In vitro testing of selected probiotic characteristics of *Lactobacillus plantarum* and *Bifidobacterium longum*

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Summary

Lactobacillus plantarum strain CCM 7039 and *Bifidobacterium longum* strain CCM 4990 were physiologically characterized using methods of model biological barriers. Tested strains, at densities of 10^8 or 10^9 KTJ.cm^{-3}, were able to survive in the presence of 0.3% or 0.5% bile salts for 4 h. Acid resistance test showed that both microorganisms tested were able to adapt and to grow in selected media at pH values of 3 to 4. *L. plantarum* showed resistance against netilmicin, gentamicin, cefotaxime, and cefuroxime. *B. longum* was resistant against netilmicin, gentamicin and cefotaxime. Both strains were able to adhere to Caco-2 cells in defined conditions.

Keywords

Lactobacillus plantarum, *Bifidobacterium longum*, probiotic characteristics

Probiotics are live microbial food additives beneficial for consumers' health as they keep, maintain or improve intestinal microbial balance [1]. Probiotics are currently defined as substances or products containing sufficient numbers of viable microorganisms which, after having been implanted or after colonisation, change the microflora within a certain anatomical location of the host, and thus can manifest their beneficial effects on health [2, 3]. Recent research however has suggested that it is not only live microorganism but also their dead forms and certain components of the cell walls of the microorganisms which are likely to influence the host [4, 5].

The commonest and the most wide-spread probiotic bacteria used in the production of traditional diary products and fermented foods include *Lactobacillus* and *Bifidobacteria* spp. [1, 4, 5].

The theoretical basis for the selection of probiotic microorganisms includes safety, functionality (survival, colonization, antimicrobial production, immune stimulation, antigen toxic activity, and pathogen prevention), and technological aspects (growth in the food, sensory characteristics, stability, viability - feasibility of their use in production process) [6, 7].

The present work represents a continuation of previous experiments [8–11] which focused on lactic fermentation of vegetable juices using *Lactobacillus plantarum* CCM 7039 and *Bifidobacterium longum* CCM 4990 also tested in the present work, to inoculate the raw material (fresh strained cabbage). The aim of the present work was to conduct the basic in vitro tests to assess the probiotic characteristics of selected microorganisms, specifically to test the resistance of the strains *Lactobacillus plantarum* CCM 7039 and *Bifidobacterium longum* CCM 4990 against bile salts; ability to survive at low pH; fermentation of saccharides; and resistance to selected antibiotics.

MATERIALS AND METHODS

Bacterial strains

Strains of the microorganisms *Lactobacillus plantarum* CCM 7039 and *Bifidobacterium longum* CCM 4990 were made available by Czech

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Collection of Microorganisms (Faculty of Sciences, Masaryk University, Brno, Czech Republic).

Culture conditions of *Lactobacillus plantarum* CCM 7039 and *Bifidobacterium longum* CCM 4990

Lactobacillus plantarum strain CCM 7039 was supplied as lyophilisate; it was reconstituted in MRS broth (broth for *Lactobacillus* according to De Man, Rogos and Sharpe, pH 5.7 ± 0.2; Merck, Darmstadt, Germany). The culture was grown at 37 °C for 24 hours. The reconstituted culture was inoculated to Petri plates with MRS agar (agar for *Lactobacillus* according to De Man, Rogos and Sharpe, pH 5.7 ± 0.2, Merck). Always freshly prepared *Lactobacillus* cultures in MRS broth were used for the experiments.

Bifidobacterium longum CCM 4990 was cultured under stricter anaerobic conditions. Prior to being added to the test medium, the strain to be cultured in MRS broth was centrifuged under sterile conditions (9 000 rpm, 5 min), subsequently washed with physiological saline, and added in this environment into the test medium, to make the initial density of approx. 10^8 KTJ.cm^{-3}.

