IN VITRO CHARACTERIZATION OF PROBIOTIC PROPERTIES OF LACTIC ACID BACTERIA FROM BULGARIAN RYE SOURDOUGHS

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ABSTRACT

Lactic acid bacteria play a key role in human health. These friendly bacteria as a part of the microflora of the gastrointestinal tract (GIT) have a beneficial influence on microbial balance. The probiotic cultures stimulate the growth of beneficial microorganisms, crowd out potentially harmful bacteria and reinforce the body's natural defense mechanisms. Recent data improve the positive effects of probiotics and stimulate research for discovering of new lactic acid bacteria strains with probiotic properties.

In present work the probiotic potential of 23 strains lactic acid bacteria, isolated from Bulgarian rye sourdoughs from different geographical regions was studied in vitro. Their affiliation to the genus Lactobacillus and Pediococcus was determined by classical phenotyping methods. Screening of the strains at selecting factors high acidity and different concentrations of bile salts was first accomplished. In addition, susceptibility to antibiotics, production of hydrogen peroxide and spectrum of antimicrobial activity were determined. After primary selection, 8 strains were chosen and they were tested in a model system for in vitro simulation of the conditions into the human GIT.

As a result, two strains with high probiotic potential were found.

Keywords: lactic acid bacteria, probiotic, sourdough

Introduction

Probiotics are defined as live microorganisms with beneficial influence on the host health (15). The use of lactic acid bacteria (LAB) as probiotics to enhance health and well-being has been proposed for many years (10). The probiotics play a key role in regulation of GIT (19). Diet, stress and infections disturb the microbial equilibrium in human body, which often leads to decrease in the population of lactobacilli and bifidobacteria (8). Uncontrolled proliferation of pathogens may lead to different clinical disorders (7). The LAB have significant importance in stabilization of the intestinal microflora and prevent the colonization of potentially harmful bacteria. Thus, they reinforce the body's natural defense mechanisms.

When selecting probiotics, different criteria have to be fulfilled. The probiotic cultures must be tolerant to acid and bile, which enables selected strains to survive, grow and perform its therapeutic benefits in the intestinal tract (13). Therefore, acid and bile tolerance is considered one of the most important properties of probiotic microorganisms. Production of antimicrobial substances such as lactic acid, hydrogen peroxide, bacteriocins is also among the criteria used to select probiotic microorganisms (9, 11, 20). The antibiotic susceptibility is a necessary characteristic for every pre-selected LAB strain (2).

The probiotic strains were isolated from food products such as raw milk and dairy products (14), fermented meat (17), as well as from human organisms (16). However, there is no data for probiotic potential of lactic microflora of rye sourdoughs. Our recent investigation on microflora of Bulgarian rye sourdoughs allowed isolation and characterization of large number LAB strains. In such complex microbial system we identified LAB related to the genera: *Pediococcus*, *Lactobacillus, Leuconostoc* and *Streptococcus* (21).

The probiotic properties of 23 strains isolated from

Bulgarian rye sourdoughs were characterized *in vitro* in present work. The aim was to determine the production of antimicrobial substances, antibiotics susceptibility and their resistance to low pH and elevated bile salt concentrations and to select putative probiotic strains.

Materials and methods

Microorganisms

Twenty three strains isolated from Bulgarian rye sourdoughs from different geographic regions were studied. The isolates were stored at -80°C in de Man, Rogosa and Sharpe medium (MRS, Merck, Germany) supplemented with 70% glycerol and pre-cultivated twice in appropriate media, before application in corresponding tests.

Resistance to low pH and bile salts

The tests were performed in modified MRS broth with pH 4 and MRS supplemented with 0,3; 0,5 and 1% (w/v) bile salts (Oxgall, Merck) for 24 h at 37°C, using a control- non-modified MRS with pH 6.5. The LAB growth was determined spectrophotometrically, by measurements of the optical density at 590nm at the 0, 1, 3, 6, 9 and 24 h. In addition, the viable cell count was determined by Koch's method at 0, 3 and 24 hour.

