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LACTOBACILLUS STRAINS BELONGING TO *CASEI* GROUP DISPLAY VARIOUS ADHERENCE TO ENTEROCYTES AND MUCUS*

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ABSTRACT

Background. The ability of lactobacilli to adhere to the surface of the intestine is an important functional characteristic which can largely determine the effective colonization of the intestinal tract by probiotics. The following study compares the adhesion efficiency of the twenty strains of *Lactobacillus* genus belonging to *Casei* group to the Caco-2 cells and gastrointestinal mucus.
Material and methods. Twenty isolates of lactobacilli belonging to *Casei* group were tested. The ability of bacterial cells to adhere to mucus was examined using adhesion assay to gastrointestinal mucus. Obtained results were compared with adhesion efficiency to Caco-2 cells. Phylogenetic relationship between isolates was analysed by rep-PCR.
Results. The results showed large differences in adhesion efficiency between strains, as well as differences in the efficiency of adhesion to the intestinal epithelial cells and mucus. Group similarity highlighted by a rep-PCR technique does not correspond with groups of similarity in terms of the characteristics of the ability to adhere to mucus or the epithelial cells of intestinal tract.
Conclusions. Strains having a high adhesion efficiency to enterocytes do not always show a high adhesion efficiency to the mucus. This may indicate the presence of different and multiple factors responsible for adhesion efficiency of *Lactobacillus* group *Casei* strains to epithelial cells and mucus.

Key words: *Lactobacillus*, adhesion, mucus, Caco-2, enterocytes, adhesines

INTRODUCTION

Bacteria of the genus *Lactobacillus* include many probiotic strains. Many of them belong to the *Casei* group which includes three species: *Lactobacillus casei*, *Lactobacillus paracasei* and *Lactobacillus rhamnosus* (Felis and Dellagio, 2007). Each probiotic strain, according to the criteria that define this group of microorganisms, must have a beneficial effect on the human organism. It should be derived from the gastrointestinal tract of healthy person and demonstrate the ability to survive extreme environment prevailing in some parts of the gastrointestinal tract (Guidelines..., 2002). A desirable feature of probiotic is also a high efficiency of intestinal colonization, which prolongs the time of its beneficial effect on the host. This is particularly important in relation to the function of immunomodulating effect of probiotics and its antagonism with respect to pathogenic microorganisms (Grangette et al., 2005; Hisbergues et al., 2007). Bacteria can colonize

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the intestine where they can settle on the surface of the intestinal epithelium. Essential for successful colonization is occurrence of the phenomenon of adhesion. Multiple physical and chemical agents are involved in the process of adhesion. The process relies on macromolecules anchored to the cell surface and secreted outside the cells. Bacteria of the genus *Lactobacillus* are able to adhere directly to the surface of epithelial cells and/or mucus produced by them. Adhesins responsible for binding to molecules present on the surface of enterocytes are, for example, collagen binding protein CbsA or fironectin binding protein FbpA (Antikainen et al., 2002; Buck et al., 2005). Adhesion to intestinal mucus occurs with the participation of adhesins such as mucin binding proteins (Roos and Jonsson, 2002), or certain polysaccharides associated with the bacterial cell wall or secreted into the external environment (Darilmazl et al., 2011). SpaCBA protein complex forming fimbriae on the surface of bacterial cells is responsible for the adhesion to both mucus and enterocytes (Kankainen et al., 2009; Reunanen et al., 2012). The adhesion efficiency of different strains may show considerable differences, affecting the efficiency of beneficial effect of the probiotic.

The aim of this study was to analyse whether the adhesion efficiency of *Lactobacillus* group *Casei* to either enterocytes or gastrointestinal mucus is phylogenetically related trait.

