SELECTED PROBIOTIC PARAMETERS OF LACTIC ACID BACTERIA ISOLATED FROM THE FAECES OF VEGETARIANS AND NONVEGETARIANS

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ABSTRACT

In this work were isolated about 80 strains of lactic acid bacteria from the faeces of young (21-30 years) and older (51-60 years) vegetarians and nonvegetarians. The identification of isolated strains was based on their morphological and biochemical properties. The following probiotic properties were determined: survival at low pH value, and bile salt hydrolase activity. The isolated strains belong to lactobacilli, bifidobacteria, enterococci and propionibacteria. All strains were negative in bile salt hydrolase activity, but the growth in the presence of bile was not inhibited. The results from the study of survival at low pH showed considerable variability in both dietary groups regardless the age of probands.

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Key words: gastrointestinal tract, probiotic bacteria, vegetarians, nonvegetarians

INTRODUCTION

The human gastrointestinal microflora represents an ecosystem of the highest complexity. In the gastrointestinal tract exists variability in bacterial numbers and populations. The human large intestine consists of 400-500 different cultivable species. The groups consist of different rods and cocci, such as bacteroides, bifidobacteria, eubacteria, lactobacilli, peptostreptococci, enterococci, coliforms, ruminococci, methanoges bacteria and acetogens. A number of different factors are able to affect the composition of the colonic microbiota, e.g. composition of the diet, aging, stress, health status, and environmental cicrcumstances (1-6).

Selected strains of the lactic acid bacteria (LAB) belonging mainly to the genera *Lactobacillus* and *Bifidobacterium* have been used many years in the food production, predominantly in the manufacture of fermented dairy products and have GRAS (Generally Recognized As Safe) status (3, 7, 8). In the two recent decades lactobacilli and bifidobacteria are being used as probiotics. Probiotics according to the World Health Organization (WHO) are defined as „live microorganisms which, when administered in adequate amounts, confer a health benefit on the host“ (1, 9). Criteria for the selection and assessment of probiotic lactic acid bacteria included: human origin, nonpathogenic behavior, resistance to technologic processes (ie, viability and activity in food or dietary supplements), resistance to gastric acidity and bile toxicity, adhesion to gut epithelial tissue, production of antimicrobial substances, ability to modulate immune responses, and ability to influence metabolic activities (eg, cholesterol assimilation, lactase activity, and vitamin production) (3, 7, 10, 11,12).

Numerous studies reported health-promoting properties of lactic acid bacteria in animals and humans. These properties include: enhancing the bioavailability of minerals (calcium, iron, manganese, copper and phosphorus), synthesis of several vitamins (folic acid, riboflavin, nicotinamide, pyridoxine, vitamin B_{12}), prevention and treatments of gastrointestinal disorders (diarrhea, gastrointestinal and urogenital infections), treatments of hypercholesterolaemia, and lactose intolerance, reduction in pro-carcinogenic enzymes, stimulation of the immune system, and treatment of food-related allergies (1, 3-6, 9, 13-18).

In this study, we isolated ca. 80 strains of lactic acid bacteria from the human faeces of vegetarians and meat-eaters. The strains were identified and tested for probiotic properties such as survival at low pH value, and bile salt hydrolase activity.

MATERIAL AND METHODS

Chemicals. Analytical grade chemicals were obtained either from Mikrochem, Slovakia, Lachema, Czech Republic, and Merck, Germany while bacteriological media were obtained from Merck, Germany, Biokar Diagnostics, France, or Oxoid, England respectively.

Microorganisms. Lactic acid bacteia (LAB) were isolated from the human faeces of 4 vegetarians and meat-eaters.

Strains isolation and cultivation. LAB were isolated from the human faeces by appropriate dilutions with saline, plated on MRS and Rogosa Bios agars and incubated anaerobically at 37°C for 2-3 days. Isolated colonies were picked up and transferred to MRS broth. They were propagated twice and streaked on MRS agar to check the purity of the isolates. All media were sterilised at 121°C for 20 min (19, 20).

Morphological and biochemical tests. Isolates from human faeces were identified according to the methods described in literature. The cultures were examined microscopically and morphological characteristics noted. Key features for identification were Gram-staining, catalase test, growth at 15 and 45°C, gas production from glucose in MRS-broth using Durham tubes, and determination of the sugar fermentation spectrum using API 50 L Lactobacillus identification system (BioMérieux, France) and ANAEROtest 23 (Pliva-Lachema Diagnostika, Czech Republic). For all tests bacterial overnight cultures were prepared. The lactic acid bacterial overnight cultures were prepared in MRS-broth at 37°C for 16-18 h (19, 20).

