African Journal of Microbiology Research Vol. 1 (6) pp. 065-078, November 2007
Available online http://www.academicjournals.org/ajmr
ISSN 1996-0808 ©2007 Academic Journals

Full Length Research Paper

Characterization of selected strains from *Lactobacillus acidophilus* and *Bifidobacterium bifidum*

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Accepted 31 October, 2007

The presented study aims to characterize *Lactobacillus acidophilus* DSM 20079 and DSM 20242 and *Bifidobacterium bifidum* DSM 20082, DSM 20215, DSM 20239 and DSM 20456 strains. Within the framework of the performed investigations, the author evaluated their biochemical properties, resistance to antibiotics, antagonism towards selected pathogens as well as the effect on their survivability of different environmental conditions with special emphasis on the conditions prevailing in the human gastrointestinal tract. The performed experiments revealed significant differences between the examined bacterial species as well as between strains of the same species. All the examined strains were characterized by diverse biochemical properties, resistance to antibiotics and showed antagonistic action against *Helicobacter pylori* bacteria. The tested strains of the *L. acidophilus* bacteria were found to exhibit antagonistic activity against *Escherichia coli* and *Salmonella enteritidis* bacteria, although the observed activity was smaller in comparison with the antagonistic activity against *H. pylori*. These data confirm reports about the antagonistic effect of probiotic bacteria on the growth of pathogenic bacteria. All the examined bacterial strains fulfil the basic criterion expected from probiotic strains, that is, are capable of surviving in the '*in vitro*' conditions of the gastrointestinal tract, at low pH and in the presence of bile salts.

Key words: *Bifidobacterium bifidum*, *Lactobacillus acidophilus*, probiotic, antibiotic resistance, acidity resistance, bile tolerance, antagonistic activity.

INTRODUCTION

Widespread interest in probiotic bacteria that can be observed nowadays results from their medicinal properties reported both for human and animal subjects. It is worth stressing, however, that such positive properties are observed only for certain strains.

Probiotics have been employed in the feeding of farm animals such as pigs, poultry, ruminants as well as fish for a long time and they were expected to replace antibiotics or supplement their use. In addition, numerous literature data reported beneficial effects of probiotic bacteria on the human organism. Probiotic bacteria are characterized by numerous antagonistic traits in relation the gram (+) and gram (-) bacteria, including pathogenic bacteria. The mechanism of this process consists in the competition for the place of adhesion to the epithelium of the gastrointestinal tract, struggle for nutrients, stimulation of the resistance of the organism and production of antibacterial substances. Substances which trigger off the so called non-specific inhibition of pathogen development are, primarily, lactic and acetic acids, hydrogen peroxide and bacteriocins (Bielecka et al., 1998_a). It is also assumed that probiotic bacteria exhibit an activity in controlling *Helicobacter pylori*, that is, bacteria responseble for chronic gastric and duodenal ulcer diseases. *In vivo* and *in vitro* experiments on mice confirmed a considerable influence of *Lactobacillus acidophilus* on the inhibition of the development and reduction of survivability of *H. pylori* (Coconier et al., 1998). Lactic acid bacteria as well as products in which they occur show anti-carcinogenic action (Fooks et al., 1999; Hirayama and Rafter, 2000; Raftel, 2003). Lactic acid bacteria take part in alleviating symptoms of lactose intolerance (Kamaly, 1997; Fooks et al., 1999; Zubillaga et al., 2001). Probiotic bacteria are also believed to reduce the risk of the occurrence of bacterial intestinal disorders and prevent diarrhoea (McNaught and MacFie, 2001; Wilcox, 2003). More

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over, they are characterized by anti-cholesterol activities which consist in the capability of these cultures to assimilate this compound (Fooks et al., 1999; McNaught and MacFie, 2001). Among functions which exert a beneficial influence on the human organism is their capacity to activate and enhance the immunological system of the host which increases the resistance of the organism to infections (Gill, 1998; Hoerr and Bostwick, 2000). Lactic acid bacteria are also known to play an important role in the prevention of osteoporosis and caries and the attenuation of allergic reactions. This explains the increasing interest in probiotics by manufacturers of functional food articles with the aim to enrich the natural microflora of the gastrointestinal tract.

Bacteria which constitute part of functional food articles must be capable of settling the gastrointestinal tract of the host. It is, therefore, reasonable to carry out initial selection of probiotic bacterial strains on the basis of their resistance to the unfavorable physiological factors in the gastrointestinal tract (Holzapfel et al., 1998). Survival time is important in the selection of bacteria strains to be used as probiotic adjuncts (Bolin et al., 1997). According to the guidelines for the evaluation of probiotics in food reported by a Joint FAO/WHO working group, two of the currently most widely used *in vitro* tests are resistance to gastric acidity and bile salts, as based on the both survival and growth studies (Pinto et al., 2006).

This study undertook an attempt to elaborate the environmental characterization of selected bacteria from the *L. acidophilus* and *Bifidobacterium bifidum* species.

MATERIALS AND METHODS

Bacterial strains and growth conditions

The following six bacterial strains were used in this investigation: *L. acidophilus* (DSM 20079 and DSM 20242) and *B. bifidum* (DSM 20082, DSM 20215, DSM 20239 and DSM 20456), which were obtained from a museum collection of strains from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). The following bacterial strains were used to investigate the antagonistic properties of the above-mentioned strains of bacteria: *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *H. pylori* DSM 4867, *Salmonella enteritidis* SL 5319 as well as the test strain of the *L. acidophilus* ATCC 4356 bacteria. The above mentioned strains were obtained from the American Type Culture Collection (ATCC), DSMZ and the National Institute of Hygiene Collection in Warsaw, Poland (NIH).

L. acidophilus was grown in MRS medium (Merck) and *B. bifidum* in Medium 58 (DSMZ) anaerobically at 37ºC. The inoculum of the tested bacteria was prepared from strains stored on a substrate with glycerol at the temperature at -70°C using appropriate media. Bacteria were proliferated for 24 hours and further culturing was carried out after increasing the volume of the medium for the next 48 hours anaerobically at the temperature at 37°C. The cells of the strains cultured in medium, were collected by centrifugation (15 min; 5000 × g; at 4°C) and suspended in sodium chloride solutions (0.85 g/100 ml).

Bacteria prepared in this way provided the inoculum which was used at the amount of 10% (v/v). The number of live (cfu/ml) bacteria was determined using the Koch's plate method.

Characteristics of properties of potentially probiotic bacteria

The performed experiments aimed at characterizing the capability to metabolize saccharides, resistance to antibiotics, antagonistic effect of bacteria against selected microorganisms, resistance to bile salts and survivability in the environment with different pH of all 6 strains of potentially probiotic bacteria.

The biochemical profile of *Lactobacillus and Bifidobacterium* strains

Carbohydrate assimilation profiles were obtained by commercial test API 50 CHL (bioMérieux, Warsow, Poland). The tubes (49 carbohydrates) were felt with the inoculated API 50 CHL Medium with *L. acidophilus* or *B. bifidum* and incubated anerobically at 37ºC for 48 h.

Antibiotic resistance test (Kędzia and Koniar, 1980; Zhou et al., 2005)

Bacteria sensitivity to antibiotics was determined using the ring method (ring diameter – 6 mm) which consisted in the diffusion of the antibiotic into the substrate overgrown with the bacterial strain. Strains were tested with regard to their sensitivity to gentamicin (10 µg), kanamycin (30 µg), neomycin (30 µg), streptomycin (10 µg), erythromycin (15 µg), nitrofurantoin (300 µg), penicillin G (10 units), ampicillin (10 µg), carbenicillin (100 µg), colistin sulphate (10 µg), vancomycin (30 µg), nalidixic acid (30 µg), rifamicin (5 µg), tetracycline (30 µg), oxytetracycline (30 µg), chloramphenicol (30 µg) (Mast Diagnostics, Mast Group Limited, Merseyside, U. K.). Plates were incubated at 37ºC for 48 h in conditions suitable for the tested strain of bacteria. Zones of inhibition were measured in millimetres.