Test "*In vitro* digestion"

The resistance to specific conditions occurring on the GIT, such as digestive enzymes (pepsin, pancreatin), pH change from pH 2 in stomach to pH 8 in duodenum and to bile was evaluated in a model system simulating the conditions into GIT. The survival of the LAB isolates was studied in different nutrient media as followed: MRS (pH 2), MRS with 2% (w/v) bile salts, MRS (pH 2) with 0,3% (w/v) pepsin and MRS (pH 8) with 0,1% (w/v) pancreatin in different time points by scheme (**Table 1**).

TABLE 1

Scheme of samples collecting for determination of survivability of the isolates in test "*in vitro* digestion"

Medium	Time points for analysis							
	1min	60min	90min	180min	240min	6 h	24 h	48 h
MRS, pH 2	+	+	-	+	-	-	+	-
MRS with 2% oxgall	+	-	-	+	-	+	+	+
Pepsin (pH 2)	+	-	+	+	-	-	-	-
Pancreatin (pH 8)	+	-	-	-	+	-	+	-

Antimicrobial activity of LAB strains

The agar-well diffusion method (12) was applied to determine the antimicrobial activity of cell-free cultures of selected LAB strains. The exponential cultures of *Escherichia coli HB 101*(IMb, BAS), *Enterococcus sp.* (IMb, BAS), *Bacillus subtilis* ATCC 6633, *B. cereus* (NBIMCC); *Staphylococcus aureus* strain *Smith* (NBIMCC), used as test microorganisms, were inoculated (~10^5-10^7 CFU/ml) in appropriate agar media. In order to estimate the putative effects of lactic acid and other antimicrobials produced during the fermentation, the tests were performed with fresh (acid) and with neutralized to pH 6.2-6.5 (with 5M NaOH) cell-free filtrates from 24 h and 48 h cultures of each strains. The activity was expressed as diameter (mm) of obtained zone of inhibition.

Determination of hydrogen peroxide (H_2O_2) production

A qualititative method was applied, as described by Eschenbach (5). Single colonies of LAB strains were cultured at 37°C on Petri dishes with MRS-agar in the presence of 25mg/100ml 3, 3^1, 5, 5^1 – tetramethylbenzidine (TMB, Sigma) and 1mg/100ml horseradish peroxidase (Type I-Sigma 116U/mg, Sigma). After 48 hours incubation under anaerobic conditions, on short exposure at air oxygen the colonies producing H_2O_2 turn dark blue or brick-colored and the non producing H_2O_2 colonies remain white.

Determination of resistance to antibiotics

The antibiotic susceptibility was determined by the standard Kirby-Bauer disk diffusion method (1) with the following antibiotics: (i) *beta-lactam antibiotics*– penicillin (10 E/disk), ampicillin (10 mg/disk); (ii) *aminoglycosides*– gentamycin (10 mg/disk), streptomycin (10 mg/disk); (iii) *macrolides*– eritromycin (15 mg/disk), oxacillin (1 mg/disk); (iv) *tetracyclines*– tetracycline (30mg/disk); (v) *glycopeptides*– chloramphenicol (30 mg/disk); lincomycin (15 mg/disk).

Results and Discussion

In present study was evaluated the probiotic potential of 23 LAB strains from Bulgarian rye sourdoughs. The group of isolates was randomly selected, from recently created LAB collection (21).