Tolerance of LAB to acid pH. For survival of isolates at low pH value MRS-broth was adjusted to pH 3,0 with hydrochloric acid, sterilised, and the survival determined at 37°C for 3 hours. The inoculation number of LAB was ca. 10^6 cfu/ml.

Bile salt hydrolase activity assay. Bile salt hydrolase activity was tested according to method of Daskevicz and Feighner. Bile salt plates were prepared with 0,5 % (wt/vol) of the sodium salt of taurodeoxycholic acid (TDCA) and inoculated with overnight culture by using a 10 µl culture. MRS agar plates were used as control. The plates were incubated anaerobically at 37°C for 72 h (21).

RESULTS AND DISCUSSION

Isolation and identification of strains

Lactic acid bacteria were isolated from the human faeces of probands involved in the project. The probands (n = 240) were divided according to the dietary patterns into groups of vegetarians (n = 136) and meat-eaters (n = 104). The group of vegetarians included vegans, lactoovovegetarians and semivegetarians. The probands were men and women at age from 21 to 60 years. Four samples of faeces were choosen for isolation of lactic acid bacteria. Two samples were from the age group of 21-30 years old probands: one sample labelled as ZVI from 23-years old vegetarian women (18 isolated strains), and the sample labelled as ZNI (19 isolated strains) from 26-years nonvegetarian women. The other two samples were from the age group of 51-60 years old probands: 54-years old vegetarian women, sample labelled as ZVII (19 isolated strains), and 51-year old nonvegetarian man, sample labelled as MNI (20 isolated strains).

The isolates were examined microscopically and morphological characteristics noted. The identification methods included

Gram-staining, catalase test, growth at 15 and 45°C, gas production from glucose in MRS-broth using Durham tubes, and determination of the sugar fermentation spectrum using API 50 L Lactobacillus identification system (BioMérieux, France) and ANAEROtest 23 (Pliva-Lachema Diagnostika, Czech Republic) (19, 20).

The isolated strains on the agar plates formed round, small or larger, white to creamy colonies with typical mild lactic acid aroma. All isolated strains were Gram-positive, catalase-negative, non-sporing rods, cocci or bifidobacteria, often arranged in star-like or „V" patterns, typically called „bifido" arrangements (19).

Total number of lactic acid bacteria of all 240 samples is summarised in Table 1. Lactobacilli and bifidobacteria counts in all age groups were nearly 5 or 6 logarithmic orders. Significantly higher amounts of lactobacilli and bifidobacteria were found in women. Surprisingly, the highest number of lactobacilli and bifidobacteria were found in women older age groups (41-50 or 51-60).

Tab. 1 Number of lactobacilli and bifidobacteria in faecal microflora of probands (log cfu/g)

Age group 21-30		
Faecal microflora	**NV**	**V**
Lactobacilli and bifidobacteria	6,03 ± 0,47	5,47 ± 0,34
Men	**NV**	**V**
Lactobacilli and bifidobacteria	5,87 ± 0,40	5,39 ± 0,20
Women	**NV**	**V**
Lactobacilli and bifidobacteria	5,78 ± 0,30	5,51 ± 0,10
Age group 31-40		
Faecal microflora	**NV**	**V**
Lactobacilli and bifidobacteria	5,26 ± 0,50	5,28 ± 0,40
Men	**NV**	**V**
Lactobacilli and bifidobacteria	4,97 ± 0,20	6,03 ± 0,50
Women	**NV**	**V**
Lactobacilli and bifidobacteria	5,59 ± 0,10	4,83 ± 0,47
Age group 41-50		
Faecal microflora	**NV**	**V**
Lactobacilli and bifidobacteria	5,58 ± 0,50	5,81 ± 0,31
Men	**NV**	**V**
Lactobacilli and bifidobacteria	4,80 ± 0,80	5,40 ± 1,20
Women	**NV**	**V**
Lactobacilli and bifidobacteria	6,60 ± 1,40	6,60 ± 1,70
Age group 51-60		
Faecal microflora	**NV**	**V**
Lactobacilli and bifidobacteria	5,90 ± 0,50	5,29 ± 0,54
Men	**NV**	**V**
Lactobacilli and bifidobacteria	5,00 ± 1,50	5,10 ± 1,40
Women	**NV**	**V**
Lactobacilli and bifidobacteria	6,50 ± 1,80	5,40 ± 2,12

Legend: V – vegetarians, NV – nonvegetarians; cfu – colony forming unit

The counts of lactic acid bacteria in four chosen faeces samples are summarised in Table 2. The results showed differences in the counts of LAB within the ages of probands, and their dietary patterns. Significantly higher amounts of LAB were demonstrated in nonvegetarians. Based on the informations from the dietary questionnaires, meat-eaters consumed lactic acid fermented products such as yogurt and cheese more frequently and in higher amounts as vegetarians.

