

## LACTOBACILLUS SPP. BELONGING TO THE CASEI GROUP DISPLAY A VARIETY OF ADHESINS

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### ABSTRACT

**Introduction.** Adhesion of bacteria from the genus *Lactobacillus* to the gastrointestinal epithelium is, to a considerable degree, dependent on the interactions between adhesins found on the surface of bacterial cells and elements found within the epithelium. A significant role in these interactions is played by bacterial proteins exposed to the cell wall surface, which are capable of binding to molecules of substances comprising the extracellular matrix of the intestinal epithelium.

**Materials and methods.** In order to analyze the extracellular proteome of intestinal bacteria in terms of the presence of cell adhesion molecules, a total of twenty strains from the *Lactobacillus* spp. group *Casei* were tested. The analyses were conducted using SDS PAGE, 2-D electrophoresis, Western blot and mass spectrometry. An experiment was also conducted to assess the adhesion capacity of the tested strains to cervical epithelial cells (HeLa).

**Results.** The tested strains varied in their adhesion efficiency to HeLa cells, ranging from 0.5% to 29%. Using electrophoretic methods a total of 54 extracellular protein fractions were distinguished in these strains, additionally identifying potential adhesion molecules (e.g. a surface antigen of the NLP/P60 family and a small heat shock protein/chaperonin).

**Discussion.** The identification of these proteins in the extracellular proteome of *Lactobacillus* spp. isolates may suggest that they serve currently unknown functions on the cell surface, including those connected with the interactions between bacteria and the intestinal epithelium. Such analyses may provide insight into new factors promoting probiotic adhesion to various types of epithelial cells.

**Keywords:** *Lactobacillus*, adhesion, protein, epithelium

### INTRODUCTION

Bacteria from the genus *Lactobacillus* have been thoroughly investigated due to their considerable potential probiotic activity, which has a positive effect on human health. An important functional characteristic of probiotics is connected with their capacity to attach to the epithelium, which promotes their extended positive effect on the host and determines certain parameters such as competition and exclusion of pathogenic bacteria. A decisive role in adhesion is played by

factors found on the surface of bacterial cells, referred to as adhesins. This group includes proteins, exopolysaccharides, lipoproteins and proteoglycans. In this respect, great importance is ascribed to proteins capable of binding to molecules of various substances found on the surface of the intestinal epithelium. The probiotic extracellular proteome comprises proteins participating in bacterial adhesion to epithelial cells. These proteins may be divided into two categories, one of which

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is composed of sortase-dependent proteins (SDPs). At the C-end they have the LPXTG motif, facilitating their recognition by sortase and the subsequent attachment of proteins to amino groups of the cell wall peptidoglycan (Call and Klaenhammer, 2013). The other group of adhesion proteins comprises proteins of the S-layer. This is the outermost layer found in most *Lactobacillus* strains, composed mostly of proteins which account for approx. 13% total protein content in the bacterial cell (Chen et al., 2009; Khaleghi and Kermanshahi, 2012). It was established that the common characteristic of many proteins in the S-layer is that they are connected with the SLH motif at the N-end, which permanently binds proteins with the bacterial cell wall surface (Hynonen, 2009).

Proteins of the SDP family include adhesins, which bind various molecules found on the surface of enterocytes. Some of the better known of these are the mucin-binding proteins, which are embedded in the mucus produced by the intestinal epithelium. They include e.g. large molecular (352 kDa) MUB proteins equipped with the Mub domain responsible for binding mucin molecules (Boekhorst et al., 2006). Domains belonging to the mucin-binding protein family (MucBP) are also found in other proteins involved in the adhesion of bacteria from the genus *Lactobacillus* to the epithelium.

The capacity to bind mucus was also shown for the SpaC subunit of the fimbrial SpaCBA protein complex. It is the only fimbrial adhesin, the presence and participation of which in the adhesion of *Lactobacillus* bacteria to enterocytes have been confirmed. It is not clear which amino acid sequence in the SpaC subunit structure is responsible for mucus binding. To date, the only sequences which have been detected are those resembling the Willenbrand factor domain A and domain B, which can be found in the collagen-binding protein in *Staphylococcus aureus* (Kankainen et al., 2009).

The SDP-type proteins may also be bound with elements of the extracellular matrix of intestinal epithelial cells, such as collagen (p29 from *L. fermentum* RC-14), fibronectin (FbpA from *L. acidophilus* NCFM) and laminin (Buck et al., 2005; Heinemann et al., 2000).

In the case of S-layer proteins, adhesive properties were shown *in vitro* for the SlpA protein. Silencing its encoding gene in *Lactobacillus acidophilus* reduced the adhesion efficiency of the bacteria to enterocytes (Buck et al., 2005). It is the best characterized S-layer

protein. It is equipped with a domain responsible for binding to fibronectin (de Leeuw et al., 2006). The S-layer proteins may also bind with collagen and laminin. An example of this may be provided by the CbsA protein from *L. crispatus* JCM 5810, which is equipped with both these domains. Another known function of SLPs is that it binds with elements of plasma, e.g. proteins identified in *L. brevis* OLL2772, and may bind with the human blood A antigen, also found in the intestinal mucus (Uchida et al., 2006). The adhesion of *Lactobacillus* bacteria to the intestinal epithelium may also involve proteins which do not function only as adhesins, but also serve other important functions within the cell. One of the better known proteins is GAPDH (glyceraldehyde 3-phosphate dehydrogenase), an important glycolytic enzyme. GAPDH may be secreted outside the cell wall, where it attaches to it via an electrostatic charge (Antikainen et al., 2007b). This protein is responsible for adhesion to the mucus produced by the intestinal epithelium, similarly to another glycolytic enzyme, enolase. However, enolase has the capacity to bind not only to the mucus, but also fibronectin (Castaldo et al., 2009), collagen and laminin (Antikainen et al., 2007a).

