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ORIGINAL PAPER

Exopolysaccharides Produced by *Lactobacillus rhamnosus* KL 53A and *Lactobacillus casei* Fyos Affect Their Adhesion to Enterocytes

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Abstract

Probiotics promote and help to maintain beneficial microbiota composition of the gastrointestinal tract ecosystem and have a positive impact on the host's health. Production of exopolysaccharides is an important feature of probiotic lactobacilli. It increases the chance of their survival in the gastrointestinal tract and promotes adhesion to the epithelium; therefore, exopolysaccharides are important for the process of colonization. Two lactic acid bacteria strains were used in this study: *Lactobacillus rhamnosus* KL 53A and *Lactobacillus casei* Fyos. Exopolysaccharides were isolated from bacterial cells and their monosaccharide composition was examined using liquid chromatography. The influence of exopolysaccharides on lactobacilli adhesion to enterocytes was studied after deglycosylation of the bacterial cells and incubation with the selected intestinal microbiota strains that metabolize polysaccharides – *Faecalibacterium prausnitzii* DSM 17677 and *Blautia luti* DSM 14534. Both deglycosylation and incubation with polysaccharide metabolizing strains influenced the ability of probiotic strains to adhere to enterocytes. Enzymatic deglycosylation decreased adhesion efficiency of *L. rhamnosus* KL 53A; however, co-incubation of both lactobacillus strains with *F. prausnitzii* DSM 17677 resulted in an increase of their adhesion efficiency. Exopolysaccharides are important adhesins of *Lactobacillus* spp. that influence their ability to colonize gut epithelium. Other members of gut microbiota can modify the adhesion property *in situ*; therefore the composition and metabolic state of commensal bacteria may influence their probiotic action.

Key words: bacteria, adhesion, deglycosylation, polysaccharides, Caco-2 cells

Introduction

Exopolysaccharides (EPS) are extracellular biopolymers that are produced by many species of microorganisms, including *Lactobacillus* spp. (Ruas-Madiedo and de los Reyes-Gavilán 2005). They may remain attached to the bacterial envelope or be secreted into the environment in the form of mucus (Ruas-Madiedo et al. 2006).

In terms of structure, two main groups of EPS produced by bacteria are distinguished: homo- and heteropolysaccharides. Homopolysaccharides consist of one type of monosaccharide moiety, typically glucose or fructose, and have a simple structure. Differences between their various types are related to the characteristics of the primary structure, such as the type of monomer and bonds between the monomer subunits. Heteropolysaccharides have a more complex structure. They are composed of monosaccharides belonging to at least two different types. The structure of individual heteropolysaccharides may vary considerably. The common feature for most of them is the presence of glucose, rhamnose and galactose, found at different ratios (Welman and Maddox 2003).

There are two known pathways of EPS synthesis in lactic acid bacteria, i.e. the extracellular glucosyltransferase or fructosyltransferase (GT) dependent pathway, and the Wzy/Wzx-dependent pathway (Ryan et al. 2015). The substrate used for the synthesis of homopolysaccharides via the GT dependent pathway is sucrose or another oligosaccharide containing fructose for fructan synthesis (Galle and Arendt, 2014), and the energy used in this process derives from its hydrolysis. Glucosyltransferases carry out the synthesis of glucans, while fructosyltransferases are involved in the formation of fructans (Korakli et al. 2003). Many different types of GT are encoded in the genomes of lactobacilli and only a small part of them have been biochemically characterized (van Hijum et al. 2006). The GT pathway is a relatively simple enzymatic process, which requires low energy inputs (Ryan et al. 2015).

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The Wzy/Wzx-dependent pathway is a more complex enzymatic process, with several enzymes and regulatory proteins involved in heteropolysaccharides synthesis and secretion. It initially takes place in the cytoplasm, where repeating units of heteropolysaccharides are assembled by the glucosyltransferases. The Wzx flippase translocates the repeated units into the periplasm, where polymerization by the Wzy polymerase takes place. In the last stage, polysaccharide is transported outside the cell by the OPX protein (Schmid et al. 2015).

Most stages of exopolysaccharide biosynthesis occur in the cytoplasm (Welman and Maddox 2003). Polymerization or EPS secretion into the environment takes place within the cell membrane. In some cases (e.g. dextran, levan) the synthesis of EPS can be entirely extracellular (Schmid et al. 2015).

The amount and type of synthesized heteropolysaccharides depend on the environment, in which the strain grows (De Vuyst and Degeest 1999). Depending on the composition of the medium, temperature, pH and oxygen content, lactobacilli can produce exopolysaccharides of different sugar monomers, interconnected by different types of bonds (De Vuyst and Degeest 1999; Furukawa et al. 2000).