MATERIAL AND METHODS

Bacterial strains. Twenty strains of *Lactobacillus* belonging to the *Casei* group were obtained from the culture collection of the Department of Biotechnology and Food Microbiology of Poznań University of Life Sciences (Table 1). Tested strains were derived from dietary supplements and humans' feces. Bacterial cultures were propagated in MRS liquid medium (Biocorp, Poland), at 37°C, under anaerobic conditions. Bacteria were cultured in Gene Box (Biomerieux, Poland) designed for the anaerobic microorganisms culture. AnaeroGen (Thermo Scientific, Poland) anaerobic atmosphere generators were used to obtain anaerobic conditions.

Isolation of bacterial exopolysaccharides. Bacterial strains were growth in liquid MRS medium for 18–20 hours, at 37°C in anaerobic conditions. 50 µl of bacterial cultures was plated into Petri dishes and cultured for 72 hours (at 37°C in anaerobic conditions). After this time, bacterial colonies were washed from the plates with water. OD of bacterial suspensions was standardized. Suspensions were mixed with 1M NaOH (1:1) and incubated over night (room temperature, gentle shaking). After incubation, bacterial suspensions were centrifuged (13 000 rpm, 1 min). Supernatants were mixed with ice-cold ethanol (1:2), exopolysaccharides were precipitated for 72 hours at 4°C. After this time, exopolysaccharides were centrifuged (13 000 rpm, 10 min), supernatant was throw out and polysaccharides were air-dried for 20 minutes. After drying they were frozen at –80°C.

Polyacrylamide gel electrophoresis and staining of exopolysaccharides. Polyacrylamide gel electrophoresis of bacterial exopolysaccharides was prepared according to Maniatis et al. (1975). The acrylamide stock solution contained 20% (w/v) acrylamide and 0.67% NN'-methylenebisacrylamide. Concentrated electrophoretic buffer contained 0.2M Tris/borate/0.002M-disodium EDTA, pH 8.3 (2 × Tris/borate/EDTA). Equal volumes of these solutions were mixed at room temperature. For polymerization, 0.4 ml of 10% (w/v) ammonium persulphate in water and 0.04 ml of NNN'N'-tetramethylethylenediamine were added per 100 ml of gel solution. The gels were cast between glass plates. The gels were polymerized overnight at room temperature. 10 µl of each polysaccharide sample was mixed 1:1 with 2M-sucrose and 0.2% bromophenol blue in 1 × Tris/borate/EDTA buffer and applied to the well. Gels were electrophoresed at 20 mA for 20 min and then at 40 mA for 1.5 h in 1 × Tris/borate/EDTA buffer. Precision Plus Protein™ All Blue Standard (Bio Rad, Poland) was used to estimate molecular mass of exopolysaccharides. After electrophoresis, gels were stained according to Cowman et al. (1984). The gels were stained in 0.5% alcian blue in 2% acetic acid for 45 min, then destained for 15 min in 2% acetic acid.

Adhesion of tested strains to the Caco-2 cells. Adhesion assay was performed as described previously (Schmidt et al., 2010), briefly: bacterial cells were grown in MRS broth (Biocorp) at 37°C in anaerobic conditions with methyl-[3H]-thymidyne (5 µl/ml of broth, 60–90 Ci/mmol, 1mCi/ml; Hartmann Analytic

