. bulgaricus GB* transforms the carbohydrates into lactic (L-form) and tartic acid, and *Lact. bulgaricus 144 D* - into lactic acid only. *Lact. acidophilus 2* produces a significant amount of lactic, citric and tartic acid in the culture medium.

Antibiotic resistance of the lactobacilli

Antibiotics are substances with antimicrobial action, which influence both Gram-positive and Gram-negative bacteria. They inhibit the growth or destroy the microbial cells. In order to fulfill these functions, the antimicrobial substance must penetrate the cell, conjugate with a certain cell structure, which participates in the vital processes (DNA replication and cell division) or suppress them completely.

The effect of the antibiotics on the microbial cells is associated with the inhibition of the synthesis of the cell wall (inhibition of the synthesis of the peptidoglycan or the addition of other components - β-lactam antibiotics), the transcription and replication (rifamycin) and the formation of the polypeptide chains (aminoglicosides, tetracyclines, macrolides, chloramphenicol).

When applied, they destroy not only the harmful, but also the beneficial gut microflora. The effect of 22 antibiotics - β-lactam (penicillin, ampicyline, cefamndole, ciprofloxacin, amoxicyline, oxacyllin, piperacyline, azlocylin), amino-glicoside (streptomycin, gentamycine, canamycine, lyncomycin, clindamycin, amycacin, vancomycin, tobramycin), macrolide (rifampin, erythromycin), tetracycline (tetracycline, doxicycline), aromatic (chloramphenicol) and nalidixic acid, on the growth of the selected lactobacilli was studied. The concentrations investigated were the equivalent to the actual concentration *in vivo* during antibiotic therapy. The results in Table 7 show that the lactobacilli are resistant to antibiotics.

The growth of *Lactobacillus delbrueckii subsp. bulgaricus 144* was inhibited by the effect of macrolide and aminoglicoside antibiotics and the cells of *Lactobacillus delbrueckii subsp. bul-garicus GB*-by a larger group of antibiotics - some β-lactam, aminoglicoside and chloramphenicol (Table 7).

From the investigated *Lactobacillus acidophilus* strains *Lactobacillus acidophilus* and *Lactobacillus acidophilus* A demonstrated sensitivity towards all preparations, while the growth of *Lactobacillus acidophilus 2* was inhibited by most of the β-lactam and some of the amino-glicoside antibiotics (Table 7).

The selected *Lactobacillus helveticus* H strain was resistant to all of the applied antibiotics and the growth of the cells of *Lactobacillus plantarum 226-15* and *Lactobacillus casei subsp. casei C* was inhibited by some β-lactam antibiotics, erythromycinand doxicycline.

Discussion

The conducted research on the resistance of *Lactobacillus* strains from

the national gene fund to model conditions of digestion (low and neutral values of pH and pepsin and different concentrations of bile salts) reveals the heterogeneity of their biological properties. This on its turn shows the need for selection while choosing bacterial strains with probiotic properties. The obtained results are in conformity with the studies of Donohue, Salminen and Marteau (1998) and Gasser (1994) on the selection of lactobacilli with probiotic properties.

The different *Lactobacillus* strains varied in their resistance to pH, digestion enzymes and bile salts. Resistance is due to the characteristics of both the species and the strain itself. On this basis from the investigated *Lactobacillus delbrueckii subsp. bulgaricus* strains were selected the following: *Lactobacillus delbrueckii subsp. bulgaricus* NCIMCC 26, *Lactobacillus delbrueckii subsp. bulgaricus* NCIMCC 144 and *Lactobacillus delbrueckii subsp. bulgaricus* NCIMCC GB, from the *Lactobacillus acidophilus* strains - *Lactobacillus acidophilus* NCIMCC 2, as well as the strains *Lactobacillus helveticus* NCIMCC H, *Lactobacillus casei subsp.* casei NCIMCC C, *Lactobacillus plantarum* NCIMCC 226-15.

Some of the metabolites produced by the lactic acid bacteria are lactic, citric and other organic acids, through which they acidify the medium and inhibit the growth of the pathogens. Another group of substances with antimicrobial action are the bacteiocins, which have a protein nature.

The high inhibitory activity of the selected lactobacilli against pathogens and conditional pathogens from Enterobacteriaceae, the causing agents of toxicoin-fections and intoxications is determined by their ability to produce lactic, acetic and other organic acids and bacteriocins. These results conform the investigations of Kail et al. (1992) and Tanaka et al. (1994).

The high antimicrobial activity of the Lactobacillus strains is in conformity with the results of other authors (Kail et al., 1992; Tanaka and Ohwaki, 1994) and their conclusion that the growth of the enteropathogens in the digestive tract is inhibited mainly by the lactobacilli. Therefore, they are correctly defined as the main regulators of the balance of the microflora of the stomach and the intestines.

The resistance of the cells of the different *Lactobacillus* strains to most of the applied in medical treatment antibiotics reveals the possibility for their application in the cases of disbacteriosis. On the other hand, it is better to use strains with natural polyvalent resistance as components of probiotics for the treatment of disbacteriosis. Nevertheless resistance can be induces, the natural strains are preferred.

Conclusion

As a result of the conducted investigation of lactobacilli from the Bulgarian national gene fund were selected lactic acid bacteria strains, which conform to the requirements for probiotic cultures, defined by Salminen et al. (1998) and Enikova (2004). These strains are in the basis of the invented in Bulgaria probiotics with the trademark "Enterosan", which are registered in Geneva ac-

cording to the International Convention for the EU countries.

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