The ability of lactic acid bacteria to produce EPS is commonly used in the dairy industry to improve texture and to stabilize the products (Patel and Prajapati 2013). The physiological role of EPS produced by lactic acid bacteria remains, however, little known. EPS affect the surface properties of bacterial cells. EPS play an important role in adhesion and colonization of the gastrointestinal tract by probiotics. They also promote the formation of the biofilm that facilitates colonization of the epithelium and increases the chance of bacterial survival in the gastrointestinal tract (Lebeer et al. 2009; Stack et al. 2010). EPS enhance the hydrophobicity of the bacterial cell surface, increasing the ability of bacteria to bind to the intestinal mucosa (Sun et al. 2007), and likely participate in enterocyte adhesion as ligands for epithelial surface receptors (Ruas-Madiedo et al. 2006). There are also reports that EPS protect against phage attack and phagocytosis (Gopal and Crow 1993; Comstock and Kasper 2006).

Exopolysaccharides may also act as prebiotics, i.e. non-digestible food ingredients that are beneficial for the host organism by selectively stimulation of growth and/or activity of one or more species of bacteria colonizing the gut. It has been shown that certain bacteria belonging to *Firmicutes* or *Bacteroides* in the intestinal ecosystem of humans metabolize the EPS produced by *Lactobacillus* and *Bifidobacterium* species (Rios-Covian et al. 2016). The interaction between the different species of microorganisms present in the intestines is one of the factors that influence the composition of the intestinal ecosystem (Wexler et al. 2016). This also applies to probiotic bacteria delivered to the digestive tract (O'Toole et al. 2008). Their beneficial effect on the host organism can be modified by species of native microbiota, for example by limiting their adhesion to the intestinal epithelium and thereby reducing their time within the intestine.

The aim of our work was to determine the role of EPS on adhesion of *L. rhamnosus* KL 53A and *L. casei* Fyos to the gut epithelium and to investigate whether intestinal commensal microorganisms have the ability to metabolize EPS synthesized by the probiotics, therefore *in situ* influencing their properties. Two strains *F. prausnitzii* DSM 17677 and *B. luti* DSM 14534, the representatives of the species that are common in the intestinal microbiota, have been selected for our study.

Experimental

Materials and Methods

Human and bacterial cells. Two lactic acid bacteria strains were used in this study: *L. rhamnosus* KL 53A (derived from a dietary supplement Dicoflor, Vitis Pharma, Warsaw, Poland) and *L. casei* Fyos (derived from a probiotic drink). Lactic acid were cultured in the MRS medium (Biocorp, Warsaw, Poland) at 37°C in an anaerobic jar. For the adhesion tests, the Caco-2 cell line was used (obtained from European Collection of Cell Cultures). The Caco-2 cell line derived from a colorectal cancer shows morphology and functional features of normal small intestine cells in post-confluent culture. Caco-2 cells are widely recognized as a substitute for the human intestine epithelial cells and are used to study.

Adhesion and invasion of intestinal pathogens. Caco-2 cells were cultured in Dulbecco's modified Eagle's medium (Lonza, Belgium) supplemented with 10% (v/v) heat-inactivated calf serum (Lonza, Belgium) and 1% (v/v) nonessential amino acids (Lonza, Belgium). The cells were grown in a humidified atmosphere of 10% CO_2 at 37°C. To examine the influence of the bacterial strains that metabolize exopolysaccharides on the adhesion of lactobacilli to enterocytes, two strains of bacteria that occur in the human intestines were used: *B. luti* DSM 14534 and *F. prausnitzii* DSM 17677 (Touyama et al. 2015; Rios-Covian et al. 2016). Strains were derived from DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). Bacteria were grown in Anaerobe Basal Broth (Oxoid, Basingstoke, United Kingdom), at 37°C in an anaerobic jar.

Isolation, purification and quantification of exopolysaccharides from lactic acid bacteria. Bacterial cells were grown in MRS broth at 37°C in anaerobic