Antagonistic action of tested bacteria on selected bacteria-agar slab techniques (Strus, 1998; Strus et al., 2001)

The bacteria chosen as indicators were: *E. coli* ATCC 25922, *S. aureus* ATCC 25923, *H. pylori* DSM 4867, *Salmonella* SL 5319 and *L. acidophilus* ATCC 4356. The indicator bacteria of *Escherichia*, *Staphylococcus*, *Salmonella* species after culturing in a suitable broth medium at the temperature of 37°C, aerobic conditions for 24 h were next incubated again for 4 – 8 h. The concentration of bacteria after culturing was brought to the density of 0.5 in McFarland's scale. The *Helicobacter* bacteria were incubated using appropriate atmosphere generators of the bioMérieux Company for 48 - 72 h on the substrate with agar and then they were suspended in the physiological solution of NaCl bringing the density of the suspension to 3.0 in McFarland's scale. 100 µl of the inoculum of each strain was spotted on Mueller-Hinton agar plates for *Escherichia*, *Staphylococcus*, *Salmonella* and Brucella with the addition of human blood, hemin and vitamin K_1 for *Helicobacter*. The examined antagonistic strains were incubated at the temperature at 37°C, anaerobic conditions for 24 h. At the end of the incubation time, cylinders of the constant diameter of 10 mm were cut out from agar plates. Two cylinders derived from cultures of different antagonistic bacteria were transferred onto plates with swabs of indicators strains prepared earlier. The plates were placed in a refrigerator for 4 h at the temperature at 4°C and later incubated at the temperature of 37°C in either aerobic or anaerobic conditions, depending on the requirements of the indicator species. After incubation, the diameter of the growth inhibition of the indicator strain was measured and the result was given in mm including the diameter of the cylinder itself.

Table 1. List of sugars and their derivatives (API 50CHL) metabolised at least by one of the examined bacterial strains.

Substrate	*Lactobacillus acidophilus*		*Bifidobacterium bifidum*			
	DSM 20079	DSM 20242	DSM 20082	DSM 20215	DSM 20239	DSM 20456
glycerol	-	-	+	-	-	+
D- ribose	-	-	+	-	+	+
D- galactose	-	+	+	+	+	+
D- glucose	+	+	+	+	+	+
D- fructose	+	+	+	+	+	+
D- mannose	+	+	-	+	+	+
D- mannitol	-	-	-	-	-	+
N-acetyl-glukosamine	+	+	+	+	+	+
amygdalin	+	+	-	+	+	+
arbutin	-	+	-	+	+	+
esculin	+	+	+	+	+	+
salicin	+	+	-	+	+	+
cellobiose	+	+	-	+	+	+
D- maltose	+	+	+	+	+	+
D- lactose	+	+	+	+	+	+
D- sucrose	+	+	+	+	+	+
D- trehalose	+	+	+	+	+	+
raffinose	-	+	-	-	-	-
gentiobiose	+	+	+	+	+	+
D- turanose	-	+	+	-	-	-

Acidity resistance test

The survivability of bacteria in the environment characterized by different pH was determined in the substrate by regulating and stabilizing its pH to the following values: 2; 3; 4; 5; 6; 7; 8. The applied bacterial inoculum contained 10^9 cfu/ml. The number of live bacteria (cfu/ml) was measured at definite time intervals until no live bacteria were found in the medium. The substrate pH was regulated using 1 M HCl and 0.5 M NaOH, while its stability was maintained using Titrisol (Merck) buffers of pH= 2; 3; 4; 5; 6; 7; 8. The test was replicated twice.

Bile tolerance of cultures (Gopal et al., 1996)

In this experiment, oxgall (Difco) was used as the equivalent of bile. The following concentrations of oxgall in the medium were used: 0, 0.1, 0.2, 0.3 and 0.4% (w/v) and they corresponded to tenfold higher concentrations of bile. The added bacterial inoculum was of the 10^7 cfu/ml order in the amount of 10% (v/v) of the medium and the cultures were incubated anaerobically at 37ºC for 8 h.

Bacterial growth was measured spectrometrically at 620 nm at 2 h intervals during the 8 h incubation period. Appropriate controls (MRS and Medium 58 without culture) were used as a reference blanc. To quantify inhibition of cultures by bile, a coefficient of inhibition was calculated, according to the formula:

$$C_{inh} = (A_{620\,nm}\ \text{control} - A_{620\,nm}\ \text{oxgall}) : A_{620\,nm}\ \text{control}$$

Where: $A_{620\ nm}$ control- optical density of the culture broth without oxgal. $A_{620\ nm}$ oxgall- optical density of the broth containing oxgall, measured at the same time. (Gopal et al., 1996):

Simultaneously, every two hours, the number of live bacteria (cfu/ml) was determined. The test was replicated three times.

Statistical assessment

All the bacteria for each strain were prepared in three replications and the results are mean values from these repetitions. In the course of the performed statistical analysis of results with the assistance of the Excel 2000 software, all the experimental designs were analyzed employing mean descriptive statistics, correlation coefficient and single-factorial analysis of variance for $p<0.05$.

RESULTS

Capability to metabolize saccharides

After 48 h of incubation all tested strains assimilated glucose, fructose, N-acetyl-D-glucosamine, maltose, lactose, sucrose, trehalose and gentiobiose. In addition all *B. bifidum* strains assimilated galactose. Galactose was assimilated also by *L. acidophilus* DSM 20242. Table 1 presents the list of sugars and the fermented derivatives by at least one of the characterized strains. The performed tests showed that none of the strains fermented the following substances: erythritol, D i L- arabinose, D i L- xylose, D- adonitol, β metyl-D-xyloside, L- sorbose, L-rhamnose, dulcitol, inozitol, D- sorbitol, α-metylo-D-mannosidase, α-metylo-D-glucoside, D-melibiose, inuline, D-melezitose, starch, glycogen, xylitol, D- xylose, D- tagatose, D- fucose, D and L- arabitol, gluconate, 2-keto-gluconate i 5-keto-gluconate. Apart from the fermentation of galactose, the biochemical properties of the *L. acidophillus* and *B. bifidum* species differed as to the fermentation of amygdalin, salicin, celobiose. These saccharides were

Table 2. Susceptibility of *Lactobacillus* and *Bifidobacterium* strains to antibiotics Size of inhibition areas of growth of the examined strains by selected antibiotics [mm± SD].

Antibiotic (concentration)	*Lactobacillus acidophilus*		*Bifidobacterium bifidum*			
	DSM 20079	DSM 20242	DSM 20082	DSM 20215	DSM 20239	DSM 20456
gentamicin (10µg)	0	0	0	0	0	8,60±0,85
kanamycin (30µg)	0	0	0	0	0	8,00±0,49
neomycin (30µg)	0	0	0	0	0	10,75±1,02
streptomycin (10µg)	0	0	0	0	0	11,63±0,97
erythromycin (15µg)	24,63±0,30	23,58±0,70	0	0	17,58±0,45	30,17±0,56
nitrofurantoin (300µg)	0	0	10,17±1,09	0	0	0
penicillin G (10 units)	25,73±0,26	32,80±0,71	8,05±0,53	0	0	40,00±0,33
ampicillin (10µg)	26,83±0,67	28,88±1,07	12,25±0,61	0	18,00±1,63	33,47±2,46
carbenicillin (100µg)	28,43±0,88	35,38±0,56	0	0	0	41,85±0,45
colistin sulphate (10µg)	0	0	0	0	0	0
vancomycin (30µg)	0	0	0	0	0	0
nalidixic acid (30µg)	0	0	15,33±1,68	0	0	0
rifamicin (5µg)	14,33±0,43	15,60±0,36	0	0	42,30±0,24	18,40±0,71
tetracycline (30µg)	0	19,25±0,90	0	0	0	30,05±0,80
oxytetracycline (30µg)	0	17,43±0,87	0	6,30±0,16	29,60±0,3	29,53±0,74
chloramphenicol (30µg)	22,38±0,76	25,05±0,37	0	0	22,00±1,22	27,17±1,99

fermented by strains from the *L. acidophilus* species and all species from the *B. bifidum* species with the exception of the DSM 20082 species. No identical strains from the point of the possibilities of utilizations of the saccharide substrate were found among the examined strains.