Table 1. Bacterial strains used in this study

Isolate	Species	Source
DS5	*L. casei*	Yacult probiotic drink, Yacult Europe, The Netherlands
DS8	*L. casei*	Fyos probiotic drink, Nutricia, Belgium
HI2	*L. casei*	Human stool isolate
HI3	*L. casei*	Human stool isolate
HI6	*L. casei*	Human stool isolate
HI7	*L. casei*	Human stool isolate
DS6	*L. paracasei*	Latopic probiotic supplement, Biomed-Krakow, Poland
HI1	*L. paracasei*	Human stool isolate
DS1	*L. rhamnosus*	Lactoral probiotic supplement, Biomed-Krakow, Poland
DS2	*L. rhamnosus*	Dicoflor probiotic supplement, Vitis Pharma, Poland
DS3	*L. rhamnosus*	Ellen probiotic tampon, Ellen AB, Sweden
DS7	*L. rhamnosus*	Ecovag probiotic supplement, Krotex, Poland
DS9	*L. rhamnosus*	Lakcid probiotic supplement, Biomed-Lublin, Poland
DS10	*L. rhamnosus*	Lactovaginal probiotic supplement, Biomed-Krakow, Poland
DS11	*L. rhamnosus*	Lacibios probiotic supplement, ASA, Poland
DS12	*L. rhamnosus*	Lakcid probiotic supplement, Biomed-Lublin, Poland
DS13	*L. rhamnosus*	Lakcid probiotic supplement, Biomed-Lublin, Poland
DS14	*L. rhamnosus*	Latopic probiotic supplement, Biomed-Krakow, Poland
HI4	*L. rhamnosus*	Human stool isolate
HI5	*L. rhamnosus*	Human stool isolate

GmbH, Germany). 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. Caco-2 cells (passage no. 49–52) used in the adhesion assay were prepared in PTFE filter (0.4 μm pore size) inserts for 6-well tissue-culture dishes (Merck-Millipore, Poland) by inoculating $4 \cdot 10^5$ cells/cm^2. Twenty one-day-post-confluent Caco-2 cell monolayers were washed with 1 ml HBSS then 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_2 in air. After 60 min incubation, monolayers were washed three times with sterile, HBSS to remove free bacterial cells. The amount of adhered bacterial cells was estimated from the radioactivity remaining at the Caco-2 monolayer. Each assay was performed in triplicate. Radiolabelled bacteria in amount initially added for adhesion and washed Caco-2 monolayer with adhered radiolabelled 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 by liquid scintillation in Beckmann LS6500 after addition of Hionic-Fluor scintillation cocktail (Perkin-Elmer, Poland).

Adhesion of tested strains to the gastrointestinal mucus. The lyophilized gastrointestinal mucus (cat no. M1778, Sigma-Aldrich) was dissolved in PBS with calcium and magnesium ions to concentration of 0.1 g/ml. Maxi Sorp 96-well plates (cat no. 436110, Nunc) were coated with mucus solution overnight at 4°C, then washed twice with 300 ml of Hank's balanced salt

solution (HBSS). Bacterial cultures were washed twice with PBS with calcium and magnesium ions. Bacterial suspension (50 µl with a density of $5 \cdot 10^8$ cfu/ml) was applied to the wells. Incubation was carried out for 1 hour at 37°C. After incubation, wells were washed twice with HBSS and then, 100 ml of HBSS was loaded onto each well. The amount of bacteria adhered to the mucus was determined using BAC-Titer-Glo Microbial Cell Viability Assay (Promega). The measurement of the luminescence intensity was performed using the Infinite M200 reader (Tecan).

Extraction of DNA from bacteria. DNA from bacterial cultures was isolated with Genomic Mini Kit (A&A Biotechnology, Poland) according to the manufacturer instruction.

Rep-PCR. PCR reactions were performed using two sets of primers: REP (REP1R: 5'IIIICGICGICATCIGGC; REP2I: 5'ICGICTTATCIGGCCTAC) and ERIC (ERIC1R: 5'ATGTAAGCTCCTGGGGATTCAC; ERIC2: 5'AAG TAAGTGACTGGGGTGAGCG) detecting the repetitive sequences in the bacterial genome. The reaction contained 50 ng genomic DNA, 1.5 U polymerase (Run Polymerase A&A Biotechnology), 1× reaction buffer, 200 uM each of deoxyribonucleotide triphosphates, and 0.5 mM of each primer. Initial denaturation was performed at 95°C for 1 min, followed by 35 cycles of: denaturation, 95°C, 1 min, annealing, 40°C (for REP primer), 42°C (for ERIC primer) for 1 min, and elongation 72°C, 4 min. Final elongation was performed at 72°C for 8 min. Separation of the PCR products was carried out in 2% agarose gel, stained with ethidium bromide. Gel images were analysed using Quantity One (Bio-Rad). The similarity between isolates (based on their genetic fingerprints) was calculated based on the Dice coefficient, UPGMA method and expressed graphically as a dendrogram using Data Assist™ v3.01 Software (Life Technologies).