conditions for 24 h. After this time, 100 µl of bacterial culture was plated on MRS agar and incubated at 37°C in anaerobic conditions for 72 h. Bacterial biomass was washed from the plate with deionized, sterile water. Then, one volume of 2 M NaOH was added to bacterial suspension. After incubation overnight in room temperature (22°C), sample was centrifuged (2800 × g, 20 min). Two volumes of ice-cold ethanol were added to the supernatant and incubated for 48 h at 4°C until the EPS precipitated. At the next step, samples were centrifuged (2800 × g, 30 min), supernatant was discarded and EPS were resuspended in sterile water and dialyzed for 72 h using the D-tube dialyzer Mega, 6–8 kDa (Merck, Warsaw, Poland). Then the samples were frozen at –80°C. The monomer composition of EPS was analyzed using HPLC (Rezex RSO-Oligosaccharide Ag^+ 200 × 10.0 mm column with 60 × 10.0 mm precolumn, a RID refractometric detector, water as the mobile phase, column temperature: 80°C, flow rate: 0.3 ml/min, injection volume: 10 µl, pressure: 24 bar, run time: 1 h). Before HPLC analysis samples were hydrolyzed using acidic hydrolysis. A volume of 1 ml of 25% (v/v) sulfuric acid was added per 100 mg of sample and incubated for 12 h at 4°C. Then 1 ml of 70% sulfuric acid (v/v) was added and the sample was incubated in a water bath at 55°C for 2 hours. After this time samples were cooled at room temperature and diluted with 10 ml of distilled water. The next step consisted of incubation in a water bath at 90°C for 1 h. In the last stage samples were cooled at room temperature, neutralized by adding 500 mg of calcium carbonate and filtered using a syringe filter with a 0.2 µm pore size hydrophilic PTFE membrane.

Deglycosylation of bacterial cells. Enzymatic deglycosylation of bacterial cell surface was made using the Protein Deglycosylation Mix (Bio Labs, Ipswich, Massachusetts, USA) (Table I) according to the instructions for non-denaturing reaction conditions. Before deglycosylation, the bacterial cells were labelled with methyl-[^{3}H]-thymidine (5 µl/ml of broth, 60–90 Ci/mmol, 1 mCi/ml) as described previously (Schmidt et al. 2010) and inactivated with 4% formaldehyde, as described in our previous paper (Markowicz et al. 2016) to prevent EPS synthesis during and after the deglycosylation step. Briefly, the bacterial cells were grown in MRS broth at 37°C under anaerobic conditions for 18–20 h. After this time, bacteria were washed twice with PBS and incubated in 4% formaldehyde in PBS for 24 h at room temperature. After inactivation, the bacterial cells were washed twice with PBS and resuspended in the same buffer. Inactivation effectiveness was verified by plating bacteria on MRS agar and incubation for 48 h at 37°C under anaerobic conditions. Lack of colony growth indicated complete inactivation. Three technical replicates were made (each in a triplicate). Deglycosylated bacterial cells were used in the adhesion assay to examine the influence of deglycosylation on the efficiency of adhesion to epithelial cells.

Inhibition of glycotransferases. *L. casei* Fyos was grown in the presence or absence (controls) of five different glycotransferase inhibitors: castanospermine, deoxymannojirimycin, kifunensine, swainsonine and deoxynojirimycin hydrochloride (1 mg/ml of MRS broth) for 18–20 h, 37°C under anaerobic conditions. Methyl-[^{3}H]-thymidine (5 µl/ml of broth, 60–90 Ci/mmol, 1 mCi/ml) was added to label the bacteria. After this time, bacteria were inactivated in 4% formaldehyde in PBS for 24 h at room temperature, washed two times with PBS and resuspended in HBSS. Three technical replicates were made (each in a triplicate). Inactivated bacterial cells were used in the adhesion assay to examine the influence of glycotransferase inhibition on the efficiency of adhesion to epithelial cells.

Cell surface proteome analysis. Proteomic analyses were performed using mass spectrometry. *L. casei* Fyos was grown in the presence of swainsonine (1 mg/1 ml

Table I
Composition of the Protein Deglycosylation Mix.

Enzyme	Hydrolyzed bounds	Number of enzymatic units added per 100 µl of reaction
O-Glycosidase	Catalyzes the removal of core 1 and core 3 *O*-linked disaccharides from glycoproteins.	400000 U
PNGase F	Cleaves between the innermost GlcNAc and asparagine residues of high mannose, hybrid, and complex oligosaccharides from *N*-linked glycoproteins unless α(1-3) core fucosylated	5000 U
Neuraminidase	Catalyzes the hydrolysis of terminal, non-reducing α2,3, α2,6, and α2,8 linked *N*-acetylneuraminic acid residues from glycoproteins and oligosaccharides.	500 U
β1-4 Galactosidase	Catalyzes the hydrolysis of terminal, non-reducing β1-4 linked D-galactopyranosyl residues from oligosaccharides and glycoproteins.	80 U
β-*N*-Acetylglucosaminidase	Catalyzes the hydrolysis of terminal, non-reducing β-*N*-Acetylglucosamine residues from oligosaccharides and glycoproteins.	40 U

