

## Health-promoting properties exhibited by *Lactobacillus helveticus* strains\*

Katarzyna Skrzypczak<sup>1</sup>✉, Waldemar Gustaw<sup>1</sup> and Adam Waśko<sup>2</sup>

<sup>1</sup>Department of Fruits, Vegetables and Mushrooms Technology, Faculty of Food Science and Biotechnology, University of Life Sciences in Lublin, Lublin, Poland; <sup>2</sup>Department of Biotechnology, Human Nutrition and Food Commodity Science, Faculty of Food Science and Biotechnology, University of Life Sciences in Lublin, Lublin, Poland

Many strains belonging to lactobacilli exert a variety of beneficial health effects in humans and some of the bacteria are regarded as probiotic microorganisms. Adherence and capabilities of colonization by *Lactobacillus* strains of the intestinal tract is a prerequisite for probiotic strains to exhibit desired functional properties. The analysis conducted here aimed at screening strains of *Lactobacillus helveticus* possessing a health-promoting potential. The molecular analysis performed, revealed the presence of a *slpA* gene encoding the surface S-layer protein SlpA (contributing to the immunostimulatory activity of *L. helveticus* M 92 probiotic strain) in all B734, DSM, T80, and T105 strains. The product of gene amplification was also identified in a *Bifidobacterium animalis* ssp. *lactis* BB12 probiotic strain. SDS-PAGE of a surface protein extract demonstrated the presence of a protein with a mass of about 50 kDa in all strains, which refers to the mass of the S-layer proteins. These results are confirmed by observations carried with transmission electron microscopy, where a clearly visible S-layer was registered in all the strains analyzed. The *in vitro* study results obtained indicate that the strongest adhesion capacity to epithelial cells (HT-29) was demonstrated by *L. helveticus* B734, while coaggregation with pathogens was highly diverse among the tested strains. The percentage degree of coaggregation was increasing with the incubation time. After 5 h of incubation, the strongest ability to coaggregate with *Escherichia coli* was expressed by T104. The T80 strain demonstrated a significant ability to co-aggregate with *Staphylococcus aureus*, while DSM with *Bacillus subtilis*. For B734, the highest values of co-aggregation coefficient was noted in samples with *Salmonella*. The capability of autoaggregation, antibiotic susceptibility, resistance to increasing salt concentrations, and strain survival in simulated small intestinal juice were also analyzed.

**Key words:** *Lactobacillus helveticus*, probiotics, adhesion, autoaggregation, coaggregation

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### INTRODUCTION

Bacteria inhabiting guts have an important role in maintaining health of the entire host body. To avoid negative consequences connected with undesirable changes in microflora, a proper microorganism composition in the intestinal ecosystem needs to be maintained. This can be implemented through a supply of functional bacterial strains, which are capable to resist stressful fac-

tors during technological processes and survive under environmental conditions present during passage through the stomach to the intestinal tract to be able to exert the desired health-promoting effects (Nazarro *et al.*, 2012).

*Lactobacillus helveticus* is a homofermentative, Gram-positive, rod-shaped thermophilic microorganism belonging to lactic acid bacteria (LAB). These microorganisms are used in the dairy industry as a starter predominantly employed to ferment milk in manufacturing of several cheeses. Besides their technological applications, scientific reports have demonstrated evidence that the *L. helveticus* species strains exhibit health-promoting properties (Taverniti & Guglielmetti, 2012).

Several *in vitro* studies have confirmed that some strains of *L. helveticus* meet the requirements for probiotic bacteria with common properties, i.e. an ability to antagonize pathogens, survive in the digestive tract conditions, and adhere to epithelial cells. Also, studies conducted *in vivo* in mouse models provide evidence that *L. helveticus* is able to stimulate the immune system, increases defense against pathogens, has an influence on the composition of the intestinal microbiota, and prevent gastrointestinal infections. *L. helveticus* was also demonstrated to establish synergistic interactions with other bacterial strains to antagonize pathogens (Gareau *et al.*, 2010). A protective effect was observed when *L. helveticus* was administered orally. Research suggests that *L. helveticus* may lead to health promoting effects through not only production of lactic acid, hydrogen peroxide, and other antimicrobial agents, but also by stimulating host immunity at the systemic level (Rogers, 2002). Moreover, specific enzymatic activities of *L. helveticus* help to remove allergens from food, enhance the bioavailability of nutrients, and generate bioactive peptides through protein hydrolysis (Taverniti & Guglielmetti, 2012).

Various desired health-promoting sequences with antimicrobial, immunostimulating, opioid, mineral binding, and antihypertensive activities have been isolated from products fermented with *L. helveticus* (Griffiths & Tellez, 2013). Administration of those products containing bioactive compounds might be used as a potential alternative treatment for prevention of enteric infections. (Taverniti & Guglielmetti, 2012).

The aim of this study was to investigate *L. helveticus* strains in terms of selected properties required for probiotic organisms, which might indicate their potential applications. For this purpose, molecular identification of the *slpA* gene and S-layer proteins was performed and

✉ e-mail: [skrzypczakatarzyna@wp.pl](mailto:skrzypczakatarzyna@wp.pl)

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the capability of autoaggregation and coaggregation with pathogens, adhesion capacity, antibiotic susceptibility, resistance to increased salt concentrations were compared among the analyzed strains.