Resistance/sensitivity test to bile salts

The microorganisms in culture were tested with bile salts (Fluka, Buchs, Switzerland) according to the procedures of Cebecci & Gürakan modified by us [7]. Centrifuged microorganism culture (9 000 rpm, 5 min) was replenished with physiological saline to the volume of 5 cm^3 (with the initial microorganism density being approx. 10^8 KTJ.cm^{-3} and 10^7 KTJ.cm^{-3}, respectively), and dosed to MRS broth containing the testing concentrations of bile salts (0.3%; 0.5%; or 1%), incubated in a thermostat at 37 °C for 4 h. Microorganisms were sampled and inoculated to MRS agar at hourly intervals.

Survival of lactobacilli and bifidobacteria at low pH

Cultured microorganisms were centrifuged and washed with physiological saline, re-centrifuged and replenished to 5 cm^3 with physiological saline (giving the initial microorganism density of approx. 10^8 KTJ.cm^{-3} and 10^7 KTJ.cm^{-3}, respectively), divided into 5 sterile tubes containing the test medium of the chosen pH (MRS, PBS - Phosphate Buffered Saline, physiological saline, and pH 2; 3 (and/or 3.5); 4). The cultures were incubated at 37 °C for 4 h. At hourly intervals, the cultures were sampled and inoculated, always from a different tube, to MRS agar. The procedure of the acid resistance test used was a modified reference procedure [7, 12, 13].

Saccharide utilisation test

API 50 CH test according to Rada & Petr [14] was used to test saccharide fermentation by the tested microorganisms.

The API 50 CH test (Biomérieux, Marcy - l'Etoile, France), the kit also contains API 50 CHL Medium (10 ml ampoules for *Lactobacillus* and related species) is a standardised system of 50 biochemical tests to study saccharide metabolism of microorganisms. API 50 CH plates consist of 50 microtubes each, which can be used to study the course of fermentation of substrates of the saccharide group of substances and their derivatives (each of the microtubes is intended for a different substrate). The API 50 CH test works in conjunction with API 50 CHL Medium (to which the microorganism tested is to be added), which is used to inoculate the microtubes and rehydrate the substrates. Fermentation is identified during incubation (utilisation was studied at two different temperatures: at 37 °C (ideal temperature) for 24 and 48 h; and 21 °C (the temperature at which cabbage juices are fermented) for 24 and 48 h) by colour change in the microtube from purple to yellow), due to formation of acids under anaerobic conditions, and detected by change in pH indicator present in API 50 CH Medium. The first microtube on every plate does not contain any active substrate and serves as negative control.

Sensitivity test to selected antibiotics

Reconstituted grown 16-h cultures of the microorganisms were tested for sensitivity and/or susceptibility to selected antibiotics according to the procedure of Cebecci & Gürakan [7] in our modification. For this purpose, the following Mast Discs (Mast Group, Merseyside, UK) were used: netilmicin (30 μg) – NET30, gentamicin (10 μg) – GM10, vancomycin (30 μg) – VA30, ceftriaxone (30 μg) – CRO30, cefotaxime (30 μg) – CTX30, cefuroxime (30 μg) – CXM 30, cefazolin (30 μg) – CZ30, piperacillin (100 μg) – PRL100, ampicillin (10 μg) – AP10, penicillin G (10 un.) – PG10, nalidixic acid (30 μg) – NA30, clindamycin (2 μg) – CD2, erythromycin (15 μg) E15, tetracycline (30 μg) – T30.

Four ml agar containing 200 μl 10^9 KTJ.cm^{-3} microorganisms were poured on MRS agar plates. The plates were then left to rest at the room temperature for 1 h. The antibiotic discs were distributed over the medium in Petri dishes and incubated under aerobic conditions at 37 °C for 24 h. Clear growth inhibition zones around the discs were measured and the annulus diameter was expressed in mm.

Test of adherence to intestinal epithelium

The ability of the bacteria to adhere to epithelial Caco-2 cells was studied.

Origin of Caco-2 cell cultures: the cancer cell line Caco-2 has been isolated from primary adenocarcinoma of the colon. The cells were cultured in Eagle's minimum essential medium (E-MEM – Biocom, Bratislava, Slovakia), supplemented by bovine foetal serum (10% v/v), penicillin (0.1 mg.cm^{-3}) and streptomycin (0.1 mg.cm^{-3}). The cell line was provided by Institute of Experimental Oncology, Slovak Academy of Sciences (Bratislava, Slovakia).