of MRS broth) or without swainsonine (control). After incubation for 18–20 h, 37°C under anaerobic conditions, the cell wall proteome was isolated. The bacterial biomass derived from 10 ml of overnight culture was used for isolation. Bacteria were washed with PBS and resuspended in the buffer containing 50 mM Tris pH 7.5, 1 mM EDTA, 0.1% SDS and protease inhibitors (4-(2-aminoethyl) benzenesulfonyl fluoride, 2 mM/100 ml, aprotinin, 0.3 μM/100 ml, bestatine 130 μM/100 ml, E-64 14 μM/100 ml, leupeptin 1 μM/100 ml) to extract the external proteins. After 20 min of extraction, samples were centrifuged; proteins were extracted from the supernatant by adding trichloroacetic acid to the final concentration of 1% and sodium deoxycholate to the final concentration of 0.1%. Samples were incubated overnight at 4°C, then proteins were centrifuged at 20000 × g, washed in cold acetone, dried and solubilized in the loading buffer for Laemlii SDS-PAGE. Three biological replications of the experiment were made. The protein samples were stored frozen (–80°C). Protein samples were briefly resolved in SDS-PAGE and gel slabs with proteins were sent for analysis to the Laboratory of Mass Spectrometry, IBB PAS (Warsaw, Poland), where the samples were analyzed using tandem mass spectrometry.

Lactobacilli to Caco-2 cells adhesion assay. The adhesion assay was performed using Caco-2 cell line (according to Schmidt *et al.* 2010). Bacteria were labeled with methyl-[3H]-thymidine (60–90 Ci/mmol, 1 mCi/ml; Perkin Elmer, USA) at a volume of 5 μl/ml of broth and cultured for 18–20 h. After this time, the bacteria were washed twice with sterile HBSS and resuspended in the same buffer. The monolayers of differentiated Caco-2 cells were cultivated for three weeks on the PTFE filter (0.3 μm pore size) inserts in six-well tissue-culture dishes (Merck-Millipore, Poland) after inoculation of 2×10^6 viable cells (passage no. 49–52) per insert in 2.0 ml culture medium. The Caco-2 cell monolayers were washed once with 1 ml sterile HBSS before the adhesion assay. Bacteria were added to each well (5×10^8 cfu/ml of bacteria in 2.0 ml HBSS) and incubated at 37°C in an atmosphere of 10% CO2 for 60 min. After this time the monolayers were washed three times with sterile HBSS to remove the bacterial cells that did not adhere to Caco-2 cells. The radiolabeled bacteria in the density initially added for adhesion and the Caco-2 monolayer with adhered radiolabeled bacteria were lysed in 0.9 ml of 1% SDS. To complete lysis, 0.1 ml of 1 M NaOH was added and the lysate was incubated overnight at 60°C. The radioactivity of the lysed suspension was measured by liquid scintillation in Beckmann LS6500 after addition of the Hionic-Fluor scintillation cocktail (Perkin-Elmer, Poland). The bacterial adhesion efficiency was calculated based on the number of bacterial cells present after the adhesion test, relative to the number applied initially to the membrane with Caco-2 cells. Each assay was performed in triplicate.

Co-culture adhesion assay. Adhesion of lactic acid bacteria and *B. luti* DSM 14534 or *F. prausnitzii* DSM 17677 co-cultures was tested after incubation in the modified Anaerobe Basal Broth without a carbon (therefore, the only sources of carbon for *B. luti* DSM 14534 or *F. prausnitzii* DSM 17677 were exopolysaccharides produced by lactic acid bacteria). Initial density of each bacterial strain was 10^8 cfu/ml, the same volumes of 24 h-culture of lactic acid bacteria in the MRS broth and *B. luti* DSM 14534 or *F. prausnitzii* DSM 17677 72 h-culture in the Anaerobe Basal Broth were mixed, then washed two times with PBS and cultured for 18 h at 37°C (anaerobic jar) in the Anaerobe Basal Broth without carbon sources. After that time, the density of co-culture was recorded, using spectrophotometric measurement (wavelength of 600 nm). To prepare controls, the appropriate numbers of lactic acid bacteria and *B. luti* DSM 14534 or *F. prausnitzii* DSM 17677 were incubated separately under the same conditions and mixed immediately before the adhesion assay. The number of bacterial cells in culture was approximated by comparison of spectrophotometric measurements of culture density (600 nm wavelength) and inoculations of decimal decays of bacterial suspension. Measurements and cultures were performed in triplicates (18 h of lactobacilli cultures and 48 h of *F. prausnitzii* DSM 17677 and *B. luti* DSM 14534 cultures). Three biological replications were made. Co-cultures were used in the adhesion assay. The number of adhered bacterial cells was estimated by plating decimal dilutions of lysed Caco-2 cells. Lysis of Caco-2 cells was carried out by adding 1 ml of 0.1% SDS. Then, a Viscolase nuclease (A&A Biotechnology, Gdańsk, Poland) was added to the probes (1 U/ml) to degrade DNA released from lysed Caco-2 cells. Serially diluted samples were plated on MRS agar and incubated for 48 h, at 37°C under microaerophilic conditions (to avoid growth of *B. luti* DMS 14534 or *F. prausnitzii* DMS 17677, which are strictly anaerobic strains). After that time, colonies of lactic acid bacteria were counted and the effectiveness of adhesion was calculated. Each observed colony corresponded to one bacterial cell, which adhered to Caco-2 cells. The bacterial adhesion efficiency was calculated based on the number of bacterial cells present after the adhesion test, relative to the number of bacteria incubated with Caco-2 cells. The result was the percentage of bacteria that adhered to Caco-2 cells.