RESULTS AND DISCUSSION

***Lactobacillus* strains have differentiated capacity to adhere to gastrointestinal mucus.** The ability of bacterial cells to adhere to mucus is expressed as the percentage of cells that adhered to the surface of a well coated by gastrointestinal mucus (bacteria which have not been washed off during the rinse step). Adhesion efficiency to gastrointestinal mucus ranged from 91% to less than 1% (Fig. 1). Two isolates from human stool showed very high adhesion to mucus of 91% (*L. casei* HI3) and 75% (*L. casei* HI2). Three strains from dietary supplements (*L. rhamnosus* DS1, *L. rhamnosus* DS2, *L. paraceasei* DS6) and one isolate from stool (*L. rhamnosus* HI5) showed medium adhesion efficiency (between 46% and 25%). The average capacity to adhere to gastrointestinal mucus characterised strains *L. rhamnosus* DS10 (10%), *L. casei* DS8 (9%), *L. rhamnosus* DS9 (6%), and *L. paracasei* HI1 (6%), low capacity to adhere to gastrointestinal mucus characterised *L. rhamnosus* HI4 (3%), *L. casei* HI6, *L. rhamnosus* DS3 and DS11 (2%). The remaining strains were characterised by a very low capacity to adhere or total lack of this feature (value of less than 1% of adhered cells).

Analysis of the phylogenetic relationship between tested isolates. Phylogenetic relationship between isolates was analysed by rep-PCR (Versalovic et al., 1994). This technique uses the primers binding to repeated sequences in bacterial genome. Amplicons obtained in the reaction forms patterns specific for individual strains. Used oligonucleotide primers generated 41 polymorphic bands, that allowed to determine the genetic similarity between tested isolates. Dendrogram of similarity (Fig. 2) showed that the largest range of diversity in analysed group of 20 isolates, characterized isolates belonging to *L. rhamnosus* species. Genetic fingerprints of *L. rhamnosus* DS12 and DS14 isolates were characterised by the highest coefficient of the identity at the level of 0.83. Fingerprint of *L. rhamnosus* DS9 isolate was characterised by a factor of 0.07 of identity in relation to other tested isolates. Whereas *L. paracasei* HI1 and DS6 isolates showed low genetic similarity coefficient of 0.25. The largest interspecific genetic similarity with the value of 0.68 was observed between *L. casei* (DS8, HI6, HI3) and *L. rhamnosus* DS7 isolates.

The relationship between adhesion to enterocytes, gastrointestinal and genetic similarity. In the group of tested isolates, there can be distinguished strains with large (*L. rhamnosus* DS1, *L. rhamnosus* DS2, *L. paracasei* HI1, *L. casei* HI2, *L. casei* HI3, *L. rhamnosus* HI5) and moderate (*L. rhamnosus* DS3, *L. paracasei*