The cell line was cultured in a thermostat at 37 °C in humidified atmosphere with 5% CO_2. At 5–7-day intervals, when the culture has covered the whole area, the cultures were passaged. The cells were washed with PBS solution and passaged using dry trypsin treatment with 0.25% trypsin/EDTA solution. Subsequently, the cells were blown in the medium with an automated pipette, counted in Bürker chamber, and used for experiments or further culture (at the density of 1×10^6 cells per 80 cm^2 in 10 cm^3 medium).

Procedures as described in the literature were used for the above experiments [15, 16].

RESULTS AND DISCUSSION

Resistance to bile salts represents one of the selection criteria of probiotics. Bile salt concentrations of 0.3%; 0.5%; and 1% were used. Figs. 1 and 2 show incubation time dependencies on survival of *Lactobacillus plantarum* CCM 7039 and *Bifidobacterium longum* CCM 4990 in medium containing bile salts. During the 4-h culturing time, *Lactobacillus plantarum* strain CCM 7039 showed slightly reduced cell suspension densities (KTJ.cm^{-3}) in the presence in the culture medium of all bile salt concentrations used (0.3%; 0.5%; and 1%), the reductions becoming apparent as early as after 1 h of culturing. *Bifidobacterium longum* strain CCM 4990 showed a slight increase during the second hour of culturing. Negative effects of the presence of bile salts on both strains tested were only observed at the highest concentration of the bile salts used. The strains tested (initial densities approx. 10^8 and 10^9 KTJ.cm^{-3}, respectively) are able to survive in environments containing 0.3% and 0.5%, respectively for 4 h. This time is sufficient for the microorganisms tested to survive in the digestive tract of the host, thus meeting the requirement regarding resistance to bile salts.

Another feature studied was survival of the bacteria in various environments modelling passage through the gastrointestinal system. Important for probiotic strains is that they are able to survive in the acid gastric environment. The pH value of gastric juice ranges between 1.0–2.0, rising to 5.0 or more after food consumption. The results of the tests are illustrated in Fig. 3 on the example of *Lactobacillus plantarum* CCM 7039. At higher pH values (3 and 4), both lactobacilli tested were able to adapt themselves and to grow. Conversely, both showed sensitivity to lower pH values, with the initial cell densities decreasing by approximately 5 degrees of magnitude as early as during the first hour. When comparing our results with international reports, the microorganisms tested may be claimed to have met the requirements regarding resistance to acid environments and the acid resistance criterion. Minelli et al. [17] could provide evidence for sensitivity of lactobacilli (*Lactobacillus casei* spp.) to pH value of 2.

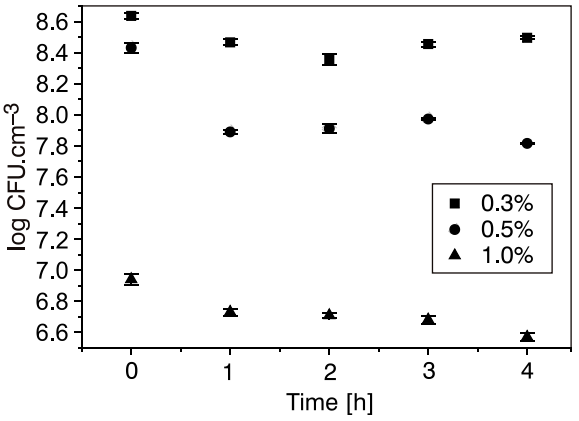

Fig. 1. *Lactobacillus plantarum* strain CCM 7039: bile salts sensitivity test.

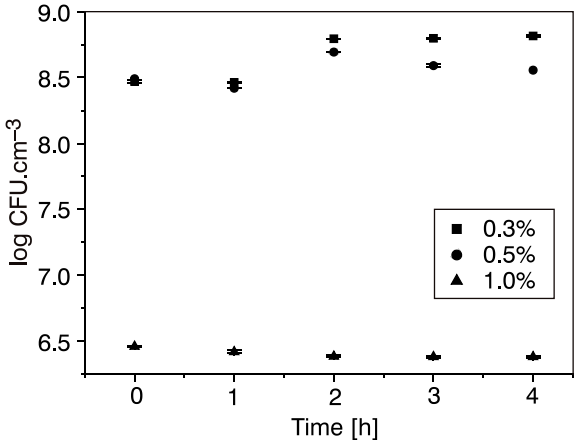

Fig. 2. *Bifidobacterium longum* strain CCM 4990: bile salts sensitivity test.

