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Prebiotic Treatment Influence the Adhesion Properties of Three *Lactobacillus* strains

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A B S T R A C T

Adhesion to intestinal epithelial cells is commonly analyzed during investigations on potential probiotic activity of certain bacteria. The aim of the present study was to investigate whether different prebiotics affect the adhesion properties of three strains lactobacilli (*Lactobacillus rhamnosus* 1010, *Lactobacillus acidophilus*11, and *Lactobacillus paracasei* 8458). Using *in vitro* model system based on cell lines derived from colon epithelial cells, we have determined the adherence ability of the three bacterial strains following treatment with 6 different prebiotics (inulin, pectin, chitosan, galacto-oligosaccharides, xylooligosaccharides and beta-glucan). Our results demonstrated significant reduction of lactobacilli adhesion after treatment with beta-glucan and chitosan. Conversely, xylooligosaccharides- and galacto-oligosaccharides-treated bacteria showed enhanced ability to adhere to enterocyte-like cell lines. Treatment with inulin did not show pronounced effect on lactobacilli adhesion. Overall, the three tested *Lactobacillus* strains displayed similar responses to the prebiotic treatment. Only pectin induced strain specific effect – reduced adhesion of *L. rhamnosus* 1010 and *L. acidophilus* 11 while the adhesion properties of *L. paracasei* 8458 were not significantly affected. These findings suggest that different types of prebiotic substances induce different mechanisms that modulate the adhesion ability and probably the probiotic activity of lactobacilli.

Introduction

Different species from the genus *Lactobacillus* are commonly used as probiotics (Zago *et al.*, 2011). By definition, probiotics are live microorganisms that confer a health benefit on the host when administered in adequate amounts (FAO/WHO, 2001). Their efficacy has been demonstrated for the treatment of gastrointestinal disorders, respiratory infections and allergic symptoms (Wohlgemuth *et al.*, 2010). The general mechanisms of action that induce these beneficial results are: production of antibacterial substances; induction of defensin production by intestinal epithelial cells; competitive exclusion of pathogenic bacteria; influence on host microbiota and pathogenic bacteria; improved intestinal barrier function; modulation of host immune (Wohlgemuth *et al.*, 2010). The predominant number of species applied as probiotics belong to the group of lactic acid bacteria (LAB), named after the main end product of their carbohydrate metabolism. LAB are Gram-

positive non-sporulating microorganisms belonging to the genera *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Aerococcus*, *Bifidobacterium*, and *Weissela* (Chukeatirote, 2003).

The intestinal microbiota can be influenced by a number of physiological and environmental factors like diet, age, genotype, health status (Lupp and Finlay, 2005). Thus, in order to beneficially modulate the gut microbial ecosystem and prevent or treat enteric disorders three main strategies are used: direct supplementation by consummation of probiotic bacteria; consummation of prebiotics that are selectively used by resident microorganisms; and combination of both approaches by application of synbiotics (mixtures of pro- and prebiotics) (Torres *et al.*, 2010).

Prebiotics are defined as selectively fermented ingredients that allow specific changes in the composition and/or activity in the gastrointestinal microbiota that confer benefits upon the host wellbeing and health (Gibson *et al.*, 2004, Roberfroid, 2007). The positive health effects of prebiotics are suggested to relate to the selective consumption by bifidobacteria and lactobacilli, which stimulates their growth and activity at the expense of other groups of bacteria in the gut (Macfarlane *et al.*, 2008). The group of commercial prebiotics includes polysaccharides (beta-glucans, pectin, inulin, chitosan) and oligosaccharides (galacto-oligosaccharides (GOS), fructo-oligosaccharides (FOS), xylooligosaccharides (XOS) and others) (Slavin, 2013, Torres *et al.*, 2010). Their beneficial health effects include protection against enteric infections; improved mineral absorption; immunomodulatory activity; trophic effects of short-chain fatty acids on the colonic epithelium; fecal bulking; decreased toxigenic metabolism of intestinal microbiota that may reduce the risk factors for colon cancer (Macfarlane *et al.*, 2008, Torres *et al.*, 2010).