## MATERIALS AND METHODS

**Bacterial strains and growth condition.** Four strains of *L. helveticus* (B734, T80, T104, and T105), kindly provided by Professor Łucja Łaniewska-Trokenheim (University of Warmia and Mazury in Olsztyn, Poland), were examined in this study. These strains were isolated from Polish fermented milk products and deposited in the Polish Microorganism Collection (Wrocław, Poland). *L. helveticus* DSM 20075 (DSMRZ, Germany) and *Bifidobacterium animalis* subsp. *lactis* BB12 (DSMRZ, Germany) were used as reference strains.

All bacterial strains were maintained in 15% glycerol stocks and stored at  $-80^{\circ}\text{C}$ . Prior to the beginning of the experiments, each bacterial strain was routinely cultured (2% v/v) in Man-Rogosa-Sharpe broth (BTL, Poland) with 0.05% cysteine addition and incubated overnight (16 h) at  $37^{\circ}\text{C} \pm 0.5$  under anaerobic conditions (Wąsko *et al.*, 2014).

**Tolerance to NaCl.** Examination of the influence of sodium chloride concentrations on bacterial growth was conducted on MRS containing 0% (control), 2%, 3%, 4%, 6%, and 8% NaCl. The growth rate of each bacterial strain was monitored by measuring optical density ( $\text{OD}_{600}$ ) using Bioscreen C (LabSystem, Finland) (Polak-Berecka *et al.*, 2013).

**Survival of bacterial strains in simulated small intestinal juice.** Components of the small intestinal juice (Macfarlane *et al.* 1998) were dissolved in distilled water, mixed thoroughly, and autoclaved (15 min/ $0.5 \text{ atm.}/117^{\circ}\text{C}$ ). During cooling, the mixture was continuously stirred to prevent coagulation. After cooling, the fluid obtained was directly supplemented with a vitamin solution containing vitamin B<sub>12</sub>, nicotinic acid amide, p-aminobenzoic acid at the concentration of 0.005 g/dm<sup>3</sup>, thiamine (0.004 g/dm<sup>3</sup>), D-biotin (0.002 g/dm<sup>3</sup>) and menadion, pantothenic acid (0.001 g/dm<sup>3</sup>) previously dissolved in 10 ml of distilled water and passed through a sterile Millipore vacuum filter with a pore size of 200 nm. Simulated small intestinal juices were freshly prepared for each experiment. After 24-h incubation at  $37^{\circ}\text{C}$  in MRS broth, 1 ml of each strain cell suspension was centrifuged ( $8000 \times g/5 \text{ min}$  at  $4^{\circ}\text{C}$ ). The pellets were washed twice in phosphate-buffered saline (pH=7.2) and resuspended to the initial volume. Washed cell suspensions (0.5 ml) were added to 4.5 ml of fresh simulated intestinal juice tempered at  $37^{\circ}\text{C}$ , mixed well, and incubated for 2 h at  $37^{\circ}\text{C}$  under anaerobic conditions with periodical shaking. 4.5 ml of sterile MRS broth inoculated with the same amount of the cell strain suspension as in the sample with simulated intestinal juice and cultured for the same period and under the same incubation conditions were the control samples for each strain. Surviving bacteria were enumerated with the pour plating method. All enumerations were carried out using the standard serial dilution method in a physiological solution, plated on MRS agar with 0.05% cysteine, and incubated at  $37^{\circ}\text{C}$  for 48 h under anaerobic conditions.

**Antibiotic susceptibility testing.** Antibiotic susceptibility of the bacterial strains was tested using the agar disc diffusion method. Six antibiotics from different groups, diverse in terms of their effect on microorganisms, were used: erythromycin and gentamicin as

inhibitors of protein synthesis; ampicillin and bacitracin as inhibitors of cell wall synthesis; nalidixic acid and rifampicin as inhibitors of nucleic acid synthesis. Biomaxima (Centrum Mikrobiologii Emapol, Poland) disks for analysis of susceptibility to each of the antibiotic tested were applied to MRS agar plates inoculated with a bacterial strain suspension. After incubation at  $37^{\circ}\text{C}$  for 24 h under anaerobic conditions, the results (average of four independent readings) were expressed as sensitive (S), when the diameter of the growth inhibition zone (clear area) was bigger than 2 cm, intermediate (I), when the diameter of the inhibition zone was up to 2 cm, and resistant (R), when no inhibition area was detected.

**Transmission electron microscopy (TEM).** For TEM, bacterial samples were fixed in 4% GA (glutaraldehyde) for 24 h ( $4^{\circ}\text{C}$ ). After fixation, the samples were washed in cacodylate buffer and postfixed for 2 h in 2% OsO<sub>4</sub>. They were then washed in a saline solution and dehydrated in an alcohol series of 30, 60, 70, 90, 96, 96%, and twice in absolute alcohol (10 min. for each change). Next, the samples were cleared in propylene oxide, embedded in epoxy resin (Agar Scientific R 1078), and sections were cut with an ultramicrotome (HM 355S, Microm). The sections were placed on a metal grid, stained with 5% uranyl acetate and Reynolds lead acetate for contrasting, and viewed in a Libra 120 transmission electron microscope (Zeiss).