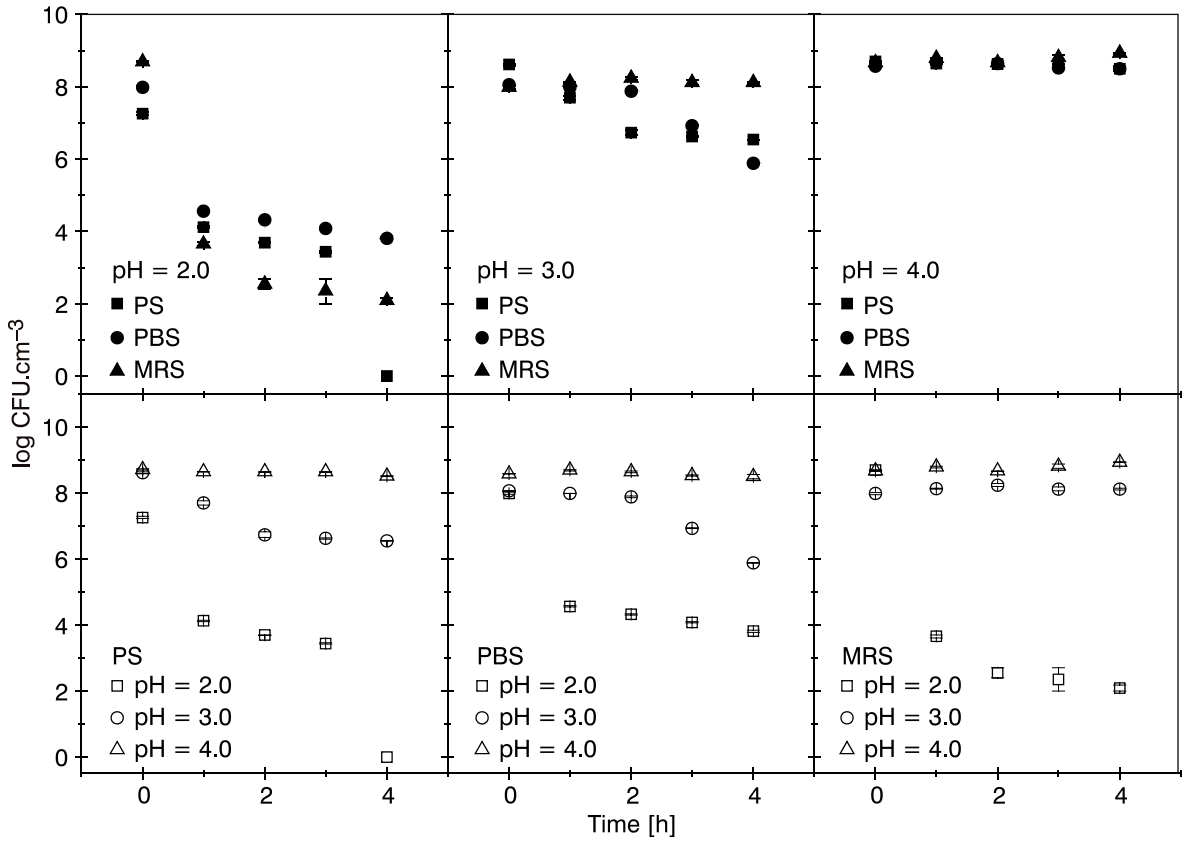

Fig. 3. *Lactobacillus plantarum* strain CCM 7039: acid resistance test to pH value of the culture medium.