The capacity to bind to the mucus is also observed in the case of the Tu factor, a protein involved in the process of translation, identified on the cell surface of *Lactobacillus* bacteria (Granato et al., 2004) as well as the GroEL chaperonin, belonging to the class of conservative heat shock proteins. Both these proteins may also have an immunomodulatory effect by stimulating the IL-8 synthesis in intestinal epithelial cells (Bergonzelli et al., 2006).

The aim of this study was to search for both known and potential adhesins involved in adhesion of twenty bacterial strains from the *Lactobacillus* spp. group *Casei* exhibiting varied adhesion rates to HeLa and Caco-2 cells and to the mucus.

## MATERIALS AND METHODS

### Bacterial strains and epithelial cells

Twenty strains of *Lactobacillus* spp. belonging to *Casei* group and originating from the collection of the Department of Biotechnology and Food Microbiology, the Poznań University of Life Sciences (Table 1) were used in the presented study. Bacterial cultures were

**Table 1.** Lactic acid bacteria isolates used in this study

Isolate designation	Strain	Source
DS1	<i>L. rhamnosus</i> KL53A	dietary probiotic supplement Lactoral, Biomed–Krakow, Poland
DS2	<i>L. rhamnosus</i> GG	dietary probiotic supplement Dicoflor, Vitis Pharma, Poland
DS3	<i>L. rhamnosus</i> LNII3	probiotic supplement Ellen, Ellen AB, Sweden
DS5	<i>L. casei</i> Shirota	probiotic drink Yakult, Yakult Europa, Germany
DS6	<i>L. paracasei</i> LOCK 0919	dietary probiotic supplement Latopic, Biomed–Krakow, Poland
DS7	<i>L. rhamnosus</i> DMS 14870	probiotic supplement Ecovag, Krotex, Poland
DS8	<i>L. casei</i> Fyos	probiotic drink Fyos, Nutricia, Belgia
DS9	<i>L. rhamnosus</i> 573/2/P	dietary probiotic supplement Lakcid, Biomed–Lublin, Poland
DS10	<i>L. rhamnosus</i> 573	probiotic supplement Lactovagnial, Biomed–Krakow, Poland
DS11	<i>L. rhamnosus</i> GR1	probiotic supplement Lacibios, ASA, Poland
DS12	<i>L. rhamnosus</i> 573/3/EN	dietary probiotic supplement Lakcid, Biomed–Lublin, Poland
DS13	<i>L. rhamnosus</i> 573/2/T	dietary probiotic supplement Lakcid, Biomed–Lublin, Poland
DS14	<i>L. rhamnosus</i> ŁOCK 0908	dietary probiotic supplement Latopic, Biomed–Krakow, Poland
HI1	<i>L. paracasei</i> HI1	human stool
HI2	<i>L. casei</i> HI2	human stool
HI3	<i>L. casei</i> HI3	human stool
HI4	<i>L. rhamnosus</i> HI4	human stool
HI5	<i>L. rhamnosus</i> HI5	human stool
HI6	<i>L. casei</i> HI6	human stool
HI7	<i>L. casei</i> HI7	human stool

cultivated in liquid MRS medium (Biocorp, Poland) at 37°C under anaerobic conditions. The HeLa cell line (obtained from the European Collection of Animal Cell Cultures – ECACC) were propagated in DMEM medium supplemented with 10% fetal bovine serum used to test bacterial adhesion to the epithelial cells.

#### Isolation of extracellular proteins

Extracellular proteins were isolated from bacterial cells obtained from the culture grown in a volume of 100 ml. Cell biomass was separated by centrifugation at 4500 rpm for 1 h and rinsed with PBS. The cells were suspended in 30 ml lysis buffer (50 mM Tris, 1 mM EDTA, 0.1% SDS) containing a protease inhibitor (Sigmafast™ Protease Inhibitor Tablets,

Sigma-Aldrich) and incubated with vortexing for 30 min. Samples were centrifuged at 4500 rpm for 1 h. Proteins were precipitated from the supernatant by adding trichloroacetic acid until 1% final concentration in presence of 0.1% sodium deoxycholate, and incubated overnight at 4°C. The next day, the precipitated proteins were centrifuged (4500 rpm, 30 min), and rinsed twice with 25% methanol. Samples were dried and stored at –80°C.

#### SDS PAGE electrophoresis of protein isolates

Protein samples were stored at –80°C in a 0.1 M Tris-HCl pH 0.8 buffer containing 10% glycerol (v/v). Prior to being transferred to the gel samples, the samples were mixed at a 1:1 ratio with loading buffer

(0.34 M Tris-HCl, 0.4% SDS, 20% glycerol, 1.716 M  $\beta$ -mercaptoethanol, 0.2% bromophenol blue, pH 6.8). Separation was carried out using commercial 12% polyacrylamide gels (Mini Protean TGX Gels, Bio-Rad) in an SDS electrode buffer (0.25 M Tris-HCl, 1% SDS, 1.92 M glycine) at a constant voltage of 200 V for 30 min. Following separation, the gels were stained overnight in a staining solution (40% methanol, 7% acetic acid, 0.025% Coomassie brilliant blue R-250) and then rinsed in a destaining solution (10% methanol and 8% acetic acid) for 3 h. Gels were documented using a gel documentation system and analyzed with the Quantity One software (Bio-Rad).

### Western blot analysis

Protein samples were separated by SDS PAGE using 12% polyacrylamide gels (Mini-PROTEAN® TGX™ Precast Gels, Bio Rad). Upon completion of electrophoretic run, the separated proteins were transferred onto nitrocellulose membrane with iBlot Gel Transfer Stacks PVDF kit (Novex) in iBlot apparatus (Invitrogen). Selected proteins were detected with antibody targeting TUFM and GAPDH (Table 2).