Tab. 2 Total number of lactic acid bacteria in the samples ZVI, ZNI, ZVII and MNI

Sample of faeces	**Lactic acid bacteria (log cfu/g)**
ZVI	4,53 ± 0,12
ZNI	5,85 ± 0,20
ZVII	0,95 ± 0,20
MNI	4,30 ± 0,32

According to the morphological and biochemical tests and microscopic observation the isolated strains were included to the genera *Lactobacillus, Bifidobacterium, Lactococcus, Propionibacterium and Enterococcus*. The classification of isolated cocci to the genus *Enterococcus* was based on the typical colour of colonies on the Slanetz-Bartley agar (dark pink colonies with metallic sheen) (19).

Lactococci and enterococci predominated in the samples ZVI and ZNI, while almost exclusively lactobacilli and bifidobacteria in the samples ZVII and MNI (older group of volunteers) were identified. Classification of isolated strains to the species was performed by API 50 L Lactobacillus identification system and ANAEROtest 23 (Tab. 3).

Tab. 3 Lactic acid bacteria species identified in the samples ZVI, ZNI, ZVII and MNI

Identified species of LAB	Percentage of occurence
Sample ZVI	
Lactobacillus paracasei ssp. *paracasei*	13,3
Lactococcus lactis ssp. *lactis*	86,7
Sample ZNI	
Enterococcus sp.	68,5
Lactobacillus plantarum	10,5
Lactobacillus paracasei ssp. *paracasei*	10,5
Lactococcus lactis ssp. *lactis*	10,5
Sample ZVII	
Bifidobacterium breve	100
Sample MNI	
Bifidobacterium breve	30
Lactobacillus catenaforme	60
Propionibacterium ovidum	10

Surviving of lactic acid bacteria at low pH

The secretion of gastric acid constitutes the main human biological barrier against most ingested microorganisms. Health beneficial lactic acid bacteria must overcome this defense mechanism. Before reaching the large intestine, probiotic bacteria must survive transit throught the stomach (7, 8, 10, 11, 22).

The survival of lactic acid bacteria at low pH value is very important characteristic for application of those cultures as probiotics. Tolerance to acid pH simulate the conditions in the stomach. The survival of the isolated strains was tested in MRS-broth adjusted to pH 3,0 at 37°C during 3 hours. The inoculating number of LAB was ca. 10^6 cfu/ml. The results showed considerable variability in both dietary groups regardless the age of probands (Fig. 1-4).

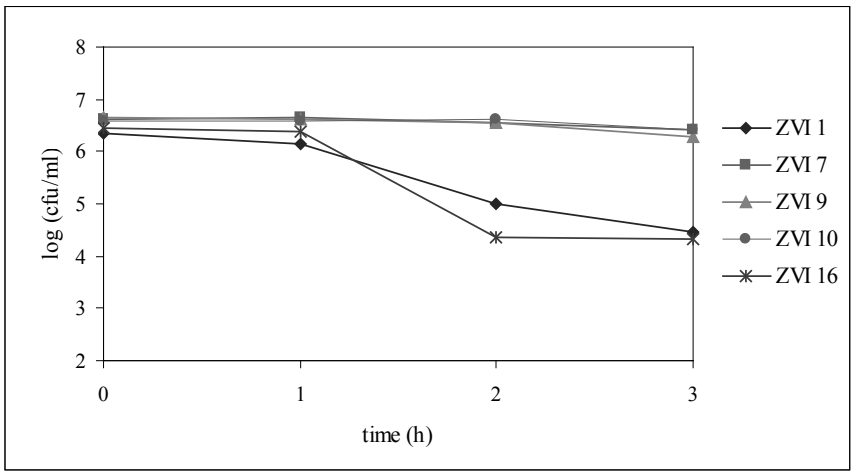

Fig. 1
Survival of the strains ZVI 1, ZVI 7, ZVI 9, ZVI 10, ZVI 16 in MRS-broth at pH 3,0 and 37 °C