Statistical analysis. The t-test for dependent groups was used to compare the results obtained for experiment evaluating the effect of deglycosylation on lactobacilli adhesion to Caco-2. One-way analysis of variance (ANOVA) was used to analyze the data on

the influence of glycotransferases inhibition on adhesion efficiency to Caco-2 and the co-culture adhesion to Caco-2. The level of statistical significance was $p < 0.05$. The statistical calculations were performed using Statistica software.

Results

***L. rhamnosus* KL 53A and *L. casei* Fyos produced acidic heteroexopolysaccharides.** The exopolysaccharides of lactic acid bacteria were isolated from cells grown on MRS agar plates under anaerobic conditions at 37°C. The HPLC analysis revealed that both *L. rhamnosus* KL 53A and *L. casei* Fyos produced acidic heteropolysaccharides that differ in their monosaccharide composition. *L. rhamnosus* KL 53A produced EPS composed mainly of arabinose, glucose and galactose at a ratio of approximately 1:2:4 with a minor addition of maltose. *L. casei* Fyos produced EPS composed mainly of glucose, galactose and arabinose at a ratio of about 1:2:4 with a minor addition of ribose. This indicated that both strains share major constituents of EPS moieties (Table II).

Table II
Monosaccharides composition of the EPS produced by *L. rhamnosus* KL 53A and *L. casei* Fyos when grown on MRS agar (anaerobic conditions, 37°C, 18 h).

Monomer	% of a monomer in EPS isolated from the strain tested	
	Lactobacillus rhamnosus KL 53A	*Lactobacillus casei* Fyos
Maltose	0.71	0
Glucose	30.9	15.7
Galactose	53.7	31.4
Arabinose	14.6	51.6
Ribose	0	1.4

Deglycosylation of bacterial cell surface with a mixture of glycolytic enzymes reduced adhesion efficiency of *L. rhamnosus* KL 53A, but not *L. casei* Fyos. Two strains of lactic acid bacteria: *L. rhamnosus* KL 53A and *L. casei* Fyos, were used in this experiment. The metabolically labelled cells were fixed with formaldehyde and subsequently treated with the deglycosylation enzyme cocktail. Deglycosylated cells were incubated with the Caco-2 cell monolayer to estimate adhesion efficiency of the treated vs. untreated cells. A significant decrease ($p < 0.05$) in adhesion efficiency was observed only in the case of *L. rhamnosus* KL 53A (Fig. 1).

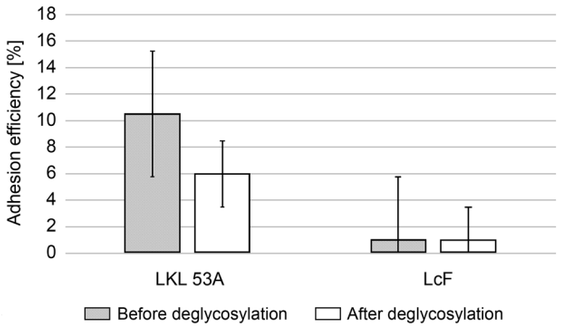

Fig. 1. Adhesion efficiency of the fixed bacteria before degycosylation and after deglycosylation. LKL 53A, *L. rhamnosus* KL 53A, LcF, *L. casei* Fyos. The bars represent average percentages of bacterial cells that adhered to differentiated Caco-2 enterocytes, the whiskers represent average adhesion ± standard error.

Inhibition of alpha-mannosidase with swainsonine increased adhesion efficiency of *L. casei* Fyos. We tested the influence of several glycotransferase inhibitors on adhesion efficiency of *L. casei* Fyos. Among the inhibitors tested (castanospermine, deoxymannojirimycin hydrochloride, swainsonine, kifunensine, and deoxynojirimycin hydrochloride) the treatement with swainsonine, an alpha-mannosidase inhibitor, resulted in a 2.8-fold increase of adhesion efficiency of the tested strain (Fig. 2). Although mannose was not found as a constituent of *L. casei* Fyos exopolysaccharide, the alpha-mannosidase encoding gene is commonly present in the genome of the species (Broadbent et al. 2012).