Stained membranes were documented using a GS-800 Calibrated Densitometer (Bio-Rad) and analyzed using the PDQuest software (Bio Rad).

### Two-dimensional (2D) electrophoresis of protein isolates

Two-dimensional separation of tested protein isolates was run using a commercial Ready Prep 2D Starter

Kit (Bio Rad) following the manufacturer's protocol. Upon completion of the separation, the gels were stained overnight with a staining buffer. They were destained with a destaining buffer for 3 h. The gels were documented using a gel documentation system and analyzed with the PDQuest 2D Gel Analysis software (Bio-Rad).

### Analysis of spots excised from 2D gels

Gels containing extracellular proteins from the tested bacterial strains were compared qualitatively and quantitatively in reference to bacterial adhesion to the epithelium using the PDQuest 2D Gel Analysis software (Bio-Rad). Ten spots differentiating weakly and strongly adherent strains were selected. Spots were cut and sent for analysis at the Mass Spectrometry Laboratory, the Institute of Biochemistry and Biophysics PAS in Warsaw to be analyzed using LC-ESI-MS/MS tandem mass spectrometry.

### Adhesion of the strains to HeLa cells

Adhesion assay was performed using HeLa cells. To label bacteria, methyl-[3H]-thymidine (60–90 Ci/mmol, 1 mCi/ml; Hartmann Perkin Elmer, USA) was added to the MRS medium at a volume of 5  $\mu$ l/ml of the broth. After 18–20 hours of growth, the bacteria were washed twice with sterile Hank's Buffered Salts Solution (HBSS) and resuspended in the same buffer. Monolayers of HeLa cells were prepared in 6-well tissue-culture dishes (Merck-Millipore, Poland) by inoculating  $2 \cdot 10^6$  viable cells per well in 2.0 ml culture

**Table 2.** Protein detection procedures using western blotting technique

Stage	Procedure using mouse antibodies	Procedure using rabbit antibodies
Blocking	rinsing in PBS containing 1% BSA, 1 h, 22°C	rinsing in TBSTT buffer (5M NaCl, 200 mM Tris HCl, 2% Triton v/v, 0.5% Tween 20 v/v, pH 7.5) containing 1% BSA, 1 h, 22°C
Primary antibodies	antibodies to TUFM proten, dilution 1:1000, incubation overnight at 4°C	antibodies to GAPDH proten, dilution 1:1000, incubation overnight at 4°C
Rinsing	washing membrane with PBS; rinsing with 2 $\times$ concentrated PBS for 5 min; rinsing with PBS containing 0.02% Tween 20 for 5 min	5 $\times$ concentrated TBSTT buffer for 5 min
Secondary antibodies	anti-mouse IgG antibodies, dilution 1:1000	anti-rabbit IgG antibodies, dilution 1:1000
Visualization	DAB (Tetrahydro-3,3'-diaminobenzidine), to obtain the desired color	DAB, to obtain the desired color

medium. The cells were cultivated to obtain 100% confluence. The cell monolayers were washed once with 1 ml sterile HBSS before adhesion assay. Bacteria at concentrations of approximately  $5 \cdot 10^8$  cfu/ml were added to each well in 2.0 ml (total volume) HBSS and incubated at 37°C in an atmosphere of 10% (v/v) CO<sub>2</sub> in air. After 2 h incubation, the monolayers were washed three times with sterile HBSS to remove free bacterial cells. The amount of adhered bacterial cells was estimated from radioactivity remaining in the HeLa monolayer. Each assay was performed in triplicate. Radiolabeled bacteria in the amount initially added for adhesion and the washed HeLa monolayer with adhered radiolabeled bacteria were lysed in 0.9 ml of 1% SDS, then 0.1 ml of 1 M NaOH was added and the lysate was incubated overnight at 60°C to complete lysis. The radioactivity of the lysed suspension was measured after the addition of Hionic-Fluor scintillation cocktail (Perkin-Elmer, Poland) in a Beckmann LS6500 counter. Adhesion efficiency was expressed as the percentage of bacterial cells remaining on the surface of epithelial cells after rinsing proportional to the number of bacterial cells initially transferred onto the epithelial cells.

## RESULTS

### Analysis of strain- and substrate-specific adhesion capacities of *Lactobacillus* spp. from the *Casei* group

Most studies on the capacity of lactic acid bacteria to adhere to epithelial cells have been conducted using cell lines functionally similar to enterocytes (e.g. the Caco-2 line) and in relation to the gastrointestinal mucus. The capacity of the tested isolates to adhere to the surface of Caco-2 cells and to the mucus was presented in our earlier papers (Markowicz et al., 2014; 2016). The results of these studies showed that the rates of adhesion to enterocytes and to the mucus are not comparable in all cases and they vary between individual tested isolates. In view of the fact that some of the tested isolates were obtained from probiotic preparations designed to normalize the vaginal microbiota (*L. rhamnosus* LNII3, *L. rhamnosus* DMS14870, *L. rhamnosus* 573, *L. rhamnosus* GR1), we decided to test the bacteria in terms of their capacity to adhere to cervical epithelial cells (HeLa). Similarly, as with

the Caco-2 cells, the tested strains showed varied adhesion rates to HeLa cells, which in many cases was not equivalent to their capacity to adhere to enterocytes or to the mucus (Table 4). It ranged from 0.5% to 29%, whereas – except for the isolate showing the greatest adhesion to HeLa cells (*L. rhamnosus* LNII3) – the other probiotic strains designed to improve the genital tract microbiota exhibited low (*L. rhamnosus* 573 and *L. rhamnosus* GR1) and moderate (*L. rhamnosus* DMS14870) adhesion rates (Fig. 1).