Resistance to antibiotics

One of the important features of probiotic strains is their resistance to antibiotics, especially when they are to be used after antibiotic therapy. The author found differentces between the experimental strains in their resistance to antibiotics and the obtained results are presented in Table 2. The DSM 20215 strain of *B. bifidum* turned out to be the most resistant to the action of antibiotics as it showed only slight sensitivity to oxytetracycline. Therefore, this strain might be useful in treatments following antibiotic therapies. All the tested strains failed to exhibit sensitivity to two peptides: colistin and vancomycin. On the other hand, the DSM 20456 strain of *B. bifidum* turned out to be the most sensitive one to the tested antibiotics – it was sensitive to 12 out of 16 tested antibiotics. From among the strains of the *L. acidophilus* bacteria, the DSM 20242 strain turned out to be more sensitive since, in comparison with the DSM 20079 strain, it showed sensitivity to the antibiotics from the tetracycline group. In addition, the DSM 20242 strain exhibited higher sensitivity to the action of the remaining antibiotics which inhibit the growth of both strains. The only exception was erythromycin in which case the area of growth inhibition of the *L. acidophilus* DSM 20079 amounted to 24.63 mm, whereas of the *L. acidophilus* DSM 20242 – to 23.58 mm.

Both *L. acidophilus* strains were resistant to colistin, gentamicin, kanamycin, nalidixic acid, neomycin, nitrofurantoin and streptomycin. Additionally, *L. acidophilus* DSM 20079 was also resistant to oxytetracycline and tetracycline.

Antagonistic effect of bacteria on selected microorganisms

Four species of pathogenic microorganisms as well as one model of probiotic bacteria were tested. The experiments were conducted in three replicates and identical results were obtained for each replicate which are shown in Table 3. The areas given in mm include the size of the cylinder with the examined strain – 10 mm. The performed experiments showed the strongest inhibitory effect of the tested bacteria on the growth of *H. pylori*. The growth inhibition zone of these bacteria by the *B. bifidum* strains reached 22 mm, while that of the *L. acidophilus* strains was bigger and amounted to 26 mm. Both strains of the *L. acidophilus* revealed a strong inhibitory impact on *S. enteritidis* and *E. coli*. What is somewhat worrying is the observed inhibitory effect of the DSM 20242 strain of *L. acidophilus* on the growth of *L. acidophilus* ATTC 4356 bacteria considered as a probiotic bacterium. In addition, the DSM 20242 strain of *L. acidophilus* inhibits

Table 3. Size of inhibition areas of growth of selected indicator strains by examined bacterial strains [mm (together with the diameter of cylinder)]

Bacteria strains	*Lactobacillus acidophilus*		*Bifidobacterium bifidum*			
	DSM 20079	DSM 20242	DSM 20082	DSM 20215	DSM 20239	DSM 20456
Escherichia coli ATCC 25922	17	17	10	11	12	10
Staphylococcus aureus ATCC 25923	12	14	10	10	12	10
Helicobacter pylori DSM 4867	26	26	22	22	22	22
Salmonella enteritidis SL 5319	23	25	10	11	12	11
L. acidophilus ATCC 4356	10	17	10	10	10	10

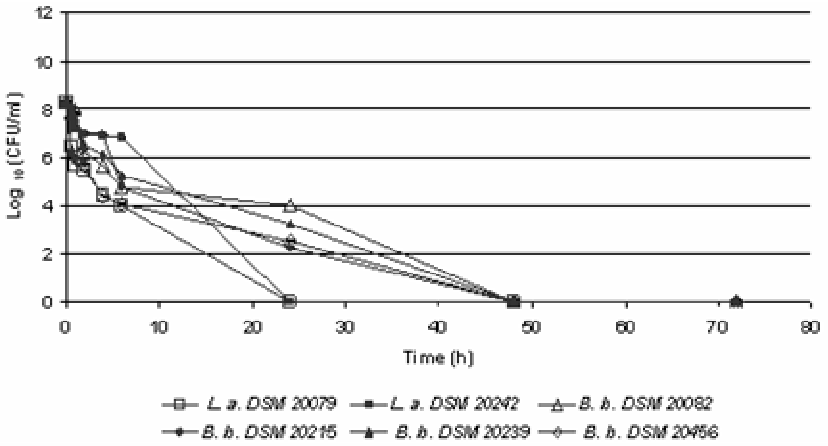

Figure 1. Survival rate of *Lactobacillus acidophilus* DSM 20079, *Lactobacillus acidophilus* DSM 20242, *Bifidobacterium bifidum* DSM 20082, *Bifidobacterium bifidum* DSM 20215, *Bifidobacterium bifidum* DSM 20239 and *Bifidobacterium bifidum* DSM 20456 in the medium of pH = 2

slightly the growth of *S. aureus*. It is evident from the data in Table 3 that strains from the *B. bifidum* species exhibit distinctly weaker properties inhibiting the growth of pathogenic bacteria. The performed tests of the antagonistic effect of the examined bacteria showed differences between the *L. acidophilus* and *B.bifidum* species but these differences were considerably smaller between the examined strains of individual species of probiotic bacteria.

Survivability in different pH environment

When characterizing the capability of the selected strains to survive in conditions of the gastrointestinal tract, we should not forget about the variable pH environment. Bacteria of the *B. bifidum* species of all the tested strains are more resistant to variable pH environment than those of the *L. acidophilus* and live longer than the *L. acidophilus* bacteria, although in the case of the environment with pH 5 and 6, these differences are less visible (Figures 1 - 7). It should also be emphasized that in the variable pH environment, differences between strains in the bacterial survivability become more apparent. In the environment with pH 2 and 3, bacteria of both strains of *L. acidophilus* survive up to the 24th h of culturing (Figures 1 and 2). Analyzing the level of live cells during the last hour, it can be concluded that *B. bifidum* DSM 20215 was the strain which was the most sensitive to the low pH of the environment of all the *B. bifidum* strains. The bacteria were found to live longer, especially in the environment whose pH was 4, 5 and 6. The *L. acidophilus* DSM 20079 bacteria lived longer in the environment with the pH of 5 and 6 than the *L. acidophilus* DSM 20242 (Figures 4 and 5). Free bacteria of both of these strains behaved differently in the environment of pH 4 (Figure 3). The *L. acidophilus* DSM 20079 strain turned out to be more sensitive. What is equally important, significant differences in the surviva-bility of free bacteria in the environment with pH 4, 5 and 6 was also observed for the individual strains of *B. bifidum* (Figures 3-5). The most favorable pH of the environment for all strains of the *B. bifidum* bacteria was 6. In the environment of pH 7 and 8, *L. acidophilus* bacteria achieved the value of 0 in the 144th hour of culturing in the case of the DSM 20079 strain and in the 168th hour of culturing – in the case of strain DSM 20242 (Figures 6 and 7). The lifetime of individual strains of the *B. bifidum* bacteria in the environment of pH 7 and 8 varied but it was sufficiently long (Figures 6 and 7). The most favorable pH for the development of the tested bacteria was