The cell surface proteome was not changed significantly by swainsonine treatment. To determine changes of the cell surface proteome after growth in the presence of swainsonine we performed the proteomic analysis by mass spectrometry. A comparison of relative numbers of identified proteins (n = 275) revealed a high correlation between control cells and those grown in the presence of swainsonine ($r = 0.957$, Fig. 3). The *L. casei* Fyos cells grown in the presence of the glycotransferase inhibitor showed a small (up to 2.25-fold) increase of several proteins: protein translocase subunit SecA, citrate lyase alpha-chain/citrate lyase, glycerol phosphate lipoteichoic acid synthase, glutamyl-tRNA(Gln) amidotransferase subunit A, the ATP synthase gamma chain, the ATP-dependent Clp protease proteolytic subunit/endopeptidase Clp, nitroreductase, 30S ribosomal protein S2, GMP synthase [glutamine-hydrolyzing]/glutamine amidotransferase, and the ATP synthase F1 sector beta subunit. This result suggests that the observed increase in adhesion efficiency after treatment with swainsonine was not a result of changes in the proteome as analyzed by mass spectrometry.

***F. prausnitzii* DSM 17677 affected adhesion of lactobacilli to enterocytes.** We tested the influence of coincubation of *Blautia luti* DSM 14534 and *F. prausnitzii* DSM 17677 with two strains of lactic acid bacteria, *L. casei* Fyos and *L. rhamnosus* KL 53A. A significant

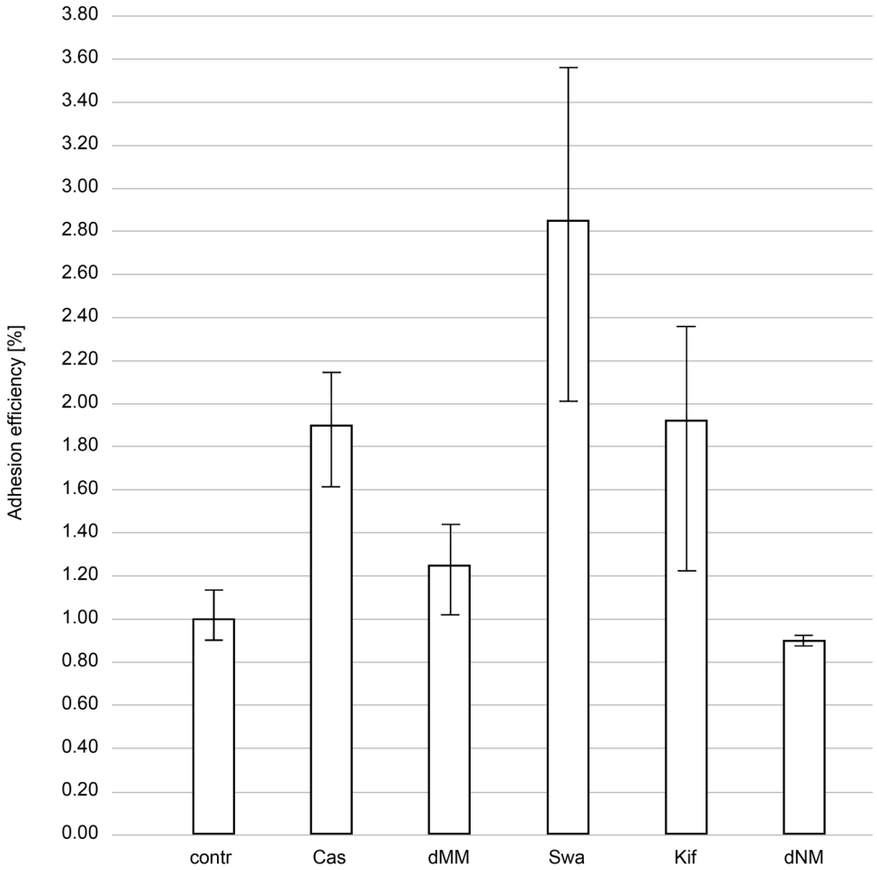

Fig. 2. Normalized to 100% adhesion efficiency of *L. casei* Fyos grown in the presence of glycosidase inhibitors: castanospermine (Cas), deoxymannojirimycin hydrochloride (dMM), swainsonine (Swa), kifunensine (Kif), deoxynojirimycin hydrochloride (dNM) and without inhibitor (contr). The bars represent average percentages of bacterial cells that adhered to differentiated Caco-2 enterocytes, the whiskers represent average adhesion ± standard error. Statistically significant changes ($p < 0.05$) are marked with asterisks (*).

increase ($p < 0.05$) in adhesion efficiency of lactobacilli was observed after incubation with *F. prausnitzii* DSM 17677 (1.3-fold increase for *L. casei* Fyos and 1.6-fold increase for *L. rhamnosus* KL 53A). *Blautia luti* caused no effect on adhesion ability of both lactobacillus strains (Fig. 4).