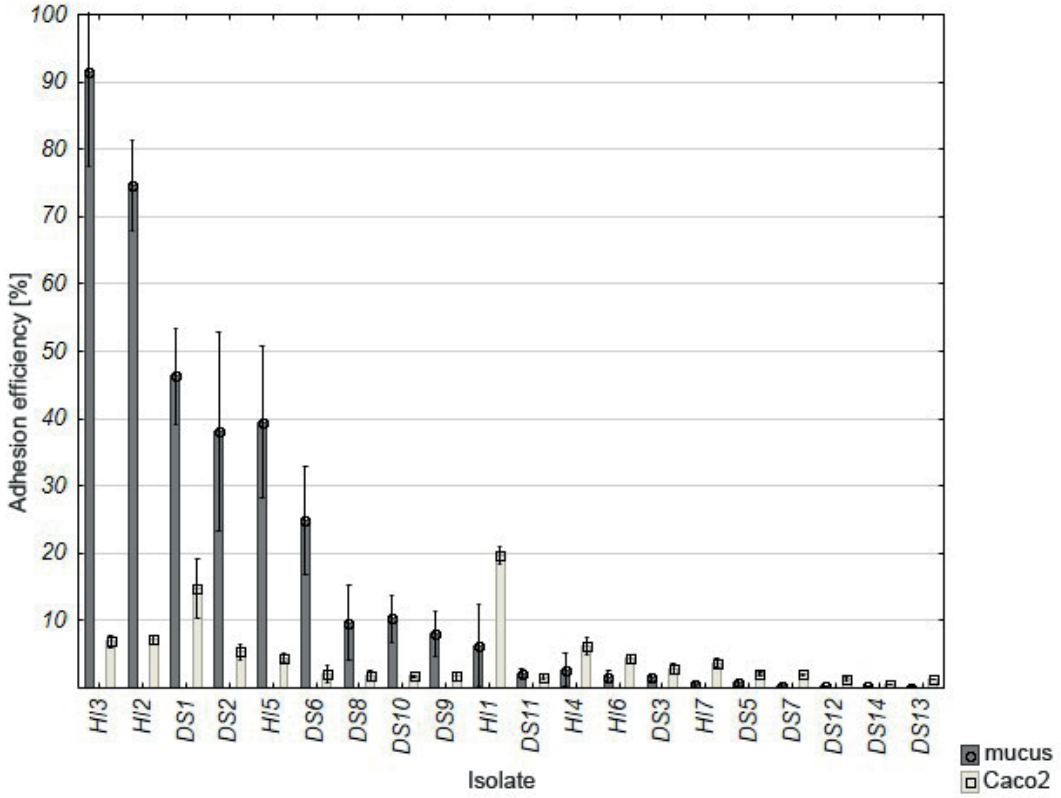

Fig. 1. Comparison of capacity of *Lactobacillus* isolates belonging to *Casei* group adherence to mucus (gray bars) and Caco-2 cells (white bars) and intestinal mucus. Bars represent average values of three measurements and whiskers represent the mean values ±1.5×standard deviation.

DS6, *L. casei* DS8, *L. rhamnosus* DS9, *L. rhamnosus* DS10, *L. rhamnosus* DS11, *L. rhamnosus* HI4, *L. casei* HI6) ability to adhere to both mucus and epithelial cell surface. These two groups have the potential to colonize the human gastrointestinal tract because of the ability to adhere to the surface of the intestines (small and large). Isolates (*L. casei* HI7, *L. casei* DS5, *L. rhamnosus* DS7) having the ability to adhere to the enterocytes (but not to the mucus) can only interact with the surface of the small intestine or in pathological cases, where the mucus layer is absent on the surface of colon. Strains characterised by weak ability of adhesion to enterocytes and mucus (*L. rhamnosus* DS12, *L. rhamnosus* DS13, *L. rhamnosus* DS14) are transit microflora with short impact on the intestinal environment. Their influence will refer mainly to the intestinal microbiome. Knowledge about these characteristics in relation to probiotic and industrial strains allows to make a better selection of strain to the expected functionality of the product (food or dietary supplement).

Comparing the *Lactobacillus Casei* group isolates adhesion efficiency to mucus and enterocytes (Caco-2 cells) showed the convergence only in some strains. The ability of tested isolates to adhere to mucus may be at least 4.5 times higher (91% of the cells of *L. casei* HI3) in comparison to adhesion efficiency to enterocytes (20% *L. paracasei* HI1). The ability to adhere to mucus and enterocytes is characterised by the Pearson correlation coefficient of $R = 0.346$. This suggests that the feature of adhesion to these two substrates is conditioned by various factors. This may be caused by presence of a variety molecules on the surface of enterocytes, which can be bound by different types of adhesins present on the surface of bacterial cells. In contrast, the potential number of adhesins involved in mucus binding process is much smaller.