Transit tolerance and viability in conditions simulated a passage in GIT

The currently available tests for the study of probiotic stains are not fully adequate to predict the functionality of probiotic microorganisms in the human body. Although, it was noted that *in vitro* bile salt resistance is in high degree of correlation with gastric survival *in vivo* (4). Thus, the resistance to gastric acidity and bile salts are between the most important *in vitro* tests in the list of WHO/FAO for the study of probiotic strains Guidelines (6). The first step in the probiotic evaluation of the group of LAB isolates was determination of their growth ability in MRS broth with pH 4 and MRS broth with 0.3, 0.5 and 1% (w/v) bile salts. The most of the strains showed good survival at the selecting conditions and the count of viable cells increase with time (**Fig. 1**). More detrimental for cells was the presence of bile salts (0.5%, 1% w/v) in the medium and the low pH**.** The behaviour of strains, monitored spectrophotometrically in above presented model media was fully confirmed by quantitative determination of vial cells number at 0 h, 3 h and 24 h. The strains brs2, brs19 and brs74 showed high sensibility at low acidity and bile salts, while the strains brs47, brs72 and brs75 not only washstand the prolonged action of these factors, but they were able to growth.

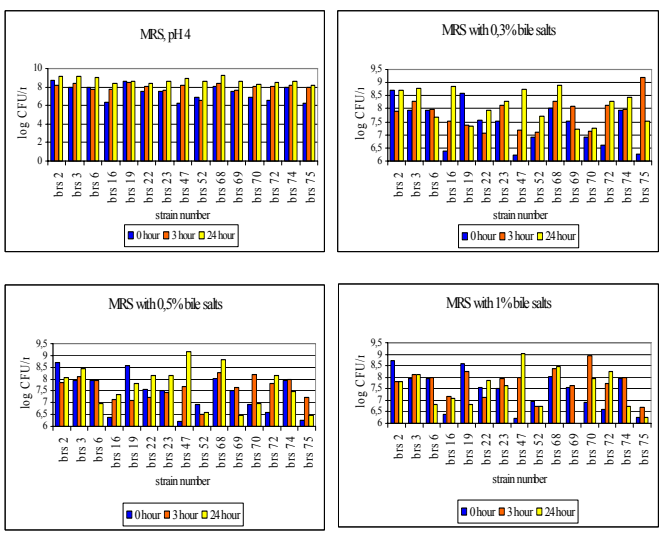

Fig. 1. Viability of lactic acid bacteria on MRS broth at 0, 3 and 24 hour after incubation by Koch's method.

Therefore eight strains brs3, brs6, brs16, brs22, brs47, brs52, brs72 and brs75, with high viability were selected and were subjected to "*in vitro* digestion" test. This test combines different factors affecting bacteria in GIT and allows estimation of their combine effect (**Fig. 2**).

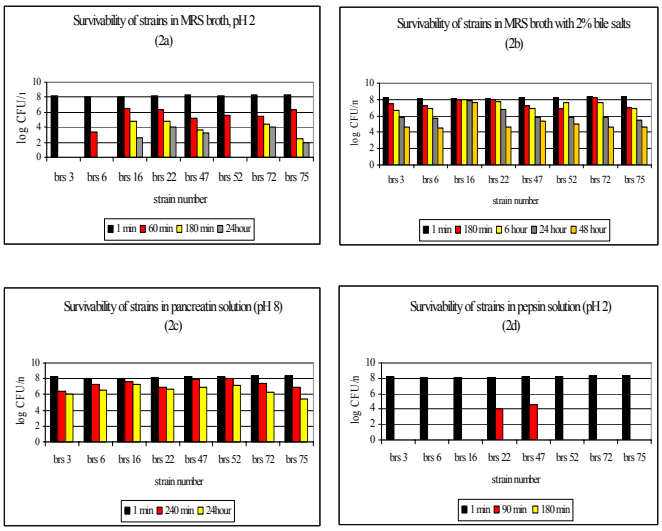

Fig. 2. "*In vitro* digestion" test of selected lactic acid bacteria from sourdoughs.