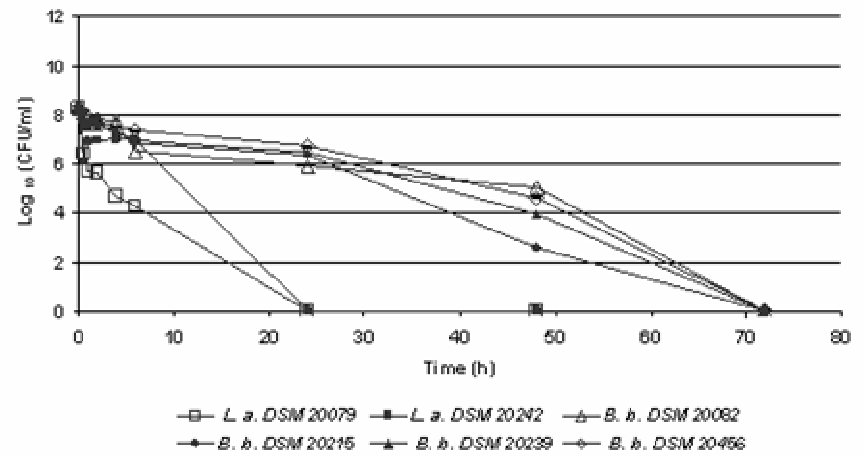

Figure 2. Survival rate of *Lactobacillus acidophilus* DSM 20079, *Lactobacillus acidophilus* DSM 20242, *Bifidobacterium bifidum* DSM 20082, *Bifidobacterium bifidum* DSM 20215, *Bifidobacterium bifidum* DSM 20239 and *Bifidobacterium bifidum* DSM 20456 in the medium of pH=3

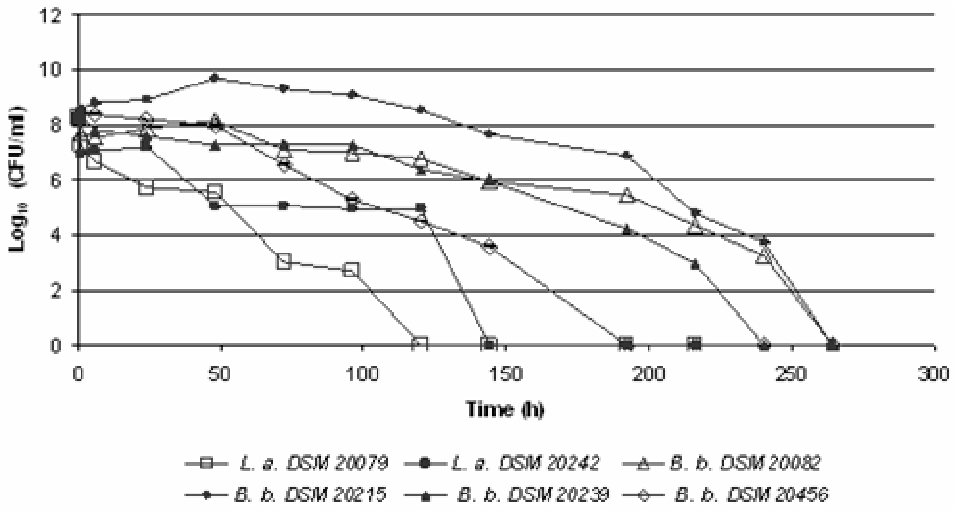

Figure 3. Survival rate of *Lactobacillus acidophilus* DSM 20079, *Lactobacillus acidophilus* DSM 20242, *Bifidobacterium bifidum* DSM 20082, *Bifidobacterium bifidum* DSM 20215, *Bifidobacterium bifidum* DSM 20239 and *Bifidobacterium bifidum* DSM 20456 in the medium of pH= 4

the environment in which pH ranged from 4 to 8. The death of bacterial cells in those conditions was caused by the lack of nutrients in the media rather than the level of their pH. It is quite probable that the replenishment of nutrients would improve the survivability of *L. acidophilus* and *B. bifidum* bacteria in these pH environmental conditions.

Resistance to bile salts

Tolerance to bile allows lactic acid bacteria to survive in the small intestine. At the beginning of the performed experiments the bacterial count was at the level of 10^6 cfu/ml and after 8 hours of incubation at the temperature of 37°C, the number of live *L. acidophilus* DSM 20079 and *L. acidophilus* DSM 20242 bacteria increased by one order of magnitude, that is, to the level of 10^7 cfu/ml for the substrates with the addition of 4% bile (Figures 8 and 9). In the case of the *B. bifidum* bacteria culture, the increase in the number of live cells occurred only in the media with the 1% addition of bile and in the case of the *B. bifidum* DSM 20456 strains, also in the case of the medium with 2% addition of bile (Figures 10 - 13). The increased absorbance was positively correlated with the

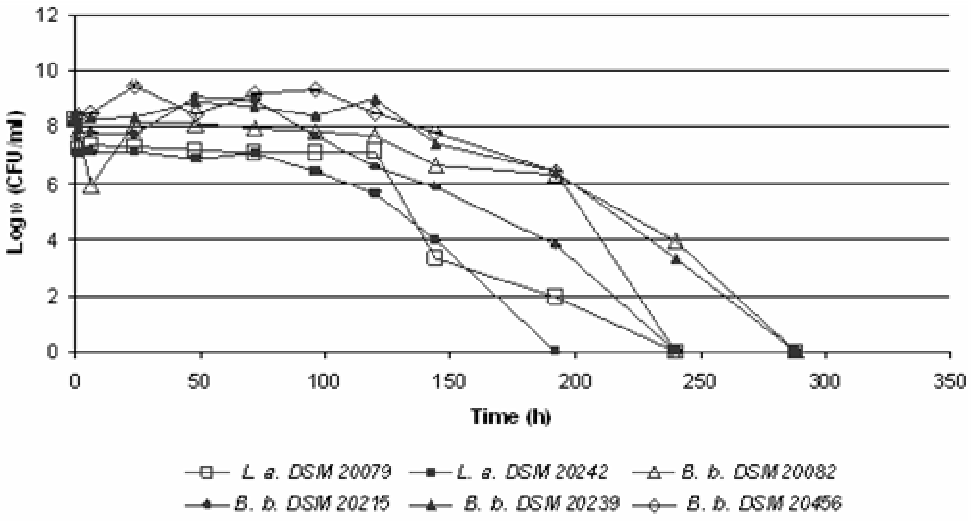

Figure 4. Survival rate of *Lactobacillus acidophilus* DSM 20079, *Lactobacillus acidophilus* DSM 20242, *Bifidobacterium bifidum* DSM 20082, *Bifidobacterium bifidum* DSM 20215, *Bifidobacterium bifidum* DSM 20239 and *Bifidobacterium bifidum* DSM in the medium of pH=5

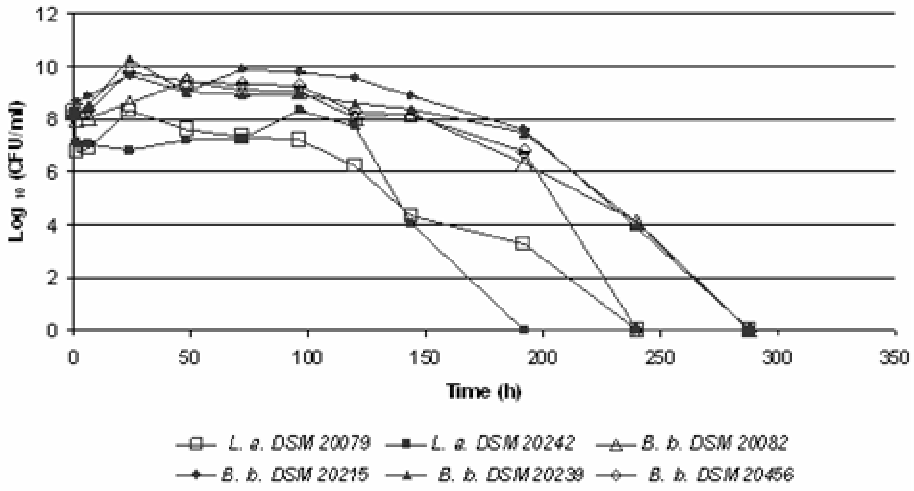

Figure 5. Survival rate of *Lactobacillus acidophilus* DSM 20079, *Lactobacillus acidophilus* DSM 20242, *Bifidobacterium bifidum* DSM 20082, *Bifidobacterium bifidum* DSM 20215, *Bifidobacterium bifidum* DSM 20239 and *Bifidobacterium bifidum* DSM 20456 in the medium of pH= 6

number of live bacteria and the values of the correlation coefficient in all cases achieved the levels ranging from 0.9 to 0.99. During the period of 8 h of culturing the absorbance was found to increase which indicated the lack of the inhibitory effect of bile on the growth of *L. acidophilus* strains. A different picture of the absorbance dynamics emerges for the strains of the *B. bifidum* species. It is evident that in the case of the control samples, that is, media without the addition of bile, a significant increase of the absorbance was observed, comparable with that of the medium with the addition of 1% bile for all strains of this species. Increased absorbance was also observed for cultures of the *B. bifidum* strain DSM 20456 supplemented with 2% bile. In media with the *B. bifidum* bacteria supplemented with higher amounts of bile, the absorbance during 8 hours of culturing remained nearly unchanged, while the amount of live bacteria either did not change or decreased.