A comparison of the adhesion capacity of individual strains of *Lactobacillus* spp. belonging to the *Casei* group in relation to the three analyzed substrates (Caco-2, HeLa and mucus; Table 4) indicates that adhesion to a given substrate is an independent trait, which suggests that it must be determined by a different type of adhesins, while strains exhibiting good adhesion to many substrates need to be equipped with many adhesins, instead of a single universal one. For this reason the aim of further analyses was to identify potential adhesins.

### SpaCBA pili expression differences in *Lactobacillus* spp. from the *Casei* group

We showed in one of our previous papers that some of the tested strains do not have the sequence encoding either the SpaCBA pilus or its individual proteins. Additionally, the present sequence of the SpaCBA operon shows polymorphism and is not expressed in all cases (Markowicz et al., 2014). For this reason the first of the undertaken analyses was to confirm expression of proteins forming the SpaCBA pilus. Based on the SDS PAGE separations of extracellular proteins from the twenty tested bacterial strains, the observed profiles of separated proteins were found to include bands with masses corresponding to individual SpaCBA protein complex subunits participating in their adhesion to the intestinal mucus (Kankainen et al., 2009; Table 3).

The results of these studies indicate that certain strains express only one or two proteins from the SpaCBA heterotrimer. In this case (*L. rhamnosus* KL53A, *L. paracasei* HI1, *L. rhamnosus* HI5, *L. casei* HI7), the expression of all the three SpaCBA proteins may confirm good adhesion capacity towards the mucus, while incomplete SpaCBA expression in the case of good adhesion to the mucus indicates potential involvement of

**Table 3.** Identification of proteins comprising the SpaCBA pilus complex being the main adhesin to the intestinal mucus in the tested isolates of *Lactobacillus* spp. group *Casei* from probiotic preparations and from human feces in SDS-PAGE separations of extracellular proteins

Code	Isolate	Protein fraction corresponding to the weight of the subunits of the Spa CBA protein complex		
		spaA (31 kDa)	spaB (21 kDa)	spaC (91 kDa)
DS1	<i>L. rhamnosus</i> KL53A	+	+	+
DS2	<i>L. rhamnosus</i> GG	–	–	+
DS3	<i>L. rhamnosus</i> LNII3	–	–	–
DS5	<i>L. casei</i> Shirota	–	+	–
DS6	<i>L. paracasei</i> LOCK 0919	–	+	–
DS7	<i>L. rhamnosus</i> DMS14870	–	–	–
DS8	<i>L. casei</i> Fyos	+	–	+
DS9	<i>L. rhamnosus</i> 573/2/P	+	–	–
DS10	<i>L. rhamnosus</i> 573	–	–	–
DS11	<i>L. rhamnosus</i> GR1	+	–	+
DS12	<i>L. rhamnosus</i> 573/3/EN	–	–	–
DS13	<i>L. rhamnosus</i> 573/2/T	+	–	+
DS14	<i>L. rhamnosus</i> ŁOCK 0908	–	–	–
HI1	<i>L. paracasei</i> HI1	+	+	+
HI2	<i>L. casei</i> HI2	+	–	–
HI3	<i>L. casei</i> HI3	–	–	+
HI4	<i>L. rhamnosus</i> HI4	+	–	+
HI5	<i>L. rhamnosus</i> HI5	+	+	+
HI6	<i>L. casei</i> HI6	+	–	+
HI7	<i>L. casei</i> HI7	+	+	+

other adhesins. This could be caused by the lack of SpaB, a protein determining the attachment of pili to the cell wall. SpaB is thought to act as a molecular switch that terminates pilus polymerization and predestines mature pili to be covalently attached to the peptidoglycan by the housekeeping sortase (Reunanen et al., 2012).

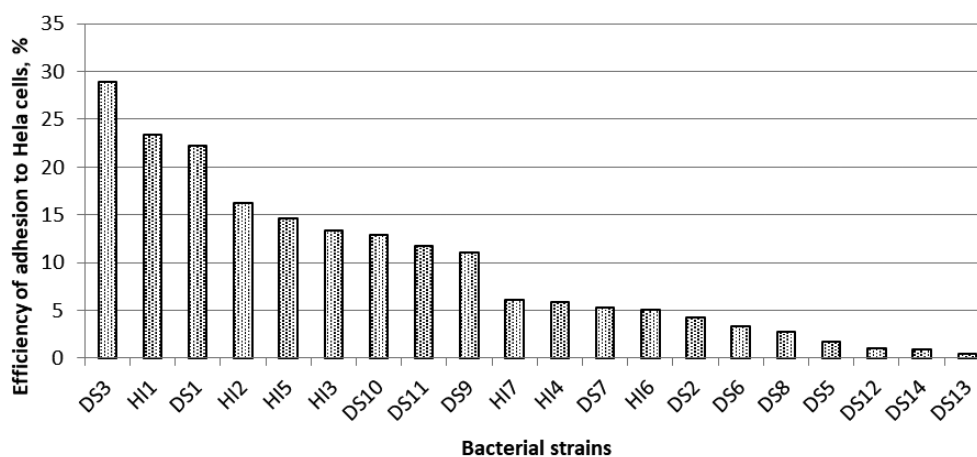
#### Detection of GAPDH and TUFM in extracellular protein fractions in the tested strains

The role of the GAPDH protein as an adhesin was confirmed in various bacterial species, including numerous bacteria from the genus *Lactobacillus*

(Kainulainen et al., 2012; Kinoshita et al., 2012; Terrasse et al., 2015). The role of the TUFM protein in promoting the adhesion of bacteria (e.g. *Lactobacillus johnsonii* NCC533, *Staphylococcus aureus*, *Mycoplasma pneumoniae*, bacteria from the genus *Leptospira*) to the epithelium has been confirmed (Granato et al., 2004; Widjaja et al., 2017; Wolff et al., 2013). Western-blot detection of GAPDH in the extracellular protein fraction of the tested isolates showed the presence of proteins with molecular masses around 22 kDa, corresponding to the molecular mass of this protein, in bacteria of the *Lactobacillus* spp. group *Casei*.