**Detection of S-layer protein genes by PCR.** DNA isolation of *L. helveticus* was performed using Genomic Mini AX Bacteria Spin (A&A Biotechnology, Gdynia, Poland) according to the attached protocol. Qualitative and quantitative assessment of the isolated DNA was performed by spectrophotometry and absorbance was measured by NanoDrop 2000c Spectrophotometer (Thermo Scientific, Germany).

SfpA gene-specific oligonucleotides F-sfp ATGAA-GAAAAATTTAAGAAT and R-sfp CACCGATCITGTAGTA (Beganović *et al.*, 2011a) were used for detection of the *sfpA* gene (HM140425). The amplification reaction was carried out in a thermal cycler (Labcyber-SensoQuest GmbH, Germany). The initial denaturation was conducted at  $94^{\circ}\text{C}$  for 5 min and followed by 25 cycles of denaturation at  $94^{\circ}\text{C}$  for 1 min, annealing at  $37^{\circ}\text{C}$  for 2 min, extension at  $72^{\circ}\text{C}$  for 2 min., and final elongation at  $72^{\circ}\text{C}$  for 8 min. PCR products were analyzed by electrophoretic separation on 1% agarose gel with addition of 0.2% Midori Green DNA Stain (Nippon Genetics Europe), run at a constant voltage of 60 V for 1 h, and visualized and photographed under UV light (GelDoc, BioRad).

**Detection of surface proteins.** The surface protein extracts were prepared according to Gatti *et al.*, (1997) with some modifications. 1 ml of the harvested cells of overnight bacterial strain cultures was washed twice in distilled sterile water and resuspended to a final volume of 1.5 ml. The suspensions were centrifuged ( $8000 \times g$ , 10 min at  $4^{\circ}\text{C}$ ). Cell wall proteins were extracted from final pellets with 0.5 ml of 0.01 mol  $\times$  l<sup>-1</sup> Tris-HCl, 0.01 mol  $\times$  l<sup>-1</sup> EDTA, 0.01 mol  $\times$  l<sup>-1</sup> NaCl, 2% SDS, pH 8 and heated at  $100^{\circ}\text{C}$  for 5 min. Next, the supernatants were centrifuged ( $11600 \times g/10 \text{ min}$  at  $4^{\circ}\text{C}$ ). Protein concentration was determined with the Bradford method.

Cell wall protein extracts thus obtained were examined on SDS-PAGE with the use of a MiniProtean apparatus (Bio-Rad) and according the Laemmli method (1970) on vertical slab gels, using a stacking gel containing 4% acrylamide and 10% resolving gel. Electrophoresis was carried out at 120 V for 60 min using the MiniProtean

apparatus (Bio-Rad). Proteins were visualized by staining with Coomassie Brilliant Blue R-250 (Sigma).

**Bacterial adhesion capacity.** Human colon adenocarcinoma cell line HT-29 (ATCC no. HTB-38) was used to assess the bacterial adhesion capacity. Cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS; Gibco™, Paisley, UK) and antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin; Sigma, St. Louis, MO) at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>. Cells were seeded onto a 24-well tissue culture plate (Nunc, Roskilde, Denmark) at a concentration of  $5 \times 10^5$  cells  $\times$  ml<sup>-1</sup>. After 24-h incubation, a monolayer was obtained. The bacterial strains were resuspended in the HT-29 growth medium at a final concentration of  $5 \times 10^7$  cells  $\times$  ml<sup>-1</sup> and 1 ml of each suspension was added to appropriate wells of the culture plate. After 2-h incubation, the monolayers were washed three times with phosphate-buffered saline (PBS with Ca<sup>2+</sup> and Mg<sup>2+</sup> ions, pH 7.4) to remove bacteria that had not attached to the HT-29 cells. Thereafter, the cells were lysed using 0.1% (v/v) Triton-X100 (Sigma, St. Louis, MO) and the number of viable adherent bacteria was determined by plating serial dilutions on MRS agar plates. The results of adhesion assays are expressed as the adhesion index for each strain (A<sub>i</sub>), which is defined as the number of bacterial cells adhering per 100 epithelial cells (Gopal *et al.*, 2001; Polak-Berecka *et al.*, 2014).

**Auto- and co-aggregation assays.** The ability of each strain to autoaggregate was assessed according to the method of Golowczyc *et al.* (2007) with a slight modification (Polak-Berecka *et al.*, 2014). Briefly, bacterial strain cultures were harvested in the stationary phase, collected by centrifugation (10000  $\times$  g for 10 min), washed twice, and resuspended in PBS (pH 7.2). In all experiments, bacterial suspension was standardized to OD<sub>600</sub>=1.0 ( $2 \times 10^8$  CFU  $\times$  ml<sup>-1</sup>). Optical density was measured in a spectrophotometer (BioRad, Germany) at regular intervals (2, 3, 4 and 5 h) without disturbing the microbial suspension, and the kinetics of sedimentation were obtained. The autoaggregation coefficient and was calculated according to Polak-Berecka and coworkers (2014).