Analyzing coefficients of growth inhibition (Table 4), it can be concluded that strains of the *L. acidophilus*

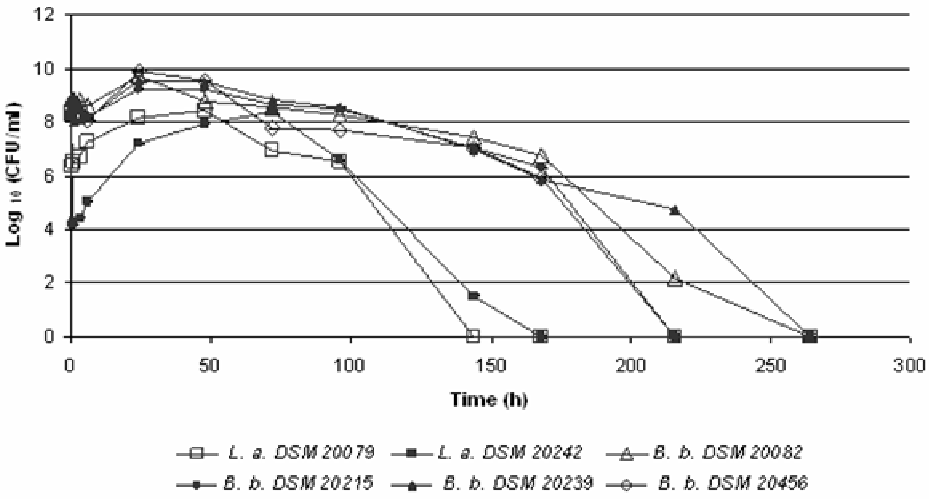

Figure 6. Survival rate of *Lactobacillus acidophilus* DSM 20079, *Lactobacillus acidophilus* DSM 20242, *Bifidobacterium bifidum* DSM 20082, *Bifidobacterium bifidum* DSM 20215, *Bifidobacterium bifidum* DSM 20239 and *Bifidobacterium bifidum* DSM 20456 in the medium of pH = 7

Table 4. Coefficient of inhibition in medium with oxgall ± SD (bile equivalent at the concentration 10 times lower).

The oxgall added (%)	Time [hours]	*Lactobacillus acidophilus*		*Bifidobacterium bifidum*			
		DSM 20079	DSM 20242	DSM 20082	DSM 20215	DSM 20239	DSM 20456
0,1%	2	0,020±0,002	0,067±0,021	0,109±0,027	0,046±0,022	0,197±0,033	0,069±0,030
	4	0,012±0,001	0,032±0,010	0,114±0,008	0,094±0,024	0,021±0,007	0,206±0,001
	6	0,020±0,001	0,014±0,025	0,078±0,001	0,165±0,007	0,148±0,010	0,168±0,009
	8	0,007±0,003	0,05±0,002	0,168±0,003	0,260±0,003	0,04±0,007	0,105±0,007
0,2%	2	0,040±0,002	0,105±0,015	0,182±0,041	0,092±0,032	0,136±0,023	0,111±0,003
	4	0,037±0,015	0,055±0,006	0,265±0,002	0,424±0,011	0,35±0,008	0,187±0,008
	6	0,039±0,015	0,042±0,011	0,392±0,018	0,565±0,004	0,578±0,002	0,042±0,004
	8	0,023±0,003	0,014±0,004	0,496±0,001	0,709±0,003	0,540±0,007	0,05±0,01
0,3%	2	0,053±0,018	0,152±0,025	0,273±0,003	0,138±0,020	0,212±0,019	0,042±0,010
	4	0,043±0,009	0,071±0,011	0,240±0,043	0,481±0,007	0,464±0,019	0,364±0,010
	6	0,055±0,017	0,062±0,018	0,313±0,009	0,676±0,005	0,689±0,007	0,437±0,004
	8	0,029±0,001	0,024±0,005	0,454±0,055	0,786±0,001	0,707±0,013	0,436±0,005
0,4%	2	0,067±0,017	0,219±0,014	0,473±0,007	0,246±0,035	0,136±0,035	0,083±0,018
	4	0,068±0,008	0,095±0,011	0,139±0,015	0,217±0,003	0,361±0,013	0,411±0,012
	6	0,064±0,008	0,118±0,012	0,265±0,01	0,488±0,007	0,496±0,003	0,587±0,004
	8	0,036±0,002	0,040±0,004	0,433±0,005	0,666±0,007	0,626±0,002	0,602±0,003

species as well as the DSM 20082 strain of the *B. bifidum* species should be considered as bile-tolerant because their growth inhibition coefficient was less than 0.5 (Gopal et al., 1996). The *B. bifidum* strain DSM 20456 was found to tolerate bile well when its concentration did not exceed 3%. In media with 4% bile, the growth inhibition coefficient in the 6th h of culturing amounted to 0.587, while in the 8th h – to 0.602. The DSM 20215 and 20239 strains of the *B. bifidum* bacteria tolerated bile well up to the 8th hour of culturing when it was applied at the concentration of 1% and at higher concentrations only up to the 4th h of culturing. Slightly lower growth inhibition coefficients, alb-

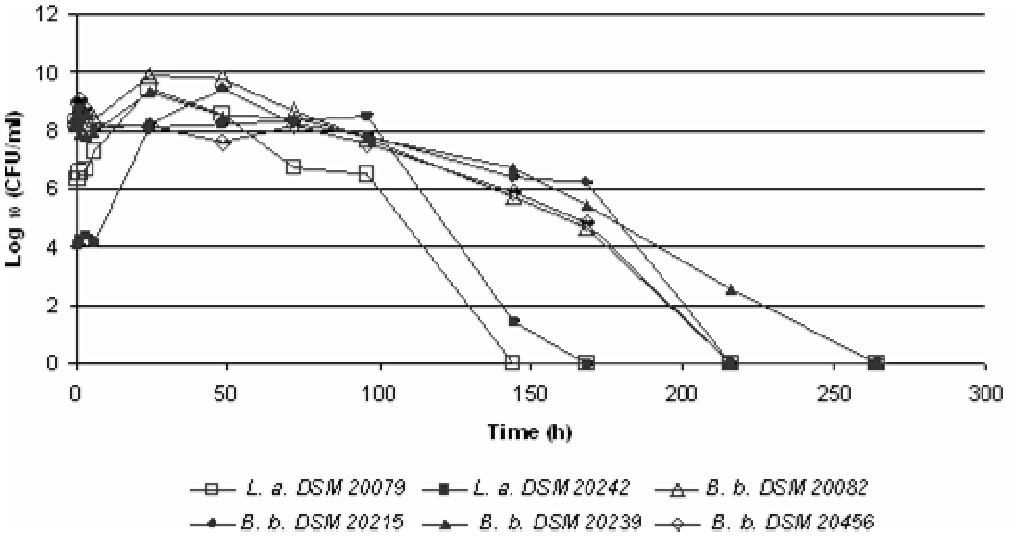

Figure 7. Survival rate of *Lactobacillus acidophilus* DSM 20079, *Lactobacillus acidophilus* DSM 20242, *Bifidobacterium bifidum* DSM 20082, *Bifidobacterium bifidum* DSM 20215, *Bifidobacterium bifidum* DSM 20239 and *Bifidobacterium bifidum* DSM 20456 in the medium of pH = 8