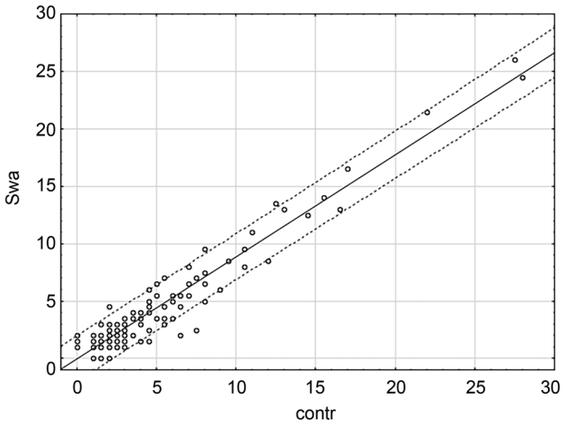

Fig. 3. A comparison of relative protein number in the *L. casei* Fyos proteomic mass spectrometry data of the control cells (contr) and of the cells grown in the presence of swainsonine (Swa).

Discussion

Many strains of intestinal microbiota exhibit the ability to degrade polysaccharides. The mechanisms they use may differ, but they always include the cell surface processing of a large glycan that precede the cellular import. *Firmicutes* produce polypeptides that bind carbohydrates and show catalytic functions. These proteins can bind EPS and release oligosaccharides, which are captured by cell wall proteins that import them into the bacterial cell (Cockburn and Koropatkin 2016). Both strains used in this study, *F. prausnitzii* DSM 17677 and *B. luti* DSM 14534, which metabolize polysaccharides (Lopez-Siles et al. 2012), belong to *Firmicutes,* but only *F. prausnitzii* DSM 17677 affected the adhesion of lactobacilli to enterocytes by enhancing this property.

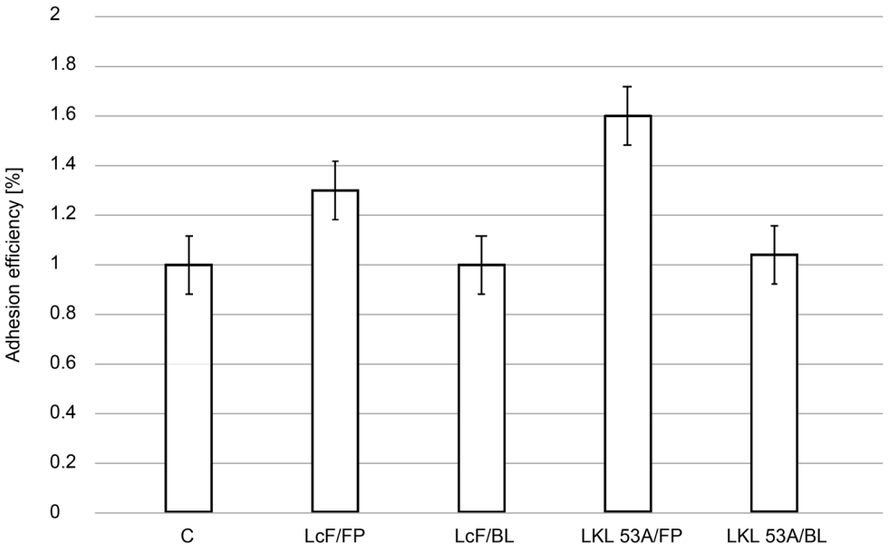

Fig. 4. Changes in adhesion efficiency to Caco-2 cells of two lactic acid bacteria strains co-cultured with anaerobic gut bacteria. Adhesion efficiency was normalized to 100%. Statistically significant changes ($p < 0.05$) are marked with asterisks (*). C – control: either *L. casei* Fyos or *L. rhamnosus* KL 53A; LcF/FP, *L. casei* Fyos co-cultured with *F. prausnitzii* DSM 17677; LcF/BL, *L. casei* Fyos co-cultured with *B. luti* DSM 14534; LKL 53A/FP, *L. rhamnosus* KL 53A co-cultured with *F. prausnitzii* DSM 17677; LKL 53A/BL, *L. rhamnosus* KL 53A co-cultured with *B. luti* DSM 14534.