VINDEROLA & REINHEIMER [6] studied the resistance of 24 strains of lactic bacteria starting cultures (*Streptococcus thermophilus*, *Lactobacillus delbruec kii* subsp. *bulgaricus,* and *Lactococcus lactis*) and of 24 strains of probiotic bacteria (*Lactobacillus acidophillus, Lactobacillus casei, Lactobacillus rhamnosus,* and bifidus bacteria) to pH 2 and/or pH 3. A weaker resistance to pH 2 was observed for lactic bacteria compared to probiotic bacteria. Only slight reductions in cell suspension densities were observed at the pH value of 3.

An important characteristic of bacteria is their ability to utilise different saccharides. This characteristic is also a feature supporting their identification. Every microorganism has a unique saccharide utilisation pattern, there nevertheless are certain „fermentation rules“ which may help classification of microorganisms. Table 1 shows the results of a 48-h test. There were only minute differences between temperatures used (21 °C and 37 °C) and between the results from tests of different duration 24 and 48 h). Results of tests of this type are relevant from mainly technological aspects: they show the ability of microorganisms to interfere with saccharide components of the given food and/or with raw materials used in the production.

Resistance or sensitivity to various antibiotics represent an important characteristic of lactobacilli and bifidobacteria strains. Table 2 shows results of resistance / sensitivity tests expressed as mean values of growth inhibition zone sizes from three parallel determinations. Altogether, from among the 14 antibiotics tested, *L. plantarum* was resistant to netilmicin, gentamicin, cefotaxime, cefuroxime. No growth inhibition zones could be observed around the discs of the aforementioned antibiotics. The largest growth inhibition zone formed around the discs of clindamycin, erythromycin, tetracycline, penicillin, and ampicillin. *B. longum* was resistant against netilmicin, gentamicin and cefotaxime. It also showed similar sensitivity to clindamycin, erythromycin, penicillin, and ampicillin. Several studies have demonstrated uniqueness of the antibiotic resistance pattern for every microorganism, even at the strain level.[7].

Adhesion of probiotic strains to intestinal surface and subsequent colonisation of the human gastrointestinal tract is considered as one of the key aspects of the probiotic effects of microorganisms. Probiotic bacteria able to adhere are able to remain in the intestinal tract over prolonged periods of time, thus having more opportunity to

Tab. 1. Test of saccharide fermentation - utilisation by microorganisms (API 50 CH).

No.	Medium (mg/microtube)		*Lactobacillus plantarum* CCM 7039		*Bifidobacterium longum* CCM 4990	
			24 h	48 h	24 h	48 h
0	0	Temoin (–)	–	–	–	–
1	GLY	Glycerol (1.64)	–/+	–/+	–/+	–/+
2	ERY	Erythritol (1.44)	–	–	–	–
3	DARA	D-arabinose (1.4)	–	–	–	–
4	LARA	L-arabinose (1.4)	+	+	–	–
5	RIB	D-ribose (1.4)	+	+	–/+	–/+
6	DXYL	D-xylose (1.4)	–	–	–	–
7	LXYL	L-xylose (1.4)	–	–	–	–
8	ADO	D-adonito (1.36)	–	–	–	–
9	MDX	Methyl-αD-xylopyranozide (1.28)	–	–	–	–
10	GAL	D-galactose (1.4)	+	+	+	+
11	GLU	D-glucose (1.56)	+	+	+	+
12	FRU	D-fructose (1.4)	+	+	+	+
13	MNE	D-mannose (1.4)	+	+	–	–
14	SBE	L-sorbose (1.4)	–	–	–	–
15	RHA	L-ramnose (1.36)	–	–	–	–
16	DUL	D-ulcitol (1.36)	–	–	–	–
17	INO	Inositol (1.4)	–	–	–	–
18	MAN	D-mannitol (1.36)	+	+	+	+
19	SOR	D-sorbitol (1.36)	+	+	–	–
20	MDM	Methyl-αD-manopyranozide (1.28)	+	–/+	–	–
21	MDG	Methyl-αD-glucopyranozide	–	–	–	–
22	NAG	N-acetylglucosamine	+	+	–/+	–/+
23	AMY	Amygdalin	+	+	–	–
24	ARB	Arbutin	+	+	–/+	–/+
25	ESC	Asculin iron citrate	+	+	+	+
26	SAL	Salicin	+	+	–/+	–/+
27	CEL	D-celobiose	+	+	–/+	–/+
28	MAL	D-maltose	+	+	+	+
29	LAC	D-lactose (of cow origin)	+	+	+	+
30	MEL	D-mellibiose	+	+	–	–
31	SAC	D-saccharose	+	+	+	+
32	TRE	D-trehalose	+	+	–/+	–/+
33	INU	Inulin	–	–	–	–
34	MLZ	D-melesitose	+	+	–	–
35	RAF	D-rafinose	+	+	–	–
36	AMD	Amidon (fibre)	–	–	–	–
37	GLYC	Glycogen	–	–	–	–
38	XLT	Xylitol	–	–	–	–
39	GEN	Genthiobiose	+	+	–	–
40	TUR	D-turanose	+	+	–	–
41	LYX	D-lyxose	–	–	–	–
42	TAG	D-tagatose	–	–	–	–
43	DFUC	D-fucose	–	–	–	–
44	LFUC	L-fucose	–	–	–	–
45	DARL	D-arabitol	–	–	–	–
46	LARL	L-arabitol	–	–	–	–
47	GNT	Gluconate potassium	+	–/+	–/+	–/+
48	2KG	2-ketogluconate potassium	–	–	–	–
49	5KG	5-ketogluconate potassium	–	–	–	–