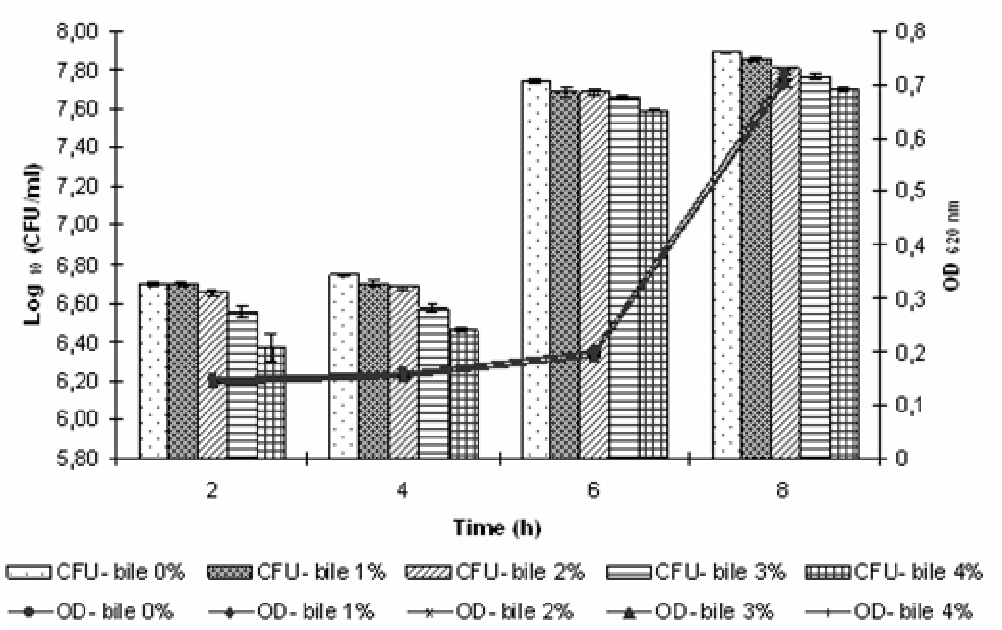

Figure 8. Survival rate of *Lactobacillus acidophilus* DSM 20079 in the medium with 1%-4% bile added and absorbance changes in bacteria cultures

eit exceeding the value of 0.5, were recorded for the DSM 20239 strain of the *B. bifidum* bacteria. Following the suggestion of Banach et al. (2001), the DSM 20215 and 20239 strains of the *B. bifidum* bacteria can be considered as "strains moderately sensitive to the effect of bile". That is how the authors characterised the strains of *L. acidophilus* RO25 and *Lactobacillus rhamnosus* „E/N", for which the growth inhibition coefficients were: 0.65 and 0.56, respectively. On the other hand, the following strains were classified by them as non-sensitive to the presence of 3% bile: *L. rhamnosus* „OXY", *L. acidophilus* LaCH5, *L. rhamnosus* PEN" and *L. rhamnosus* RO11, assuming the same criterion as Gopal et al (1996).

Our own investigation corroborated the fact that the resistance to bile is a trait characteristic for a bacterial

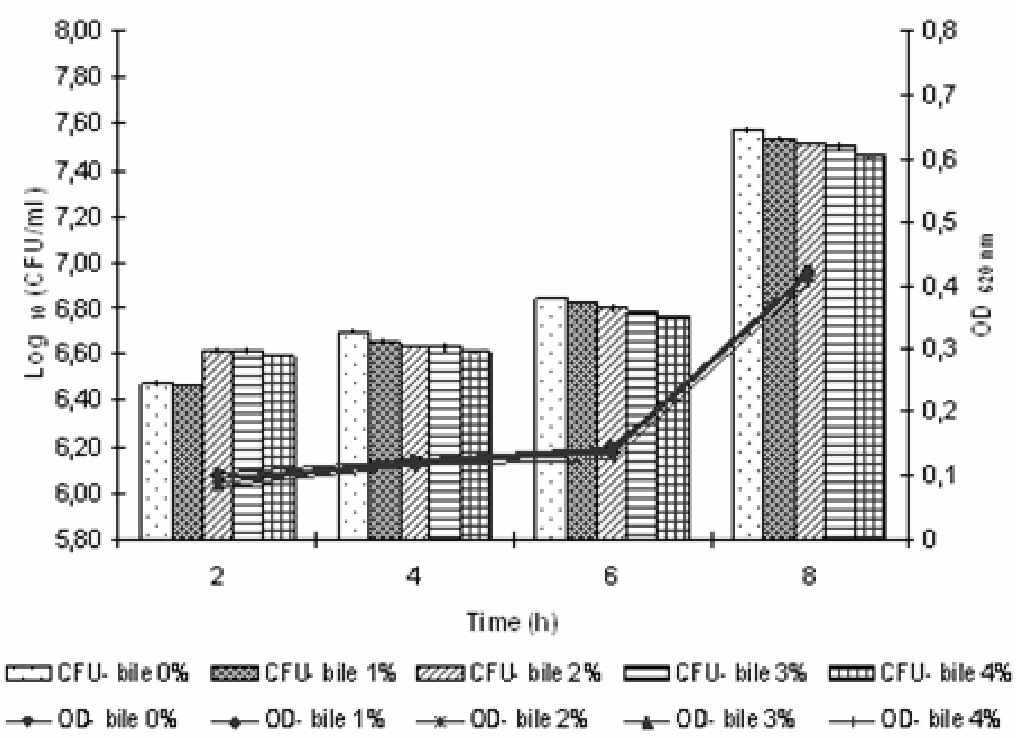

Figure 9. Survival rate of *Lactobacillus acidophilus* DSM 20242 in the medium with 1%- 4% bile added and absorbance changes in bacteria cultures

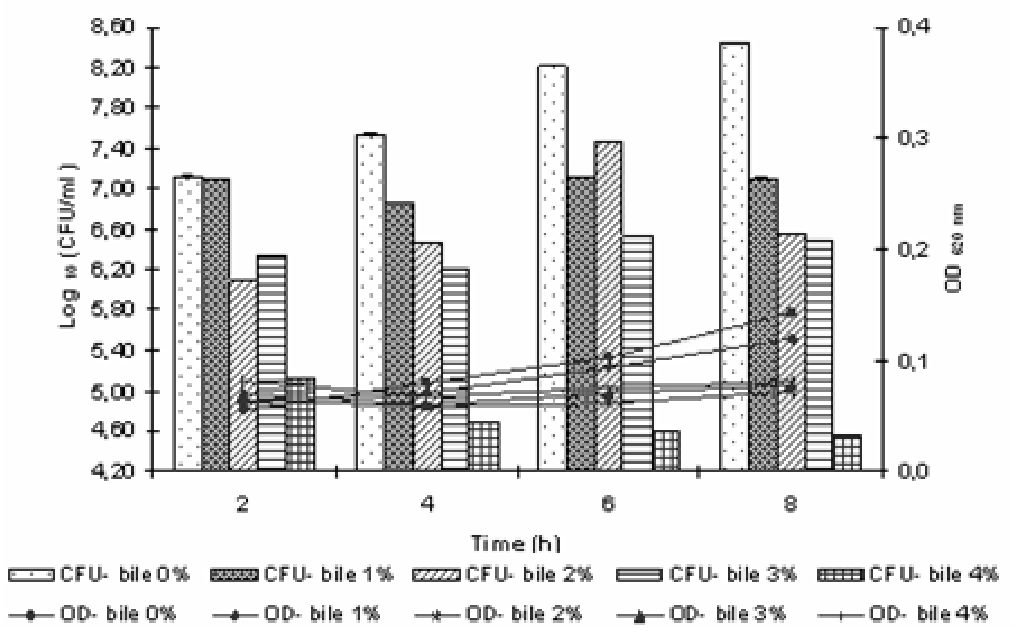

Figure 10. Survival rate of *Bifidobacterium bifidum* DSM 20082 in the medium with 1%- 4% bile added and absorbance changes in bacteria cultures.