The differences in the efficiency of adhesion to the enterocytes and mucus may also result from the nature of the interactions between bacterial and epithelial cells. It has been proven that in the process of adhesion to the mucus, electrostatic interactions plays a significant role. However their stability is low and they can easily rupture under the influence of chemical and mechanical properties (Sun et al., 2007). In addition, different strains of the genus *Lactobacillus* may synthesize different exopolysaccharides in various amounts. These are substances that affect the efficiency of adhesion of bacteria to the intestinal epithelium, however, depending on their type and concentration, the impact can be positive or negative (Landersjo et al., 2002; Ruas-Madiedo et al., 2006). Exopolysaccharide layer of the bacterial cell surface may also impede the interaction between the other adhesins present on the surface of bacterial cells, and the environment. Tested strains have ability to produce different amounts of exopolysaccharides (Fig. 2).

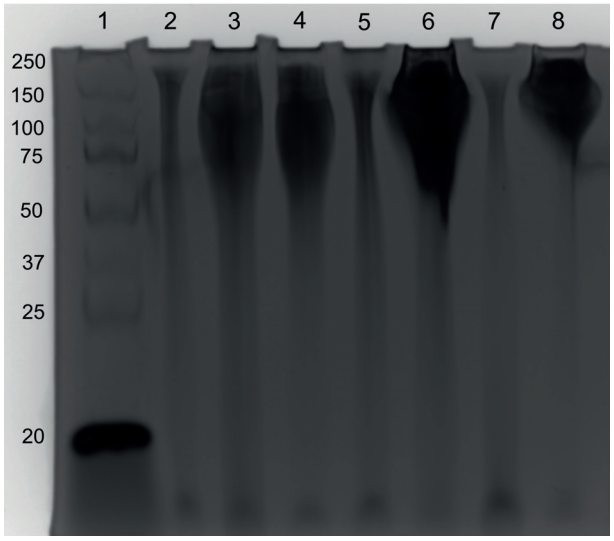

Fig. 2. Polyacrylamide gel electrophoresis of exopolysaccharides isolated from *Lactobacillus* strains. Lane 1 – molecular weight marker, lane 2 to 8 – exopolysaccharides isolated from: 2 – *Lactobacillus rhamnosus* (DS2), 3 – *Lactobacillus rhamnosus* (DS1), 4 – *Lactobacillus casei* (HI7), 5 – *Lactobacillus casei* (HI2), 6 – *Lactobacillus paracasei* (HI1), 7 – *Lactobacillus casei* (DS5), 8 – *Lactobacillus casei* (DS8)