In this study we demonstrated for the first time that *F. prausnitzii* DSM 17677 is able to affect adhesion of *L. rhamnosus* KL 53A and *L. casei* Fyos to enterocytes. *F. prausnitzii* is a phylogenetic group commonly found in human intestinal microbiome, accounting for approximately 5% of intestinal bacteria (Miquel et al. 2013). Two strains of probiotic lactobacilli used in this study synthesize acidic heteropolysaccharides, which remain attached to cell wall and most likely have an impact on adhesive ability of these bacteria. It was demonstrated by the use of deglycosylation enzymes (Fig. 1), glycosylase inhibitor treatment, and incubation with *F. prausnitzii* DSM 17677. This data shows a novel type of microbial interaction that can occur in the gastrointestinal tract and influence probiotic activity. Our finding is especially interesting in respect to the latest research of Desai et al. (2016) who demonstated that a dietary fiber-deprived gut microbiota degrades the colonic mucus barrier and enhances pathogen susceptibility.

In order to carry out experiments that required the inhibition of EPS synthesis and deglycosylation of lactobacilli, it was necessary to inactivate the bacterial cells synthesis during and after the experiments. The effect of inactivation on the adhesion of lactobacilli to Caco-2 cells has been thoroughly tested in our previous work (Markowicz et al. 2016). Formaldehyde inactivation was chosen due to the relatively low impact on the bacterial cell surface properties. It neither cause complete loss of protein activity nor reduce the adhesive properties, which was important for further analysis.

Incubation of bacteria with a mixture of deglycosylating enzymes resulted in a decreased efficiency of adhesion in *L. rhamnosus* KL 53A, which produces EPS composed mainly of arabinose, glucose and galactose with a small addition of maltose. It is likely that in this case EPS can act as an adhesion-promoting factor and when it was degraded (or partially degraded), adhesion of *L. rhamnosus* KL 53A was affected. There was no effect of enzymatic deglycosylation on *L. casei* Fyos adhesion; however, inclusion of the glycosylase inhibitor swainsonine resulted in an increased adhesion efficiency of the strain. Inhibition of EPS by synthesis could result in the exposure of surface adhesive proteins and, consequently, increased adhesion. In the case of *L. casei* Fyos, EPS can be an adhesion-reducing agent.

The deglycosylation of *L. casei* Fyos, using swainsonine, an α-(1→3)-mannosidase and α-(1→6)-mannosidase inhibitor (Table III), resulted in an increase of adhesion to enterocytes. No changes were observed in the bacterial proteome under the influence of this inhibitor. Swainsonine changed the EPS properties on the cell surface in *L. casei* Fyos but the cell surface proteome remained unchanged. However, we were unable to determine the difference in the EPS composition in the swainosine-treated and untreated cells; thus, we hypothesize that the increase of adhesion was due to the changes in EPS rather than the proteome of *L. casei* Fyos. Swainsonine affected the EPS of bacteria despite the lack of mannose in their composition, which may suggest that the α-(1→3)-mannosidase and α-(1→6)-mannosidase participate at some steps of EPS

Table III
Function of glycosidase inhibitors used to inhibit EPS synthesis in *L. casei* Fyos.

GT inhibitor	The enzyme inhibited
Castanospermine	β-glucosidase β-xylosidase α-mannosidase β-mannosidase Maltase-glucoamylase
Deoxymannojirimycin	α-(1→2)-mannosidase
Kifunensine	
Swainsonine	α-(1→3)-mannosidase α-(1→6)-mannosidase
Deoxynojirimycin hydrochloride	α-glucosidase Maltase-glucoamylase

synthesis, whereas mannose residues are not finally exposed outside of the cell. Mannose can, for example, be a precursor for EPS synthesis or be included in repeating units assembled in the cytoplasm, but not a part of the final EPS.

To the best of our knowledge this is the first report on a new type of the bacterial interaction i.e. properties of one bacterial strain influence the activity of the other strain towards their host. Enzymatic deglycosylation, EPS synthesis inhibition and incubation with *F. prausnitzii* DSM 17677 caused a different effect on adhesion of *L. rhamnosus* KL 53A and *L. casei* Fyos. The experiments have shown that both the EPS composition and their presence or absence may affect the adhesion of the LAB strains to enterocytes. Also, polysaccharides-metabolizing strains (e.g. *F. prausnitzii* DSM 17677) can affect the adhesion of lactobacilli (including probiotic strains) to the intestinal epithelium. EPS play an important role in the adhesion of beneficial lactobacilli to the intestinal epithelium; however, their synthesis pathways and functions remain poorly known when compared to those of pathogenic microorganisms. This bacterial interaction may explain differences in the host response to probiotic ingestion due to differences in the host microbiome composition.

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Authors' Contributions

CK contributed to the adhesion assays, bacterial cell cultures, isolation and hydrolysis of EPS, isolation and preparation of proteins for (MS/MS) analysis, deglycosylation assay, glycotransferases inhibition assay data collection and interpretation, writing of the manuscript. MS contributed to HPLC analysis and MTS contributed to culture of the Caco-2 cells, conception of the idea, data collection and interpretation, drafting and writing of the manuscript. All read and approved the manuscript.

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