species but also for strain. Differences between bacterial strains also depended on the concentration of bile in the applied media. All the tested strains can be considered as well tolerating bile in the concentration of up to 3%

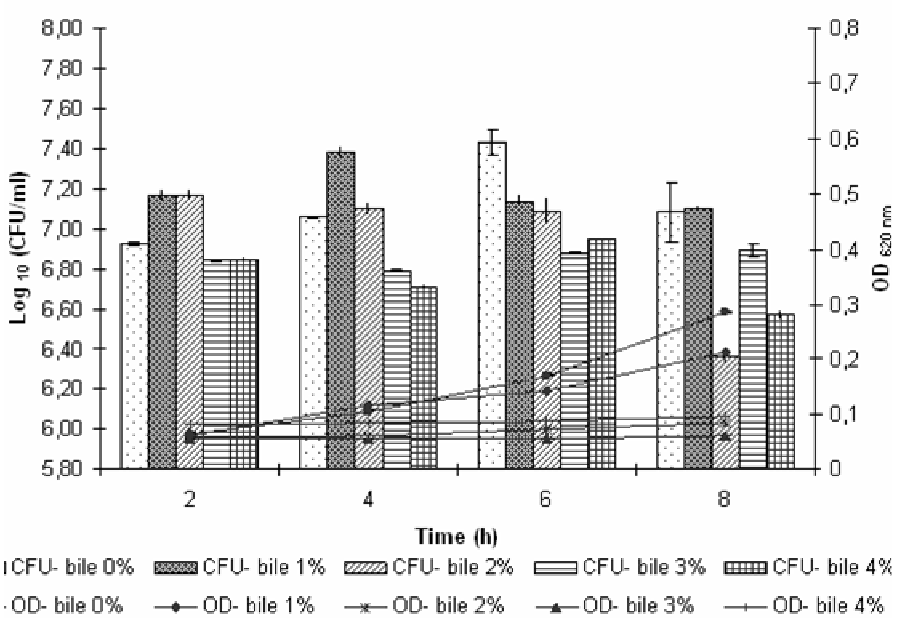

Figure 11. Survival rate of *Bifidobacterium bifidum* DSM 20215 in the medium with 1 - 4% bile added and absorbance changes in bacteria cultures

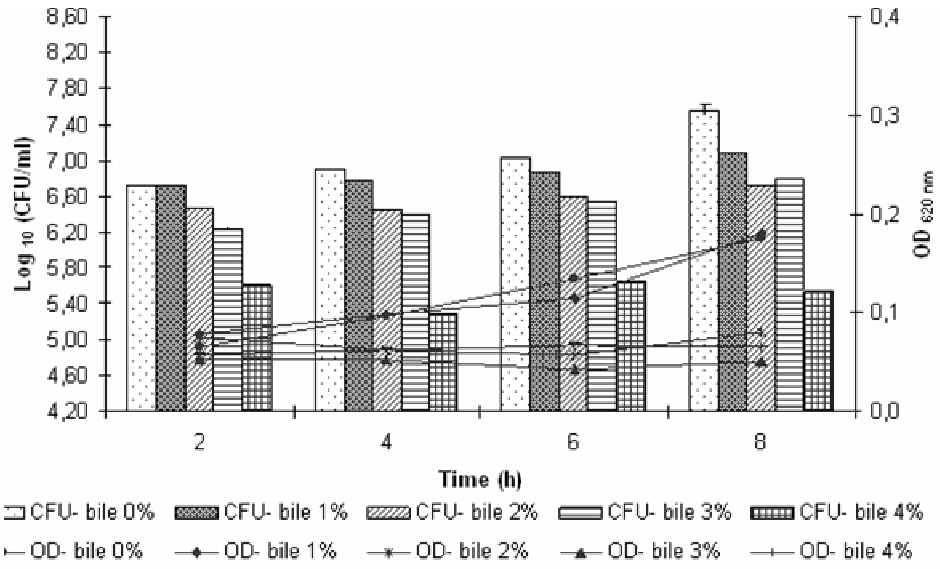

Figure 12. Survival rate of *Bifidobacterium bifidum* DSM 20239 in the medium with 1%- 4% bile added and absorbance changes in bacteria cultures.

(0.3% oxgall).

DISCUSSION

The performed investigations confirmed variations in the biochemical properties between the characterised species of bacteria but also between individual strains of the same species. Slizewska et al. (1998) carried out investigations on 23 strains of the *Bifidobacterium* genus which also demonstrated considerable variability of strains with regard to their nutrient requirements, acidifying activity as well as morphology. This confirms different nutritional requirements even within the same bacterial species which was corroborated also by our experiments. On the other hand, the performed API CH50L tests confirmed the reports that all *Bifidobacterium* bacteria are capable of fermenting glucose, galactose and fructose (Rada et al.,

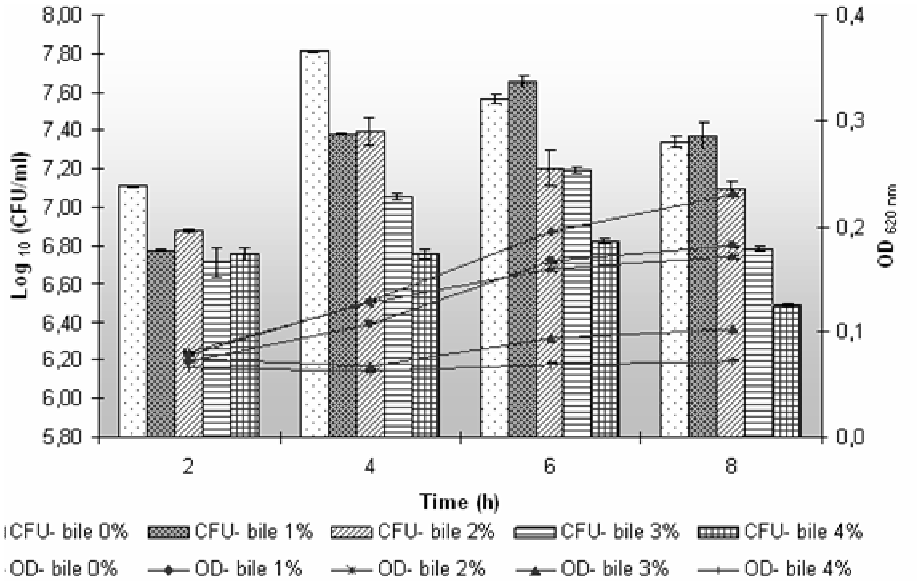

Figure 13. Survival rate of *Bifidobacterium bifidum* DSM 20456 in the medium with 1%- 4% bile added and absorbance changes in bacteria cultures.

2002).