Firstly the viability of the strains in MRS broth (pH 2) was studied (**Fig. 2a**). Despite of extremely long period of incubation-24 h, the isolates showed high resistance to low pH value, especially the strains brs22 and brs72. The acid tolerant isolates were further evaluated for resistance at high bile salt concentrations (**Fig. 2b).** All strains were able to tolerant 2% bile salts till 48 h incubation. The behaviour of the isolates incubated in pancreatin solution was similar (**Fig. 2c**). The strains showed a considerable viability at tested enzyme concentration. However, the combination of acid medium (pH 2.0) and pepsin was lethal for most of the studied strains (**Fig. 2d**). At 90th min. exposure all of the isolates, excepting strains brs22 and brs47, showed absence of living cells**.** At 180th min a lack of viable cells was proved for both strains brs22 and brs47. However, their viability in such drastic conditions for 90 min. - as much as normal the food stay in the stomach, allowed to estimate them as probiotic strains. Survival of probiotics in the GIT is under intensive study in last years. Probiotic's viability depended on the pH, the length to exposure to acid and the species and the strains used. Bezkorovainy (3) report six *L. acidophilus* strains with good viability, when they are maintained at pH of 1,5-3,0 for ≤3 h.

Antimicrobial activity

Antimicrobial activity is very important criterion for selection

of probiotic strains which are natural antagonists to the potentially harmful bacteria. Therefore the antimicrobial activity of the strains was studied against bacterial species reported as food spoilage and pathogens. With this aim filtered cells-free supernatants (fresh and neutralized) from 24 h and 48 h cultures were collected. Higher activity, expressed in mm sterile zones were observed in the assay with 24-hours cultures against spore-forming – *B. subtilis* and *B. cereus*, while against *E. coli, Enterococcus sp.* and *S. aureus* more effective were the spent cultures from late stationary phase (48 h). Probably the high level of produced lactic acid, at the end of fermentation determined such inhibitory activity for all group. In order to eliminate the effect of organic acids the cell-free filtrates were neutralized to pH 6.2-6.5 and the agar-diffusion tests were repeated with the same test-microorganisms. There was no inhibition for all indicator strains, except strain brs47, which suppress the growth of *B. cereus*. In recent studies, Simsek (18) showed that *B. subtilis* ATCC 6633 causing ropiness in bread was inhibited by strains of *L. brevis ssp. lindneri 2103*, *L. acidophilus I5* and *Pediococcus sp. E5* isolated from sourdoughs. Inhibitory isolates also exhibited quite well antimicrobial activity against other members of *Bacillus* genus.

Production of hydrogen peroxide

LAB are producers of different antimicrobials, such as lactic and other organic acids, diacetyl, hydrogen peroxide, bacteriocins or bactericidal proteins etc, which allow their domination in different ecological niches. The hydrogen peroxide production of LAB strains was studied and positive results were obtained for strains brs3, brs6, brs22, brs52, brs70 and brs75. The hydrogen peroxide is one of the substances, inhibitory for many pathogens. Thus, the ability of H_20_2 production increases the probiotic potential of LAB strains.

Antibiotic susceptibility

WHO/FAO Working group recommends to be characterized each probiotic strains be determination of antibiotic resistance patterns, because the probiotic strain could accomplish one antibiotic therapy. In this aspect the antibiotic susceptibility of each selected strains is very important. The susceptibility of the sourdough LAB isolates to action of different groups antibiotics, widely used in clinical practice, was determined. All strains were resistant to streptomycin, oxacillin and β-lactame antibiotics penicillin and ampicillin. Most of the strains showed a low susceptibility to gentamycin. Observed susceptibility to eritromycin and chloramphenicol was significant. The strains were resistant to lincomycin, except the strains brs22, brs43 and brs72, which were strongly sensitive (inhibitory zone of 40 mm).

From 23 lactic acid isolates from Bulgarian rye sourdoughs, 8 strains-brs3, brs6, brs16, brs22, brs47, brs52, brs72 and brs75 showed high resistance and ability for growth at high acidity and at bile salt concentration. Test "*in vitro* digestion" was carried out and two strains-brs22 and brs47 showed good transit tolerance. The survivability of strains brs22 and brs47 in the model system of the GIT, their resistance to often utilized antibiotics and their antimicrobial activity toward *B. cereus, B. subtilis, Enterococcus sp., S. aureus* and *E. coli* determined them as potential probiotic strains. All of the tests applied require validation with *in vivo* performance.

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