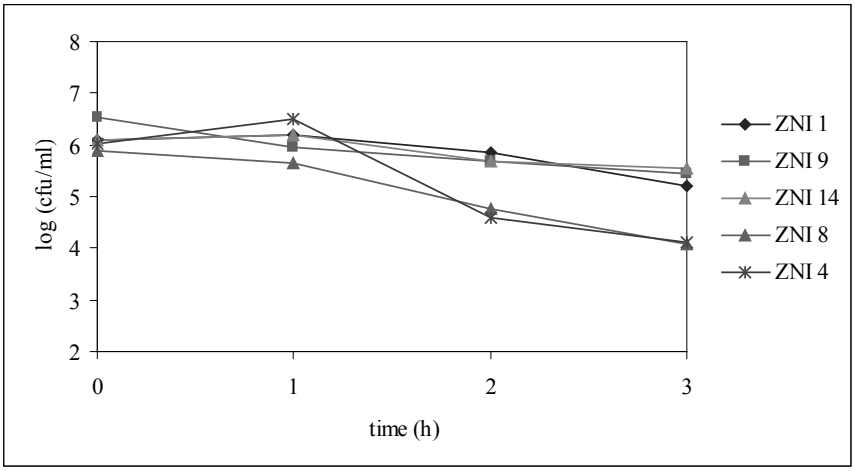

Fig. 2
Survival of the strains ZNI 1, ZNI 4, ZNI 8, ZNI 9, ZNI 14 in MRS-broth at pH 3,0 and 37 °C

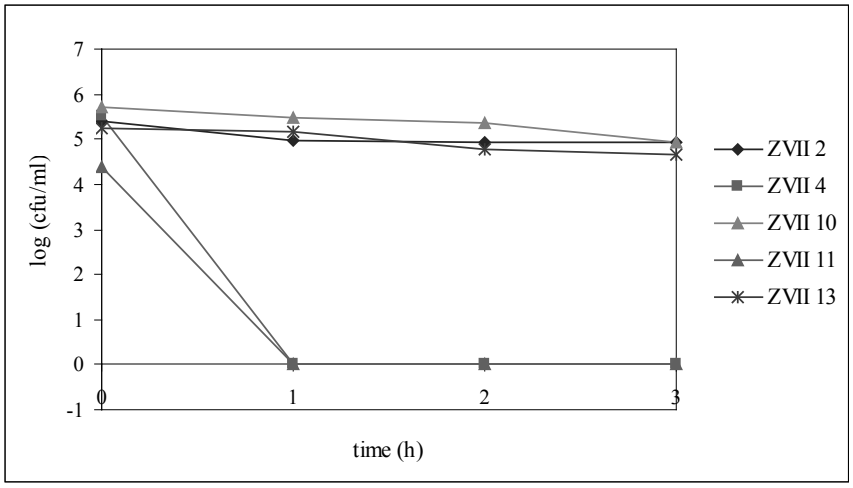

Fig. 3
Survival of the strains ZVII 2, ZVII 4, ZVII 10, ZVII 11, ZVII 13 in MRS-broth at pH 3,0 and 37 °C

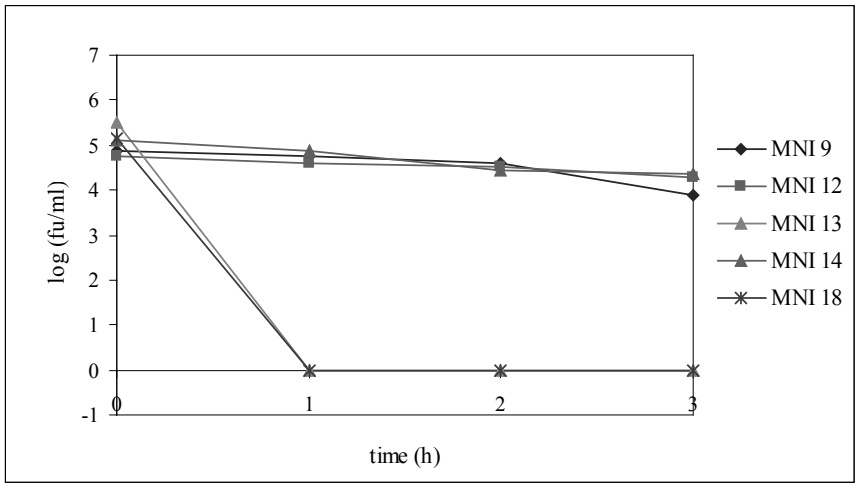

Fig. 4
Survival of the strains MNI 9, MNI 12, MNI 13, MNI 14, MNI 18 in MRS-broth at pH 3,0 and 37 °C

According to the results shown in Fig. 1, the strains ZVI 1- ZVI 16 survived at pH 3,0 very well, except the strains ZVI 1 and ZVI 16, their cells number decreased by 2 logarithmic orders. More sensitive to low pH value were the strains ZNI 1 – ZNI 19 (Fig. 2); in all tested isolates dropped the counts of bacteria by 1 or 2 log orders.