The available literature data, apart from the importance of our knowledge concerning the resistance of lactic bacteria used in human nutrition to antibiotics because of the medical significance of this fact, also emphasize the importance of our knowledge whether this resistance is natural or acquired (Kneifel, 2002). There are indications that some resistance factors can also be transferred into undesirable microorganisms by means of different molecular mechanisms. The resistance of bacteria to antibiotics depends on their genus, species, strain and phase of logarithmic growth. Moreover, antibiotic therapy may cause diarrhoea of varying course. The pathogenesis of the development of this type of diarrhoea is associated with quantitative and qualitative changes in the composition of the intestinal microflora. The application of probiotics which restore the intestinal homeostasis is widely accepted as a safe alternative of antibiotic therapy. *L. acidophilus* bacteria as one of the species commonly accepted as probiotic turned out quite effective in preventing ailments caused by the application of ampicillin, neomycin and amoxicillin (McFarland and Elmer, 1997). In the described experiments, the author applied discs with antibiotic concentrations recommended by the Committee of Experts so as to ensure the highest possible compliance of the obtained results with the minimum inhibitory concentration (MIC) (the lowest antibiotic concentration inhibiting completely the growth of bacteria) values obtained in the dilution method. This, in turn, makes it possible to refer results to the mean antibiotic level determined in the blood serum at normal dosage (Kędzia and Koniar, 1980). Differences between the tested strains in their resistance to antibiotics were recorded but the type of the resistance was not determined. A key requirement for probiotic strains is that they should not carry transmissible antibiotic resistance genes (Zhou et al., 2005). Also Delgado et al. (2005) confirmed high sensitivity differences of bacteria from the *Lactobacillus* genus to antibiotics. In the case of vancomycin, MIC for the *L. acidophilus* bacteria was less than 2 µg/ml of the medium, while for the *L. rhamnosus, Lactobacillus plantarum, Lactobacillus casei, Lactobacillus paracasei, L. plantarum, Lactobacillus parabuchneri, Lactobacillus brevis and Lactobacillus vaginalis* bacteria, it reached over 256 µg/ml of the medium.

The main cause of the *L. acidophilus* and *L. casei* bacteria activities against *H. pylori* is pH changes associated with the production of lactic and acetic acids by the above-mentioned bacterial strains. It is widely believed that the non-dissociated acetic acid plays the main role in the anti-infectious action of bifidobacteria. It was the discovery that the majority of the *L. acidophilus* strains produce bacteriocins that explained the importance of these bacteria for the gastrointestinal tract. It was found that probiotic strains are characterised by anti-microbe activity because they manufacture peptides of inhibitory properties (Strus et al., 2001; De Vuyst, 2002). Strus et al. (2001) demonstrated antagonistic properties of bacteria from the Lactobacillus genus isolated from faeces of healthy newborn babies fed mother's milk against *H. pylori, Campylobacter coli, Campylobacter jejuni* and *Clostridium difficile*. In this way they confirmed that the antagonistic activities of *Lactobacillus* strains against anaerobic bacteria are fairly widespread, although considerable differences in the strength of the antagonistic impact occur. Investigations carried out so far have shown the antagonistic influence of *Lactobacillus* bacteria against colonization by *H. pylori* in '*in vitro'* models as well as in

clinical experiments. Fermented milk containing *Lactobacillus johnsonii* La1 was used successfully in patients infected with *H. pylori* (Schiffrin and Blum, 2001). Numerous literature studies confirm our observations concerning the antagonistic effect of the examined strains against *H. pylori* but it seems important to undertake in future further studies aiming at the elucidation of the mechanism of this activity.

Beneficial action of lactic fermentation bacteria in the human organism becomes apparent when they live in unfavorable conditions existing both in the acidified products and in the gastrointestinal tract. Characteristic features of the gastrointestinal tract include, among others, low pH of the gastric juice as well as high concentrations of bile salts (Klaenhammer and Kullen, 1999). The performed experiments confirmed the fact that the tested bacteria from the *B. bifidum* species, in the model conditions of changing pH, are characterised by higher survivability than the bacteria from the *L. acidophilus* species. It is also observed that the capability to survive in different pH of the environment is also a characteristic feature for bacterial strains. All the tested bacterial strains were characterised by significantly longer survivability in the environment of pH characteristic for the individual segments of the gastrointestinal tract. In the case of the oral cavity, its pH ranges from 6.2 to 7.4, depending on the speed of saliva secretion (Keller, 2000). The lifetime of all the tested bacterial strains in these conditions exceeds 198 h. Similar pH conditions prevail also in the bowels in which the undigested parts of food reach the large intestine within the period of 8 to 9 h (Ganong, 1994). Therefore, all the strains are also resistant to pH values prevailing in the human intestines much longer than the period during which they would have to remain there together with food. It is true that bacteria are exposed to low pH of 2 to 3 in the stomach but it should be remembered that the environment of this part of the gastrointestinal tract is buffered by food. Although the time the food remains in the stomach is maximally 4 hours, bacteria are exposed to low pH for only several dozen minutes (Ganong, 1994). The analysis of the above-presented diagrams clearly shows that all strains of the tested bacteria survive in the environment of pH 2-3 longer than 23 hours. This means that all these strains fulfil the basic criterion required from probiotic bacteria, namely that they should survive in the environment characteristic for the individual pH environments inhibit the metabolism activity and growth sections of the human gastrointestinal tract.

Low pH environments inhibit the metabolism activity and growth of *L. acidophilus* and are harmful to the bacterial cells, reducing their viability. The results of Bolin et al. (1997) experiments indicated that the studied strains showed different survival abilities in the pH ranging from 1.5 to 6.5. *L. acidophilus* strains B and V-74 showed better resistance to the acidic conditions than *L. acidophilus* CH-2 and CH-5.

Accordingly to the analysis of variables affecting bacterial survival during the passage through a stomach model, results of Pinto at al. (2006) showed that it is necessary not only to test the tolerance to low pH, but the action of enzymes like pepsine and lysozyme. This aspect will be taken under consideration in the consecutive stages of our investigations.

Despite the fact that the bile concentration is not determined precisely, in their investigations numerous authors applied substrates containing from 0.15 to 0.3% oxgall (Banach et al., 2001; Goldin and Golbach, 1992; Pinto et al., 2006). Buck and Gilliland (1994) examined the tolerance to bile of the *L. acidophilus* bacteria isolated from faeces. None of the isolated 12 bacterial isolates showed higher tolerance to bile in comparison with the model strain of *L. acidophilus* ATCC 43121. The growth level of absorbance for all 12 strains ranged from 2 to 2.8 h on the MRS medium supplemented with 0.3 oxgall. *L. acidophilus* ATCC 43121 tolerated bile much better and this strain was found to grow faster than the remaining examined strains but this strain was isolated from the intestinal chyme of pigs and cannot be applied in the human diet. In our own studies, the time of growth absorbance for the two strains of *L. acidophilus* was longer and amounted to 8 hours.

Also Bielecka et al. (1998_b) observed considerable variability between strains of *Bifidobacterium* derived from humans with regard to their survivability in low pH and in the presence of bile salts. In the group of 17 strains isolated from adults, about 30% were completely resistant to the examined factors, while in the group of 18 strains derived from infants – only 5%. Populations of sensitive strains died in low pH during the period of 40 minutes, whereas in the presence of bile salts – it took them 3 h to perish. The selection of tested strains on the basis of unfavorable physiological factors yielded positive results.

The performed investigations showed higher resistance of the *L. acidophilus* bacteria to bile concentration corresponding to the concentrations prevailing in the human gastrointestinal tract in comparison with the *B. bifidum* bacteria in the *in vitro* model conditions. Nevertheless, the *B. bifidum* bacteria are more resistant to low environmental pH as well as the pH found in the further segments of the human gastrointestinal tract in comparison with the *L. acidophilus* species in '*in vitro*' experiments. Therefore, differences between bacterial species are also determined by the character of analyzed properties.

Recapitulating, the tested strains of potentially probiotic bacteria fulfill the basic criteria required from probiotic strains, namely the capability to survive in the '*in vitro*' conditions of the gastrointestinal tract – in low pH and presence of bile salts. The examined strains were characterised by differing biochemical properties as well as varying resistance to antibiotics. All the examined strains exhibited antagonistic activities against *H. pylori* bacteria. In addition, the tested strains of the *L. acidophilus* bacteria showed antagonistic activities against *E. coli* and *S.*

enteritidis bacteria. All the above-mentioned information indicates that they can become good candidates for probiotics.

ACKNOWLEGMENTS

We thank Dr. M. Strus and Professor P. B. Heczko from Jagiellonian University Medical College, Cracow, Poland for their helping with test for antagonistic activity of bacteria strains.

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