**Table 4.** Proteins differentiating isolates of *Lactobacillus* spp. group *Casei* with varying adhesion properties detected in extracellular protein fractions

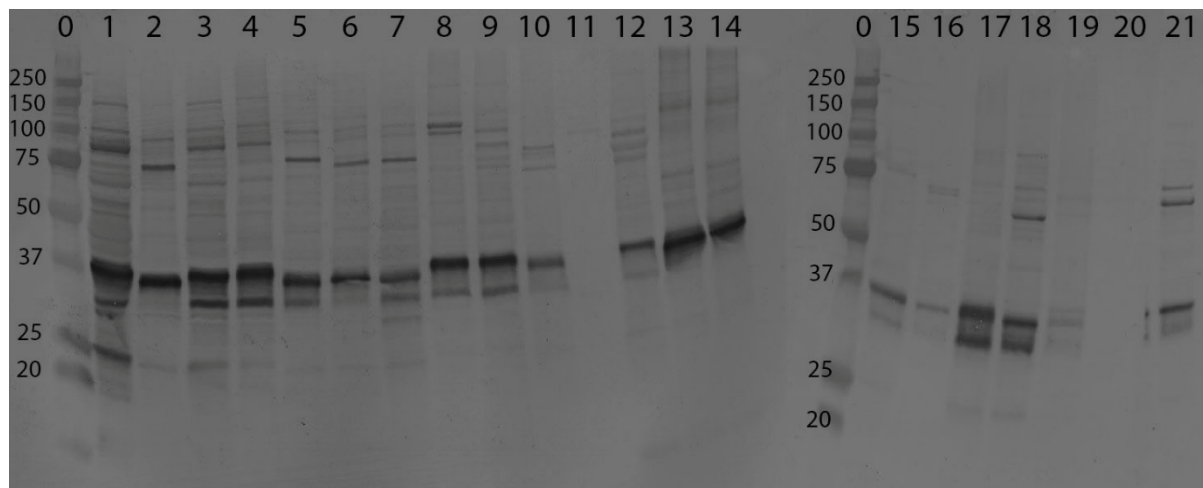
Code	Isolate	Differentiating proteins (gi number)
DS1	<i>L. rhamnosus</i> KL53A	surface antigen NLP/P60 (gi 258509011) 50S ribosomal protein 5L (gi 585118682)
HI1	<i>L. paracasei</i> HI1	FKBP-type peptidylprolyl isomerase (gi 301066386) molecular chaperone/small heat shock protein (gi 489702078) nucleoid DNA-binding protein (gi 116494867)
DS8	<i>L. casei</i> Fyos	cold-shock protein (gi 585117967) cold-shock protein CspA (gi 521378955) LGG_02452 hypothetical protein (gi 258509447); LSEI_2467 hypothetical protein (gi 116495911)
DS2	<i>L. rhamnosus</i> GG	surface antigen NLP/P60 (gi 258509011)
HI6	<i>L. casei</i> HI6	30S ribosomal protein S2 (gi 585117517)



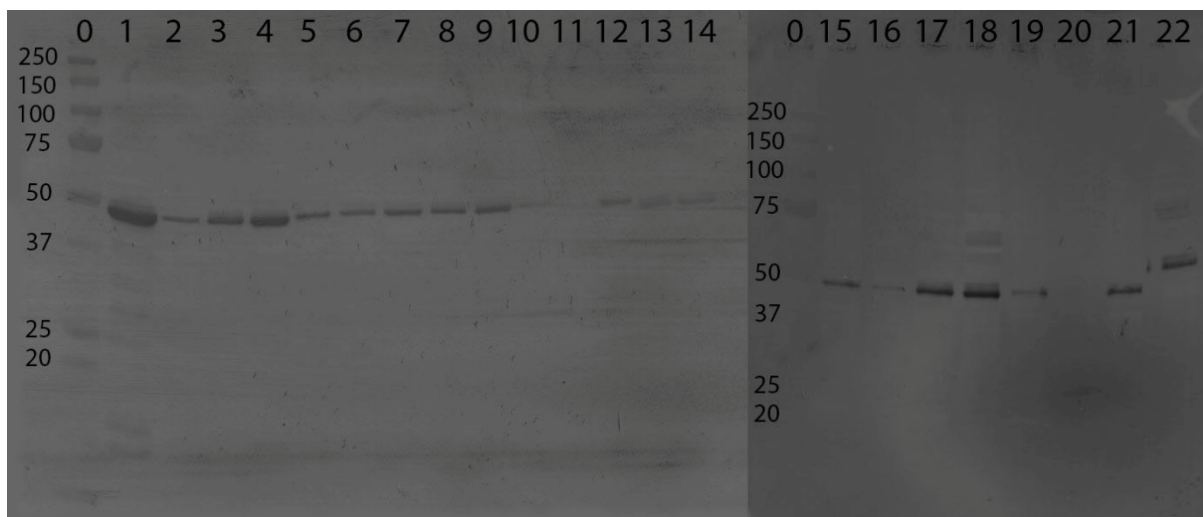
**Fig. 1.** Adhesion of lactobacilli strains to HeLa cells: DS3 – *L. rhamnosus* LNII3, HI1 – *L. paracasei* HI1, DS1 – *L. rhamnosus* KL53A, HI2 – *L. casei* HI2, HI5 – *L. rhamnosus* HI5, HI3 – *L. casei* HI3, DS10 – *L. rhamnosus*, DS11 – *L. rhamnosus* GR1, DS9 – *L. rhamnosus* 573/2/P, HI7 – *L. casei* HI7, HI4 – *L. rhamnosus* HI4, DS7 – *L. rhamnosus* DMS14870, HI6 – *L. casei* HI6, DS2 – *L. rhamnosus* GG, DS6 – *L. paracasei* LOCK 0919, DS8 – *L. casei* Fyos, DS5 – *L. casei* Shirota, DS12 – *L. rhamnosus* 573/3/EN, DS13 – *L. rhamnosus* 573/2/T