In the coaggregation assay, suspensions of bacterial cells were obtained as described above. Pathogenic bacteria were harvested in the stationary phase by 4-min centrifugation at 5000  $\times$  g and resuspended in PBS (pH 7.2). One milliliter of *L. helveticus* strain suspensions and 1 ml of pathogenic bacterial suspension at the same optical density (OD<sub>600</sub>=1.0) were mixed. Optical density was measured at regular intervals (2, 3, 4 and 5 h) in order to obtain the kinetics of sedimentation. The coaggregation coefficient was calculated in accordance with (Polak-Berecka *et al.*, 2014).

## RESULTS

The analyzed strains of *L. helveticus* exhibited diversified growth dynamics on MRS supplemented with NaCl, especially at concentrations higher than 3% (Fig. 1 A–E). Generally, with increasing salt concentrations in MRS broth, the dynamics of the growth of the strains were slower. The T105 strain was the most susceptible to the changes in the salt concentrations in the culture medium and exhibited the lowest value of optical density during the incubation time (Fig. 1E). Other strains showed similar growth curves, particularly DSM and T80 (Fig. 1B, C), while B734 showed higher sensitivity to the 4% NaCl concentration.

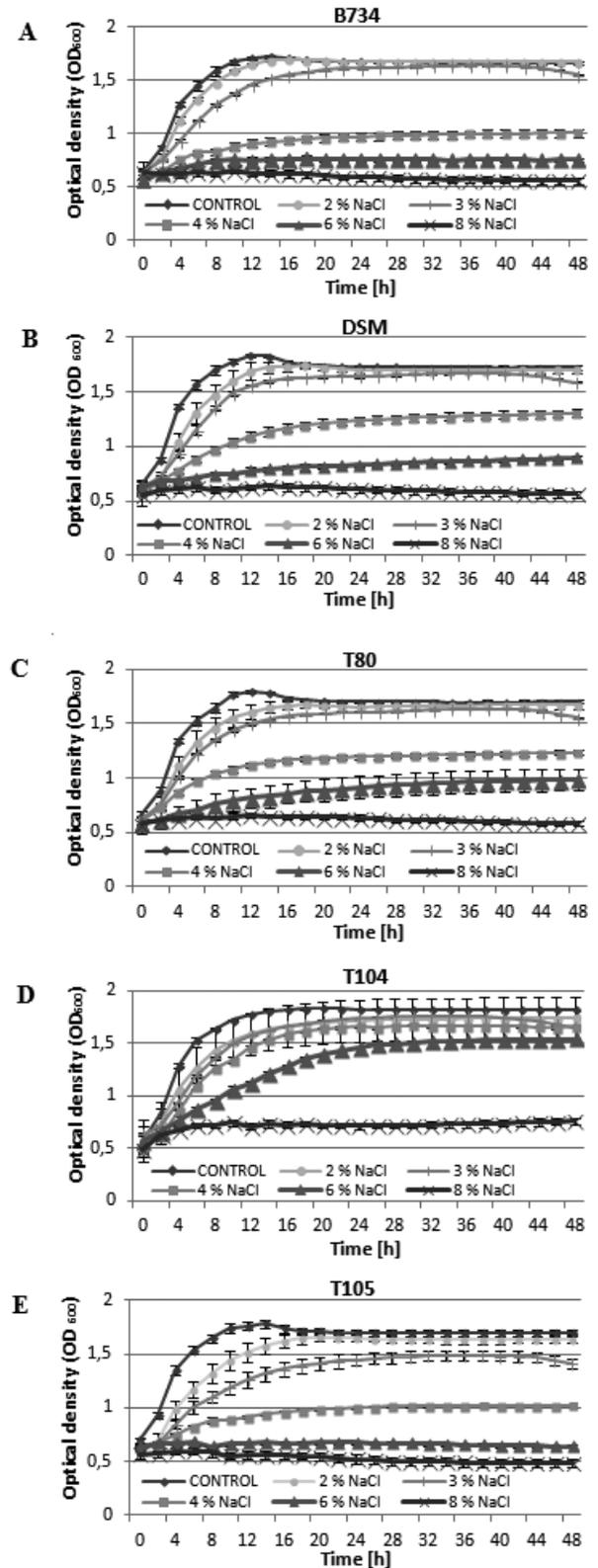


Figure 1. Effect of different NaCl concentrations on the growth capacity of *L. helveticus* strains during 48-h incubation: A, B734; B, DSM; C, T80; D, T104; E, T105.

**Table 1. Survival of bacterial strains (dilution 10<sup>6</sup>) after 24 h (and 72h for the T105 and B734 strains) digestion in simulated small intestinal juice.**

Strain of <i>L. helveticus</i>	Survival in simulated small intestinal juice (log CFU ml <sup>-1</sup> )
B734*	36.7
DSM	35.65
T80	35.65
T104	39.75
T105*	33.95

Explanatory notes: \*enumerations were carried out after 72h

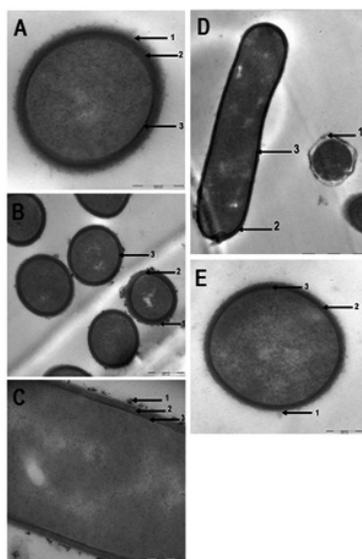
**Table 2. Antibiotic susceptibility exhibited by *L. helveticus* strains.**

Antibiotic	Concentration [µg]	Strain				
		B734	DSM	T80	T104	T105
Ampicillin	10	S	S	S	S	S
Bacitracin	10	I	I	I	I	I
Nalidixic acid	30	R	R	R	R	R
Rifampicin	5	S	S	S	S	S
Gentamicin	120	I	I	I	I	I
Erythromycin	15	S	I	S	S	S

Explanatory notes: R-resistance; I-intermediate; S-susceptible

A typical logarithmic growth phase of the strains was not registered in the medium with salt content exceeding 6%. The fastest cell mass growth was observed in the control samples.