The results obtained in this study were compared with those obtained in previous work of Markowicz et al. (2014) concerning the variation of spaCBA adhesive protein coding sequence. Most of the isolates showing high and average ability to adhere to mucus contains complete coding sequence structural proteins (spaC, spaB, spaA). They form a polymeric fimbriae, which participation in the process of adhesion to the intestinal epithelium has been previously demonstrated. They also have the ability to bind to the mucus, through the presence of the subunit SpaC mucus binding domain (Kankainen et al., 2009; Reunanen et al., 2012). Another factor differentiating ability of individual strains to adhere to the intestinal epithelium are variations in the sequences encoding the fimbrial protein structure (Toh et al., 2013) and its expression (Douillard et al., 2013; Rasinkangas et al., 2014). The spaCBA complete coding sequence is present in *L. paracasei* DS6 and *L. casei* DS8 isolates but the protein is not expressed (Markowicz et al., 2014). These strains, however, showed the ability to adhere to mucus respectively, 25% and 10%. In contrast, isolate DS10 which does not have the coding sequence of the spaCBA protein (Markowicz et al., 2014), is characterised by 10% of the ability to adhere to mucus. These data confirm that the ability to adhere to enterocytes and mucus is depend on different factors. There was no clear relationship between the phylogenetic similarity of tested strains and their ability to adhere to enterocytes and gastrointestinal mucus (Fig. 3). However, it can be seen that the strains isolated from faeces display better adhesion efficiency to the enterocytes than strains derived from dietary supplements. Such dependence has been indicated also on a larger sample of bacterial strains by Douillard et al. (2013). Four of the six strains expressing spaCBA were strains isolated from feces. The presence of such correlations may indicate environmental influence on enhancing adhesion efficiency to enterocytes. In bacteria without contact with the intestinal epithelium these abilities decrease. In this study, no clear relationship between the origin of the strain and the efficiency of adhesion to gastrointestinal mucus can be seen.

Primers used in rep-PCR technique, following by literature data are very useful in differentiating isolates within one species of bacteria (Versalovic et al., 1994). Comparison of genetic fingerprints of tested isolates