In older age group (Fig. 3 and 4) was the ability to survive at acid pH significantly lower. Only ca. 50 % of isolates from the faeces of 54-years old vegetarian women (isolates ZVII 1-ZVII 19) survived at pH 3,0 and 20 % isolates from the faeces of 51-years old nonvegetarian man (MNI 1- MNI 20). However, these strains showed low viability, they grown very slow and prepared overnight cultures did not reach the desired number of cells.

Nevertheless, strain viability and maintenance of desirable characteristics during product manufacture and storage is a necessity for probiotic strains. Ability to multiply rapidly is the important factor for technical application of the strain (3, 11, 12).

The obtained results suggest that most of these human isolates could successfully transit the human stomach and may be capable of reaching the colon. However, the bifidobacteria predominated in the group of older probands, proved less acid resistant than the lactobacilli and lactococci occured in the younger group. These results are in agreement with the data reported by Dunne et al. (7).

Bile salt hydrolase activity

Bile salt hydrolase (BSH) activity is a commonly observed phenomenon. It has been suggested, that the BSH enzyme might be a detergent shock protein that enables lactic acid bacteria to survive the intestinal bile stress. As such, BSH active bacteria may have a competitive advantage over other bacteria for surviving in the small intestine. The ability of strains to hydrolyze bile salts has often been included among the selection criteria for probiotic strain, and a number of bile salt hydrolases have been identified and characterized. However, recent data indicate, that microbial BSH activity could be potentially detrimental to the human host (23).

All isolated strains of LAB were tested for BSH activity on MRS agar plates supplemented with 0.5 % (wt/vol) TDCA. Bile salt hydrolase bacteria deconjugated taurine-conjugated bile salt producing deoxycholic acid. The deconjugation activity of bacteria is manifested a) in copious amounts of deoxycholic acid precipitated around active colonies and diffused into the surrounding medium, b) bile salt hydrolase-active strains produces opague white colonies without precipitate halos. Bile salt hydrolase-inactive strains produced similar colony types on plates with or without TDCA. The results showed, that hydrolysis of bile acid salts was negative in all studied strains, but the growth of strains in the presence of bile salt was not inhibited (21).

CONCLUSION

The human gastrointestinal microflora represents an ecosystem of the highest complexity, which may be influenced by many factors including diet composition. Health beneficial bacteria also called „probiotics“ include predominantly strains belonging to the genera *Lactobacillus* and *Bifidobacterium*. The amounts of these bacteria decrease with age particularly in the 7th and 8th decades of life. In

this study, lower numbers of lactobacilli and bifidobacteria in the dietary group of vegetarian were determined. Based on the informations from the dietary questionnaires, meat-eaters consumed lactic acid fermented products such as yogurt and cheese more frequently and in higher amounts than vegetarians. So far as is known, commercial probiotic bacteria have not been able to permanently colonize the human intestinal tract. Thus, regularly consumption of products with probiotics is recommended.

The first criterion for selection of probiotic bacteria is human origin of the strain. This is based on the observation, that bacterial species present in the intestinal flora could have a better chance of survival in their native environment. Moreover, higher portion of strains of human origin were found to be tolerant to low pH and high bile in comparison to other strains.

A competitive component of lactic acid bacteria against several bacteria is production of metabolic compounds (including organic acids, fatty acids, hydrogen peroxide, diacetyl, and bacteriocins) with antimicrobial effects. In many studies, inhibitory effects of several probiotic bacteria against a range indicator bacteria, including strains of *Listeria*, *Bacillus*, *Clostridium, Staphylococcus, Clostridium*, *Pseudomonas*, *Salmonella, Campylobacter, Candida, Enterococcus*, *Enterobacter, Streptococcus*, and *Escherichia coli* were demostrated.

Safety assessment is also an essential phase in the selection and evaluation of probiotics. Large number of *Lactobacillus* and *Bifidobacterium* strains have GRAS (Generally Recognized As Safe) status due to their long history of safe use in foods. Nevertheless, it is important to confirm the safety of novel probiotic cultures. European Food Safety Authority and WHO have made recommendations focused on the assessment of the safety of probiotics. These recommendations refer to the use of probiotics as dietary supplements in the wider „healthy“ population and cannot be directly applied to the use of probiotics in a hospital setting.

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