The GAPDH protein was found on the surface of 19 tested isolates. This protein was not detected only in the extracellular protein fraction obtained from the *L. rhamnosus* 573 isolate (Fig. 2). Similarly, Western-blot detection of TUFM showed the presence of this

protein (of approx. 44 kDa) in most isolates except for *L. rhamnosus* 573 (Fig. 3). The common occurrence of GAPDH and TUFM in the fractions of most analyzed isolates, including those exhibiting no adhesion to the tested substrates, indicates that probably they serve no



**Fig. 2.** Detection of GAPDH protein in extracellular proteins of lactobacilli using western blotting technique: 0 – molecular weight marker, kDa, 1 – *L. casei* HI7, 2 – *L. rhamnosus* GR1, 3 – *L. casei* Fyos, 4 – *L. rhamnosus* HI5, 5 – *L. paracasei* HI1, 6 – *L. rhamnosus* KL53A, 7 – *L. rhamnosus* HI4, 8 – *L. casei* HI2, 9 – *L. casei* HI6, 10 – *L. rhamnosus* LNII3, 11 – blank, 12 – *L. paracasei* LOCK 0919, 13 – *L. casei* HI3, 14 – *L. rhamnosus* 573/3/EN, 15 – *L. rhamnosus* 573, 16 – *L. casei* Shirota, 17 – *L. rhamnosus* 573/2/P, 18 – *L. rhamnosus* GG, 19 – *L. rhamnosus* DMS14870, 20 – *L. rhamnosus* LOCK 0908, 21 – *L. rhamnosus* 573/2/T. Created using Adobe Photoshop CC



**Fig. 3.** Detection of TUFM protein in extracellular proteins of lactobacilli using western blotting technique: 0 – molecular weight marker, kDa, 1 – *L. casei* HI7, 2 – *L. rhamnosus* GR1, 3 – *L. casei* Fyos, 4 – *L. rhamnosus* HI5, 5 – *L. paracasei* HI1, 6 – *L. rhamnosus* KL53A, 7 – *L. rhamnosus* HI4, 8 – *L. casei* HI2, 9 – *L. casei* HI6, 10 – *L. rhamnosus* LNII3, 11 – blank, 12 – *L. paracasei* LOCK 0919, 13 – *L. casei* HI3, 14 – *L. rhamnosus* 573/3/EN, 15 – *L. rhamnosus* 573, 16 – *L. casei* Shirota, 17 – *L. rhamnosus* 573/2/P, 18 – *L. rhamnosus* GG, 19 – *L. rhamnosus* DMS14870, 20 – *L. rhamnosus* LOCK 0908, 21 – *L. rhamnosus* 573, 22 – *L. rhamnosus* 573/2/T. Created using Adobe Photoshop CC



significant role in the adhesion of *Lactobacillus* spp. group *Casei* either to the epithelium or to mucus.

### Identification of potential adhesins using mass spectrometry

The SpaCBA pili are considered to be the main adhesin determining adhesion to the mucus. In contrast, little is known about adhesins to epithelial cells. Differences in the adhesion of certain strains to Caco-2 and HeLa cells indicate that it is not only determined by other adhesins, but that these adhesins vary depending on the epithelium type. In order to identify potential adhesins, the extracellular protein fractions in isolates of *Lactobacillus* spp. group *Casei*, varying in their adhesion capacity, were separated by 2D-PAGE. Protein gel images were compared using the PDQuest software (Bio Rad). Proteins differentiating tested bacterial isolates were selected as a result of comparative analysis in terms of the presence and intensity of individual

protein spots. Among the identified differentiating points, those with the strongest variation were selected for analysis by mass spectrometry. They originated from isolates of *L. rhamnosus* KL53A (SpaCBA+), which had very strong adhesive properties to all tested substrates, *L. rhamnosus* GG (SpaC+), which had weak adhesion to the HeLa epithelial cells, strong adhesion to the Caco-2 cells and very strong adhesion to mucus, *L. paracasei* HI1 (SpaCBA+), which had very strong adhesive properties to epithelial cells, but did not adhere to the mucus, *L. casei* HI6 (SpaCA+), which had a weak adhesive capacity to epithelial cells, but did not adhere to the mucus, or *L. casei* Fyos (SpaCA+), which showed no adhesion to HeLa epithelial cells, weakly adhered to Caco-2 cells and adhered well to the mucus (Table 5). In the extracellular proteomes of the five isolates, eight differentiating proteins were detected. The results of the identification of differentiating proteins are presented in (Table 4).