The diversity of the bacterial strains was also exhibited by their survivability in simulated small intestinal juice (Table 1). The T104 strain was the least sensitive to the simulated digestion conditions, as the most numerous colonies were obtained (39.75 log CFU ml<sup>-1</sup>). For T105 and B734, the incubation time had to be prolonged for another 24 hours. These strains demonstrated weaker re-



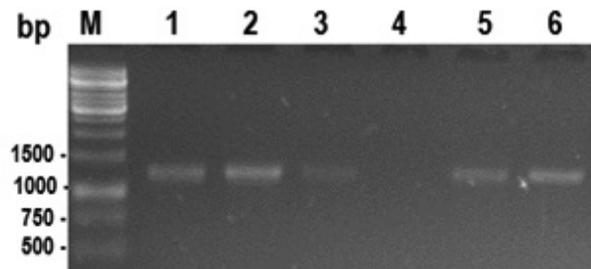
**Figure 2. *L. helveticus* cells displayed under transmission electron microscopy:** A: B734; B: T105; C: DSM; D: T104; E: T80; 1: S-layer; 2: cell wall; 3: cell membrane.

generation ability and a slower growth rate; after 48 h, only an initial phase of colony formation was observed. The DSM and T80 strains exposed to digestion by simulated small intestinal juice demonstrated the same level of cell survival (35.65 log CFU ml<sup>-1</sup>).

The antibiotic susceptibility disc diffusion test (Table 2) revealed that the tested strains exhibited phenotypic resistance only to nalidixic acid.

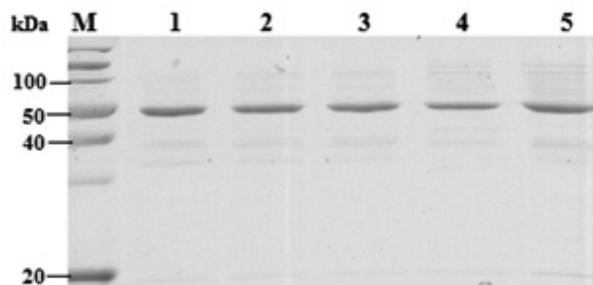
TEM images indicate the presence of the S-layer in all strains (Fig. 2). Microscopic results referring to the S-layer are confirmed by the gel (Fig. 3) displaying electrophoretic separation of amplification products of the *slpA* gene encoding the surface S-layer protein SlpA. A single 1.2 kb PCR product was obtained from *L. helveticus* B734, DSM, T80, T105 and the probiotic strain of *Bifidobacterium animalis ssp. lactis* BB12, whereas this gene was not identified in T104. All strains were further examined for the presence of the cell surface S-layer using SDS-PAGE, which revealed a potential S-layer protein with molecular weight of approximately 50 kDa in all strains (Fig. 4).

The adherence ability of the analyzed strains was examined *in vitro* as the number of bacterial cells adhering per 100 epithelial cells (HT-29). The adhesion index (A<sub>x</sub>) varied from 0.26 to 20 (Fig. 5), which implies high diversity of the strains in terms of the analyzed feature. Only T80 and T104 demonstrated Ax at a similar level of 0.82 and 0.91, respectively. The



**Figure 3. Agarose gel electrophoresis of the amplified *slpA* gene encoding the surface S-layer protein SlpA of the *Lactobacillus helveticus* strains.**

Lanes: 1: B734; 2: DSM; 3: T80; 4: T104; 5: T105; 6: *Bifidobacterium animalis* subsp. *lactis*. BB12 (probiotic strain) and M: molecular size DNA marker.



**Figure 4. SDS-PAGE surface protein profiles of *L. helveticus* strains.**

Line: 1: B734; 2: DSM; 3: T80; 4: T104; 5: T105; M, molecular weight protein standards.

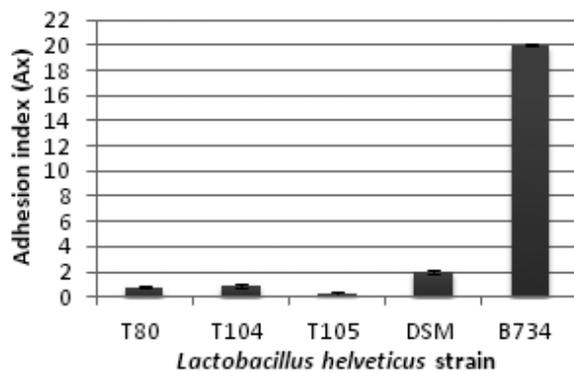


Figure 5. *In vitro* adhesion of *Lactobacillus helveticus* T80, T104, T105, DSM, and B734 to HT-29 cells. Standard deviation was  $\pm 0.1$ .

strongest adherence ability was exhibited by *L. helveticus* B734 ( $A_x=20$ ), which was ten times greater than that of the reference strain, whereas the lowest value of adhesion index was noted for the T105 strain.