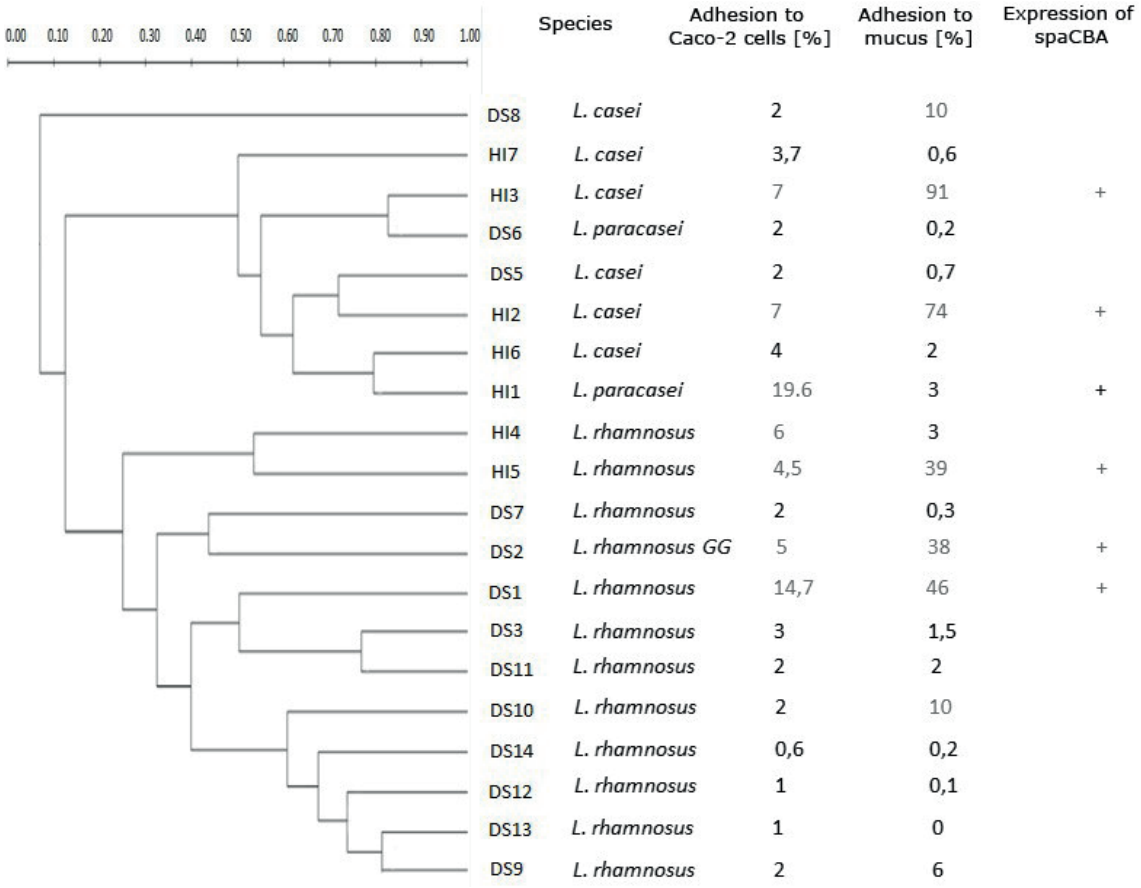

Fig. 3. Relationship between adhesion to enterocytes and gastrointestinal mucus, presence of spaCBA surface protein (data obtained in previous work of Markowicz et al. (2014)) and genetic similarity of lactobacilli strains. Dendrogram of genetic similarity between the tested human stool isolates (HI1-HI7) and dietary supplements isolates (DS1-DS13) of *lactobacilli* belonging to *Casei* group. The phylogenetic tree was obtained on the basis of genetic fingerprints of studied isolates, using the REP and ERIC primers, based on the Dice coefficient using the UPGMA method. The scale and value of the nodes describe the similarity of isolates

confirmed the close genetic relationship of species: *L. casei*, *L. rhamnosus* and *L. paracesei* belonging to the Casei group (Felis and Dellaglio, 2007).

CONCLUSIONS

Tested strains of lactobacilli belonging to *Casei* group have different adhesion efficiency to Caco-2 cells and gastrointestinal mucus. High level of adhesion efficiency can decide about enhanced ability to colonize the gastrointestinal tract and prolonged impact on the intestinal environment. Strains having a high adhesion efficiency to enterocytes do not always have a high adhesion efficiency to the mucus. This may indicate the presence of different and multiple factors responsible for adhesion to epithelial cells and mucus, including exopolysaccharides. There may also occur interactions between various adhesive agents, which can change the efficiency of bacterial adhesion. The presence of complete and unchanged sequence coding spaCBA protein complex in some bacterial strains has a positive effect on their adhesion efficiency. These studies indicated that the group similarity highlighted by a rep-PCR technique do not correspond with

groups of similarity in terms of the characteristics of the ability to adhere to mucus or the epithelial cells of intestinal tract. In order to obtain a broader picture of the relationship between the ability to adhere to the intestinal epithelium, and variety of genomic nucleotide sequences of the lactobacilli from *Casei* group, it is necessary to analyse a larger number of isolates of diverse origin.

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ZRÓŻNICOWANIE ZDOLNOŚCI BAKTERII Z RODZAJU *LACTOBACILLUS* NALEŻĄCYCH DO GRUPY *CASEI* DO PRZYLEGANIA DO ENTEROCYTÓW I ŚLUZU PRZEWODU POKARMOWEGO

STRESZCZENIE

Zdolność bakterii *Lactobacillus* z grupy Case do przylegania do powierzchni jelit jest istotną cechą funkcjonalną, która w znacznym stopniu może decydować o skuteczności probiotyku. W proces adhezji są zaangażowane liczne czynniki obecne na powierzchni komórek bakteryjnych oraz nabłonka jelitowego. Złożony charakter zjawiska adhezji sprawia, że dokładne poznanie jego mechanizmów jest trudne i wymaga licznych badań. W pracy porównano wydajność adhezji dwudziestu szczepów bakterii *Lactobacillus* z grupy *Casei* do komórek Caco-2 oraz do śluzu przewodu pokarmowego. Uzyskane wyniki wykazały duże zróżnicowanie wydajności adhezji między poszczególnymi szczepami, jak również różnice w wydajności adhezji bakterii do komórek nabłonkowych jelit i śluzu.

Słowa kluczowe: *Lactobacillus*, przyleganie, Caco-2, enterocyty, śluz, adhezyny

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