**Table 5.** A comparison of adhesion rates to cervical epithelium (HeLa), enterocytes (Caco-2) and intestinal mucus of tested isolates of *Lactobacillus Casei* spp. to HeLa and Caco-2 cells, and to intestinal mucus

Code	Isolate	HeLa	Caco-2	Intestinal mucus
1	2	3	4	5
DS1	<i>L. rhamnosus</i> KL53A	+++	+++	+++
HI2	<i>L. casei</i> HI2	++	++	+++
HI5	<i>L. rhamnosus</i> HI5	++	+	+++
HI3	<i>L. casei</i> HI3	++	++	+++
DS2	<i>L. rhamnosus</i> GG	+	++	+++
HI1	<i>L. paracasei</i> HI1	+++	+++	–
HI4	<i>L. rhamnosus</i> HI4	+	++	–
HI6	<i>L. casei</i> HI6	+	+	–
DS10	<i>L. rhamnosus</i> 573	++	–	++
DS9	<i>L. rhamnosus</i> 573/2/P	+	–	+
HI7	<i>L. casei</i> HI7	+	+	–
DS3	<i>L. rhamnosus</i> LNII3	+++	+	–
DS11	<i>L. rhamnosus</i> GR1	++	–	–
DS7	<i>L. rhamnosus</i> DMS14870	+	+	–

**Table 5 – cont.**

1	2	3	4	5
DS8	<i>L. casei</i> Fyos	–	+	++
DS6	<i>L. casei</i> Shirota	–	+	–
DS5	<i>L. paracasei</i> LOCK 0919	–	+	–
DS12	<i>L. rhamnosus</i> 573/3/EN	–	–	–
DS13	<i>L. rhamnosus</i> 573/2/T	–	–	–
DS14	<i>L. rhamnosus</i> LOCK 0908	–	–	–

Very strong adhesion (+++), strong adhesion (++) , weak adhesion (+), very weak or no adhesion (–).

The strains were divided into four groups according to quartiles of adhesion efficiency to HeLa cells, Caco-2 cells and intestinal mucus. First quartile ( $\leq 3.4\%$ , worst function), second quartile (3.5% to 11%), third quartile (11.1% to 16.4%), and fourth quartile ( $\geq 16.5\%$ , best function).

## DISCUSSION

Analyses were performed on twenty bacterial strains belonging to the *Lactobacillus* spp. group *Casei*. Thirteen strains were isolated from probiotic pharmaceutical preparations – *L. rhamnosus* KL53A, *L. rhamnosus* GG, *L. paracasei* LOCK 0919, *L. rhamnosus* 573/2/P, *L. rhamnosus* 573/3/EN, *L. rhamnosus* 573/2/T, and dietary supplements to promote alimentary tract function – *L. casei* Shirota, *L. casei* Fyos, as well as pharmaceutical preparations for vaginal microbiota normalization – *L. rhamnosus* LNII3, *L. rhamnosus* DMS14870, *L. rhamnosus* 573, *L. rhamnosus* GR1. The other seven strains were isolates from human feces (*L. paracasei* HI1, *L. casei* HI2, *L. casei* HI3, *L. rhamnosus* HI4, *L. rhamnosus* HI5, *L. casei* HI6, *L. casei* HI7).

The tested strains showed varied adhesion potential towards cervical epithelial cells (Fig. 1), which differed from the adhesion potential towards Caco-2 intestinal epithelium cells and towards the intestinal mucus (Markowicz and Schmidt, 2015). Five of the tested strains – *L. rhamnosus* KL53A, *L. casei* HI2, *L. rhamnosus* HI5, *L. rhamnosus* GG and *L. casei* HI3, showed very good or good adhesion properties towards HeLa and Caco-2 cells as well as the intestinal mucus. Three other strains, i.e. *L. paracasei* HI1, *L. rhamnosus* HI4 and *L. casei* HI6, adhered well to HeLa and Caco2 cells, but not towards the intestinal mucus. Isolates of *L. rhamnosus* 573 and *L. rhamnosus* 573/2/P adhered well to HeLa cells and the mucus. Isolates from feces, *L. casei* HI7, and three strains

isolated from pharmaceutical preparations for vaginal microbiota normalization, *L. rhamnosus* LNII3, *L. rhamnosus* GR1 and *L. rhamnosus* DMS14870, adhered well only to HeLa cells. Only one isolate, *L. casei* Fyos, was characterized by good adhesion only to the mucus (Table 4). Our studies showed that individual isolates differing in their adhesion properties to various substrates also differ in their extracellular proteomes. This shows the diverse adaptation of these strains to selected ecological niches.

The strains derived from gynecological pharmaceutical preparations showed greater adhesion capacity towards HeLa cells in comparison to Caco-2 cells, while most of them exhibited no adhesion to the intestinal mucus. The considerable variation in adhesion capacity between strains and in relation to the tested substrates within a given strain may suggest differences in adhesion factors on the surface of the strains, which predispose them to adhesion to a specific substrate type. Additionally, relationship was observed between the origin of the isolate and adhesion efficiency. Bacteria isolated from feces showed, on average, more effective adhesion than bacteria derived from pharmaceutical preparations or dietary supplements. A similar dependence was shown in studies conducted by Douillard et al. (2013).

In successive analyses, the differing adhesion capacity of the isolates towards HeLa and Caco-2 cells, and towards the intestinal mucus, led to a search for differences in the extracellular proteomes of *Lactobacillus* isolates.

In earlier studies (Markowicz et al., 2014), in six out of the twenty isolates (*L. paracasei* HI1, *L. rhamnosus* KL53A, *L. casei* HI2, *L. rhamnosus* HI5, *L. casei* HI3, *L. rhamnosus* GG) expression of SpaCBA fimbrial adhesin was confirmed at the mRNA level. SpaCBA is a protein complex, the involvement of which in adhesion was shown for the first time by Kankainen et al. (2009). For this reason its presence in some strains was considered in the analysis of the results from this study. Proteins with molecular masses corresponding to those of individual SpaCBA complex subunits were detected in band images from SDS-PAGE separations of extracellular protein mixtures of individual isolates (Table 2). The results were consistent with the analysis of the SpaCBA complex expression at the mRNA level from a previous study (Markowicz et al., 2014) in three out of six isolates (*L. paracasei* HI1, *L. rhamnosus* HI5, *L. rhamnosus* KL53A). The presence of proteins corresponding to the mass of one of the three subunits – SpaA in the *L. casei* HI2 isolate and SpaC in the isolates *L. casei* HI3 and *L. rhamnosus* GG were detected in the other isolates. However, this result does not necessarily indicate a lack of these subunits, as it may have been caused by an incomplete dissociation of the SpaCBA complex subunits, and as a result – the presence of bands with a non-specific molecular mass. In view of the above, an interesting finding was the lack of adhesion capacity of *L. paracasei* HI1 and *L. casei* HI7 to mucus, where identification of the SpaCBA complex proteins was positive.