The ability to coaggregate is a desirable probiotic property facilitating competitive exclusion of pathogenic microorganisms from the intestinal epithelium. It appears to be a strain-specific feature (Table 3). The values of the measured parameter increased with the longer incubation time. The strongest ability to coaggregate after 5 h was exhibited by the T104 strain with *Escherichia coli*, T80 strain with *Staphylococcus aureus* (43.17%), DSM with *Bacillus subtilis*, and B734 with *Salmonella*.

The autoaggregation capacity was increasing with incubation time (Fig. 6). The highest values were achieved

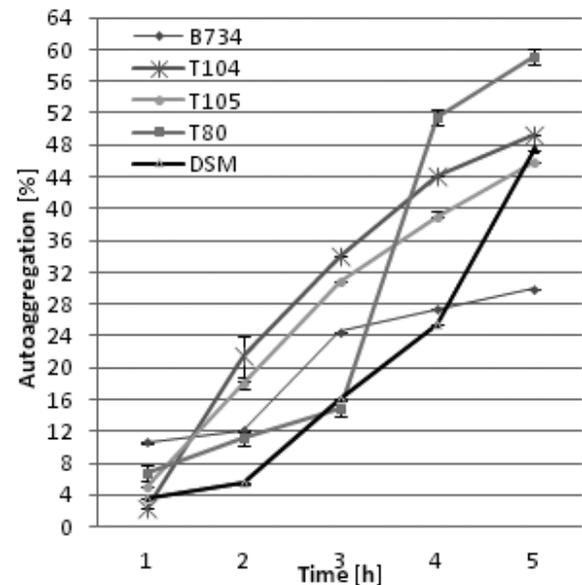


Figure 6. Autoaggregation abilities demonstrated by *L. helveticus* strains

for the T80 strain at 4 h and 5 h of incubation, i.e. 51% and 59%, respectively, whereas the weakest ability to autoaggregation was demonstrated by the B734 strain.

## DISCUSSION

*Lactobacillus helveticus* is considered as a multifunctional LAB, able to produce bacteriocins and generate bioactive

Table 3. Coaggregation percentage of *L. helveticus* strains with the representatives of pathogenic bacteria in 1 h time intervals.

Pathogen	<i>L. helveticus</i> strain	Coaggregation (%)				
		Time (h)				
		1	2	3	4	5
<i>E. coli</i>	T80	8.59 $\pm$ 0.006	10.15 $\pm$ 0.004	17.33 $\pm$ 0.035	19.38 $\pm$ 0.002	27.54 $\pm$ 0.003
	T104	5.18 $\pm$ 0.007	11.43 $\pm$ 0.002	12.78 $\pm$ 0.001	14.29 $\pm$ 0.003	32.95 $\pm$ 0.005
	T105	6.91 $\pm$ 0.014	8.95 $\pm$ 0.008	13.50 $\pm$ 0.009	13.92 $\pm$ 0.002	16.41 $\pm$ 0.005
	B734	8.67 $\pm$ 0.002	14.13 $\pm$ 0.011	14.42 $\pm$ 0.004	15.69 $\pm$ 0.005	28.07 $\pm$ 0.017
	DSM	7.34 $\pm$ 0.092	10.89 $\pm$ 0.010	15.50 $\pm$ 0.036	18.51 $\pm$ 0.009	17.81 $\pm$ 0.002
<i>S. aureus</i>	T80	6.48 $\pm$ 0.012	12.31 $\pm$ 0.006	11.27 $\pm$ 0.002	16.94 $\pm$ 0.006	48.17 $\pm$ 0.008
	T104	7.68 $\pm$ 0.002	9.76 $\pm$ 0.013	22.60 $\pm$ 0.002	26.40 $\pm$ 0.002	32.38 $\pm$ 0.004
	T105	4.60 $\pm$ 0.008	7.46 $\pm$ 0.012	19.82 $\pm$ 0.006	20.62 $\pm$ 0.005	43.92 $\pm$ 0.001
	B734	7.25 $\pm$ 0.003	8.16 $\pm$ 0.006	16.44 $\pm$ 0.001	26.89 $\pm$ 0.002	42.56 $\pm$ 0.003
	DSM	4.98 $\pm$ 0.012	8.10 $\pm$ 0.006	15.36 $\pm$ 0.003	17.80 $\pm$ 0.002	31.43 $\pm$ 0.030
<i>B. subtilis</i>	T80	9.44 $\pm$ 0.006	11.42 $\pm$ 0.003	12.61 $\pm$ 0.002	18.98 $\pm$ 0.002	22.41 $\pm$ 0.001
	T104	5.44 $\pm$ 0.004	11.66 $\pm$ 0.006	21.53 $\pm$ 0.004	25.00 $\pm$ 0.005	30.06 $\pm$ 0.005
	T105	8.55 $\pm$ 0.006	9.08 $\pm$ 0.001	12.86 $\pm$ 0.002	21.29 $\pm$ 0.002	27.10 $\pm$ 0.002
	B734	5.05 $\pm$ 0.002	9.26 $\pm$ 0.002	9.75 $\pm$ 0.003	13.57 $\pm$ 0.004	18.37 $\pm$ 0.002
	DSM	8.81 $\pm$ 0.009	9.17 $\pm$ 0.008	12.46 $\pm$ 0.013	21.55 $\pm$ 0.005	31.23 $\pm$ 0.005
<i>Salmonella</i>	T80	11.54 $\pm$ 0.004	12.46 $\pm$ 0.018	16.39 $\pm$ 0.013	17.11 $\pm$ 0.001	22.45 $\pm$ 0.002
	T104	3.39 $\pm$ 0.005	13.48 $\pm$ 0.005	15.29 $\pm$ 0.004	20.37 $\pm$ 0.003	23.44 $\pm$ 0.004
	T105	1.92 $\pm$ 0.001	10.14 $\pm$ 0.001	12.23 $\pm$ 0.002	18.56 $\pm$ 0.004	26.48 $\pm$ 0.004
	B734	1.26 $\pm$ 0.001	1.30 $\pm$ 0.001	1.49 $\pm$ 0.003	12.30 $\pm$ 0.002	33.17 $\pm$ 0.007
	DSM	2.74 $\pm$ 0.005	11.99 $\pm$ 0.014	13.93 $\pm$ 0.006	19.81 $\pm$ 0.017	23.97 $\pm$ 0.005