Tab. 2. Sensitivity test to selected antibiotics.

Antibiotic	Growth inhibition zone [mm]	
	Lactobacilus plantarum CCM 7039	*Bifidobacterium longum* CCM 4990
Netilmicin (30 μg)	–	–
Gentamycin (10 μg)	–	–
Vancomycin (30 μg)	1.3	4.3
Ceftriaxone (30 μg)	0.3	2.2
Cefotaxime (30 μg)	–	–
Cefuroxime (30 μg)	–	0.5
Cefazolin (30 μg)	0.5	0.5
Piperacillin (100 μg)	4.0	4.0
Ampicillin (10 μg)	5.8	5.3
Penicillin (10 un.)	6.3	6.5
Nalidixic acid (30 μg)	4.5	4.3
Clindamycin (2 μg)	10.8	10.3
Erythromycin (15 μg)	9.3	9.6
Tetracycline (30 μg)	7.3	4.9

show metabolic and immunomodulatory effects than do strains without the ability to adhere. Adherence was therefore thought to be the prerequisite for probiotics to efficiently induce effects on the immune system and to stabilise the intestinal mucosal barrier. Also, adhesion may provide the mechanism of competitive elimination of pathogens from intestinal epithelium [18].

Adhesion of the tested microorganisms to the surface of Caco-2 cells was studied. To test the ability of the *Lactobacillus* and the *Bifidobacterium* to adhere to Caco-2 cells, the strains were cultured for 2 hours and subsequently inoculated to Caco-2 cells kept at 37 °C in a thermostat in humid atmosphere with 5 % CO_2. The initial densities of the bacteria were 2.8×10^8 KTJ.cm^{-3} and 3.9×10^8 KTJ.cm^{-3}, respectively; after 2-h incubation with Caco-2 cells, the densities increased to 7.6×10^4 KTJ.cm$^{-3,}$ and 1.1×10^5 KTJ.cm^{-3}, respectively. After 2 hours of culturing, 3% of the bacterial cells initially present were bound to Caco-2 cells. In other words, the tested microorganisms are able to adhere to Caco-2 cells under the conditions used. In vitro adhesion experiments suggest that there are differences in the adhesion potential of different probiotic strains [19] and that strains showing adhesion ability to CaCo-2 cells do not necessarily need to adhere to the mucosa with the same effectivity.

CONCLUSION

Selected characteristics of the physiological functionalities of *Lactobacillus plantarum* CCM 7039 and *Bifidobacterium longum* CCM 4990 were tested under specified conditions.

In vitro tests of the above microorganism strains did not support their classification among probiotics. Results of selected in vitro microbiological tests nevertheless suggested that the tested microorganisms show some abilities ascribed to probiotic microorganisms. The characteristics of the strains mentioned are assumed to be further studied.

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