Isolates in which the expression of the SpaCBA complex was detected should exhibit the ability to adhere to the intestinal mucus due to the presence of the mucus-binding domain on the SpaC subunit, and to Caco-2 cells (Reunanen et al., 2012). This dependence may be observed in five out of six isolates characterized by very efficient adhesion to Caco-2 cells, mucus and HeLa cells. Isolates *L. paracasei* HI1 and *L. casei* HI7 show slight adhesion to the intestinal mucus, despite expression of the SpaCBA complex, which may have been caused by changes in the sequence of the SpaC subunit responsible for mucus binding. Instability of the coding complex subunit sequence was shown in a previous study (Markowicz et al., 2014) and it has also been reported by other researchers (Douillard et al., 2016; Sybesma et al., 2013). The lack of mucus binding may also be caused by factors hindering

access of fimbriae formed by the SpaCBA complex to the mucus and the epithelium, such as exopolysaccharides produced by certain *Lactobacillus* strains (Dertli et al., 2015).

In this study, effective adhesion to the intestinal mucus and Caco-2 cells in the isolates exhibiting no expression of the SpaCBA complex was also found. The *L. casei* Fyos isolate adhered effectively only to the intestinal mucus, while in the case of the *L. rhamnosus* 573 and *L. rhamnosus* 573/2/P isolates it adhered additionally to HeLa cells. An efficient adhesion to HeLa and Caco-2 cells, but not towards mucus, was observed for *L. rhamnosus* HI4 and *L. casei* HI6. This may prove the presence of other adhesins and adhesion mechanisms promoting adhesion to a specific type of the epithelium or mucus in some of the tested isolates of *Lactobacillus* spp.

It was decided to identify potential, currently unknown adhesion proteins in the tested strains using two-dimensional electrophoresis, followed by the identification of proteins differentiating the extracellular proteomes by mass spectrometry. In the five isolates, a total of eight differentiating proteins were identified (Table 3).

The NLP/P60 surface antigen was identified independently in two isolates, *L. rhamnosus* KL53A and *L. rhamnosus* GG, which exhibit very high or high adhesion rates to HeLa and Caco-2 cells, and to the intestinal mucus. They are also equipped with the SpaCBA complex. However, the protein spot corresponding to that, in which the NLP/P60 antigen was detected, was not found in the other isolates producing SpaCBA. The NLP/P60 antigen affects probiotic immunomodulatory properties of the *L. rhamnosus* strain GG. This strain has two homologous proteins, p75 and p40, which exhibit cell wall hydrolase activity, and contains the NLPC/P60 domain. These proteins influence probiotic properties of a strain by stimulating the Akt kinase activity in the intestinal epithelium, and they show antiapoptotic properties (Gilad et al., 2011; Yan et al., 2007), as well as the capacity to bind mucins, collagen and to adhere to enterocytes (Bäuerl et al., 2011). The cold shock proteins (Csp), identified in the *L. casei* Fyos isolate may serve as adhesins for the mucus, as the strain adheres effectively only to the mucus. The homologous proteins serve the role of a virulence factor in *Brucella melitensis* (Wang et al., 2016)

and in *Listeria monocytogenes* (Loepfe et al., 2010). Proteins from the small cold shock protein family, detected in the efficiently adhering HI1 isolate, affect the cell membrane fluidity, as well as the physico-chemical properties of the cell surface, in *L. plantarum* WCFS1 (Capozzi et al., 2011). They may also influence induction of IL-6 synthesis by *Lactobacillus* bacteria, serving a protective and anti-inflammatory function for the intestinal epithelium (Reilly et al., 2007).

The other proteins, 30S ribosomal protein S2, 50S ribosomal protein 5L, FKBP family peptidyl-prolyl isomerase, protein binding nucleoid DNA and hypothetical proteins LGG\_02452 and LSEI\_2467 (resembling altronate oxidoreductase), are intracellular proteins, serving enzymatic and regulatory functions. They are not specific to the extracellular proteome. Nevertheless, we may not exclude their participation in interaction mechanisms between bacteria and the intestinal epithelium as a result of their multifunctionality (the so-called protein moonlighting), which has already been shown for i.e. TUFM and GAPDH. After being transported outside the bacterial cell, intracellular proteins may serve new functions, different than their primary ones, e.g. take the role of virulence factors (Henderson and Martin, 2011). A particularly interesting protein in this respect is the 30S ribosomal protein S2, identified in the *L. casei* HI6 isolate, which does not express the SpaCBA protein, but adheres well to Caco-2 and HeLa cells, as well as the cold shock protein CspA, the hypothetical protein LGG\_02452 and the hypothetical protein LSEI\_2467 identified in the *L. casei* Fyos isolate, which adheres well only to the intestinal mucus.

Identification of the above-mentioned proteins in the extracellular proteome of *Lactobacillus* spp. isolates may thus suggest that they serve currently unknown functions, including those connected to the interactions of bacteria with the intestinal epithelium. These studies may provide insight into new factors promoting the adhesion of probiotics to various types of epithelial cells. More detailed analyses, including of isolates derived from bacteremia cases, may also facilitate the identification of proteins responsible for their invasiveness. If this can be demonstrated, the classification of these proteins as virulence factors should be considered.

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