peptides in fermented dairy products, which enhances the importance of *L. helveticus* as health-promoting culture in functional food products (Giraffa, 2014).

The health-promoting properties of *L. helveticus* have been reviewed by Taverniti & Guglielmetti (2013), who provide evidence that this species positively influences human health. However, the beneficial health effect of bacterial activities is strictly dependent on the specific strain and the dose used (Perdigon *et al.*, 2002; Ongol *et al.*, 2008).

This study is an attempt to compare several basic requirements imposed on probiotic organisms. The preliminary investigations aim to determine and compare the desirable potential exhibited by the analyzed *L. helveticus* strains, which might be used in dairy applications. Salt tolerance is one of the several factors that must be taken into account in the initial probiotic starter screening. Functional starter cultures have to be able to survive and maintain their health-promoting properties throughout technological processes (Beganović *et al.*, 2014). Salt addition influenced microbial growth and most of the analyzed strains were similarly inhibited by NaCl, with the exception of T104, which was much more resistant. This may affect the possibility of the application of the strains in production of food products, e.g. 4% w/v represents the maximum level of NaCl usually added during sauerkraut fermentations (Beganović *et al.*, 2014) and most of the strains tested showed a high tolerance to the salt content.

The ability to survive under conditions prevailing in the gastrointestinal tract and the capacity to antagonize enteropathogens is advantageous for probiotic bacteria to adhere to the luminal epithelium (Beganović *et al.*, 2014). The *in vitro* study revealed that the survivability in simulated small intestinal juice and cell capacity of regeneration varies among the tested bacteria. The T104 strain appears to be the most suitable to survive these digestion conditions.

*Lactobacillus helveticus* belonging to the group of lactic acid bacteria (LAB) possess Generally Recognized as Safe (GRAS) status (Giraffa, 2014). Nevertheless, the antibiotic resistance pattern of potential probiotic strains must be determined in order to exclude the use of microorganisms containing transferable antibiotic-resistance genes; moreover, absence of antibiotic resistance is considered as a safety prerequisite for selection of a probiotic strain (EFSA, 2008). The analyzed *L. helveticus* strains were sensitive to most of the tested antibiotics but displayed phenotypic resistance to nalidixic acid, which specifically inhibits DNA synthesis. Research conducted by Wiatrzyk *et al.* (2013) revealed that most strains belonging to *Lactobacillus* and *Bifidobacterium* exhibit especially high sensitivity to  $\beta$ -lactam antibiotics (except for some cephalosporins) and significant resistance to aminoglycosides and nalidixic acid. All probiotic strains isolated from investigational medicinal products and analyzed by the scientists were resistant to nalidixic acid (quinolone).

The complete mechanisms of adhesion are not thoroughly understood; however, there is evidence that bacterial cell-surface associated proteins, e.g. S-layer proteins in lactobacilli, could be involved in adhesion through autoaggregation (Kos *et al.*, 2003; Frece *et al.*, 2005; Mobilil *et al.*, 2009; Beganović *et al.*, 2011b). The role of the S-layer in the adherence of *L. helveticus* M92 to mouse and pig intestinal epithelial cells has been demonstrated (Kos *et al.*, 2003; Frece *et al.*, 2005). Studies have confirmed that several species of the genus *Lactobacillus* possess surface S-layer protein (SlpA). Due to their structural regularity and the unique self-assembling proper-

ties, S-layers have potential for many biotechnological applications (Avall-Jääskeläinen & Palva, 2005; Hynönen & Palva, 2013). PCR amplification with specific primers used to amplify the *slpA* gene revealed the presence of a single 1.2 kb product in the B734, DSM, T80, and T105 strains. The amplicon mass obtained is in accordance with the results obtained by Beganović *et al.*, (2011a). Delcour *et al.* (1999) have suggested that the SlpA protein may have the highest probability of the interaction with immune cells associated with the gut.

Lactobacilli S-layer proteins with stable tertiary structures range from 40 to 60 kDa (Lebeer *et al.*, 2008), which is in accordance with the surface protein profiles obtained and presence of a protein with a molecular weight of approximately 50 kDa, moreover the presence of the S-layer in all strains has also been confirmed by the TEM image.

It has been proven that the S-layers of *Lactobacillus* species interact with the receptors on the host epithelial cells and block receptor sites on the mucosal surfaces for the adherence of pathogenic species (van der Mei *et al.*, 2003; Liu *et al.*, 2010). The S-layer protein extract of *L. helveticus* R0052 was able to ameliorate the pathogenesis of *E. coli* O157:H7; moreover, some research indicates that the S-layer protein extract incorporated on epithelial cells prior to infection with *E. coli* O157 maintained their cellular integrity and barrier function (Sherman *et al.*, 2005; Johnson-Henry *et al.*, 2007).

Adhesion to the intestinal epithelium is another criterion to be fulfilled by a probiotic culture. This feature is important for preventing immediate washing out of the strain by intestinal peristalsis (Beganović *et al.*, 2014). Carrying out of probiotic effects is performed by bacterial adhesion, which is a primary requirement for colonization of GIT and an important prerequisite for competitive exclusion of enteropathogens and capability of immunomodulation of the host organism (Kos *et al.*, 2003; Buck *et al.*, 2005; Beganović *et al.*, 2011b). Adherence and colonization of *Lactobacillus* strains in the intestine is the fundamental requirement for bacteria to demonstrate beneficial effects on human health (von Ossowski *et al.*, 2010). Human enterocyte-like HT-29 cell cultures have been used as a model system for *in vitro* methods to determine the adherence ability of *Lactobacillus* strains to intestinal epithelial cells (Servin & Coconnier, 2003). Microorganisms that possess probiotic characteristics vary in their ability to adhere to colonic mucosa (Matto *et al.*, 2006), which is also confirmed by the results presented here i.e. the *in vitro* analysis performed for the *L. helveticus* strains.

The study suggests that adhesion to intestinal mucosa is a significant bacterial property related to the beneficial effects of lactobacilli during intestinal inflammation (Castagliuolo *et al.*, 2005). It was also reported that probiotic microorganisms with a higher adhering capacity could adhere to colonic mucosa (Gueimonde *et al.*, 2006). This property is essential for colonization of the gastrointestinal tract by probiotic lactic acid bacteria and ensures their functional stability in the intestine (Rosenfeldt *et al.*, 2003). Based on these reports, the B734 strain might be chosen as having the highest potential for adhesion in the human gastrointestinal tract, but this ability is ultimately determined by many other factors.

Coaggregation is part of the competitive exclusion mechanism, which, combined with the antimicrobial activity of the probiotic strain, might be supportive in treatment of infectious diseases (Beganović *et al.*, 2011a). Coaggregation is an important factor in elimination of pathogens from the gastrointestinal tract, i.e. in mecha-

nisms preventing pathogen adherence to host intestinal epithelial tissue (Todorov *et al.*, 2008). It has been proven that *Lactobacillus* is able to form a barrier through coaggregation that prevents colonization by undesirable microflora (Ferreira *et al.*, 2011). The coaggregation capacity might be a preliminary screening determinant for administration of probiotic bacteria to humans. The *L. helveticus* strains tested showed varying degrees of coaggregation depending on the pathogen and time. The highest percentage values were exhibited by the T80 strain coaggregating with *S. aureus* (48.17%), while T105 reached the lowest values (16.41%) with *E. coli* samples. Gueimonde *et al.*, 2006 noted a very high specificity in the inhibition of adhesion and displacement of enteropathogens by lactobacilli and suggested that case-by-case assessment should be done in order to select strains with the ability to inhibit or displace a specific pathogen.

Vlková and coworkers (2008) reported that auto-aggregation and coaggregation abilities might be used together for selection of probiotic bacteria. Both processes are crucial in biofilm formation, which protects the host from pathogen colonization (Ocaña & Nader-Macías, 2008). Taking into account those two determinants, the T80 strain appears to have the greatest ability to auto-aggregate after a 5-h incubation and exhibits the highest degree of coaggregation with pathogenic *S. aureus*. It has been suggested that the surface-bound protein influences adherence and autoaggregation abilities of strains (Nikolic *et al.*, 2010; 2012). In turn, Ramiah *et al.*, (2008) concluded that other factors were responsible for these properties. Thus, further studies are required to clarify the differences between strains in the capability of auto-aggregation and coaggregation.

## CONCLUSIONS

Appropriate assessment of microorganisms with different methods is essential for selection and evaluation of maximum survival of strains exhibiting a health-promoting potential. This is of great importance for development of starter cultures' formulations for functional food production. The preliminary *in vitro* studies reveal that the analyzed *L. helveticus* strains have a great potential, especially the T80 strain, but further studies are needed to confirm the targeted health-promoting properties like immunomodulation or antagonist activities. The *Lactobacillus helveticus* strains tested might be used in dairy applications and bring an additional functional value to the final products.

## Conflicts of interests

The authors declare no conflict of interests.

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