In the present study, we aimed to define the effect of different prebiotics on the adhesion properties of three lactobacilli strains with potential probiotic activity. Adherence to the intestinal surface is considered an important prerequisite for probiotic bacteria to interact with the host and exert their beneficial effect (Van Tassell and Miller, 2011). Bacterial adhesion affects retention time in the human gastro-intestinal tract and therefore significantly contributes to interactions between bacteria and their hosts. Thus, adhesion ability is a main feature investigated in relation with the activity and probiotic properties of bacteria (Turpin *et al.*, 2012). Difficulties to investigate bacterial adhesion *in vivo*, especially in humans, have stimulated the development of *in vitro* models for preliminary screening of potentially adherent strains (Duary *et al.*, 2011). Cell lines derived from human intestinal epithelium are intensively used for initial evaluations of binding abilities of different bacterial species, including lactobacilli. Caco-2 and HT-29 are the most commonly applied cell lines for this aim (Duary *et al.*, 2011, Mousavi *et al.*, 2016). They are able to form a homogenous polar monolayer of mature cells that resemble morphologically and physiologically enterocytes *in vivo* (Rousset, 1986, Van Tassell and Miller, 2011). Although resected human intestinal mucosal tissueis considered as a more reliable approach, we chose to use enterocyte-like cell lines in our studies in order to circumvent disadvantages like restricted availability of experimental material, need for immediate processing, as well as contamination with other cell types, bacteria and/or endotoxins. In addition to the commonly applied Caco-2 and HT-29 cells, we included in our experiments the mucin-producing cell line LS 180 (McCool *et al.*, 1994), also derived from human colon

adenocarcinoma, with the aim to compare adhesion levels of LAB to mucin-producing cells and cells that does not secrete mucins. Our results confirmed previous findings for increased bacterial adhesion to mucus-producing cells in culture (Bernet *et al.*, 1994, Gopal *et al.*, 2001).

The original contribution of this work however is the finding that different prebiotics affect specifically the adhesion properties of lactobacilli: galacto- and xylooligosaccharide treatment significantly increases the binding of *L. rhamnosus* 1010, *L. acidophilus*11 and *L. paracasei* 8458 to Caco-2, HT-29 and LS 180 cells, while incubation with beta-glucan and chitosan reduces dramatically lactobacilli adhesion.

Materials and Methods

Propagation and maintenance of *Lactobacillus* strains

Three strains of lactic acid bacteria (LAB) were purchased from the Bulgarian National Bank for Industrial Microorganisms and Cell Cultures (NBIMCC) namely *Lactobacillus acidophilus* (NBIMCC 11), *Lactobacillus rhamnosus* (*Lactobacillus casei* subsp. *rhamnosus*; NBIMCC 1010; ATCC7469; NCIMB8010), *Lactobacillus paracasei* subsp. *paracasei* (NBIMCC 8458; ATCC 25302). The LAB were obtained as lyophilized mass and stored at 4ºC until establishment of cultures.

All strains were grown in MRS broth (deMan, Rogosa, Sharpe broth; Merck, Germany) at 37ºC for 18–20 h and sub-cultured twice prior treatment with prebiotics followed by co-culture with human colon adenocarcinoma cell lines.

Lactobacilli stocks were stored at -80ºC in MRS broth containing 20% glycerol.

Cell lines and culture conditions

Three human colon adenocarcinoma cell lines were obtained from the European Collection of Authenticated Cell Cultures (ECACC, Salisbury, United Kingdom): Caco-2 (ECACC 86010202; ATCC HTB-37; cell line derived from a primary colonic tumor), HT-29 (ECACC 91072201; ATCC HTB-38; cell line isolated from a primary colorectal tumor) and LS 180 (ECACC 87021202; ATCC CCL 187; mucin-secreting cells derived from Dukes type B colon adenocarcinoma (McCool *et al.*, 1994)). All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Invitrogen, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Sigma-Aldrich, Germany) and antibiotics of 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma-Aldrich, Germany). Cultures of either of the three cell lines were established in 25 cm^2 flasks (TPP, Switzerland) and the cells were maintained at 37ºC, 5% CO_2, 95% atmospheric air in a humidified incubator. When 70-80% confluency was reached the cells were harvested using 0.25% trypsin/0.53 mM EDTA solution and placed in 75 cm^2 culture flasks.

Prebiotic treatment

The following prebiotics were used in the experiments: barley beta-glucan (Sigma-Aldrich, Germany), galacto-oligosaccharide (Yakult, Tokyo, Japan), xylooligosaccharide (Shandong Longlive Bio-technology, China), inulin (Haya Labs, USA), chitosan (Equilibra, Italy), and apple pectin (Sigma-Aldrich, USA). All substances were dissolved in sterile DMEM without antibiotics and FBS. Prebiotic stock solutions were stored at 4ºC until use.

Lactobacillus cultures in exponential growth phase were centrifuged at 4000 rpm/min for

15 minutes. After removal of the supernatant, the cells were resuspended in DMEM without antibiotics and FBS. Then, the cells were divided in 7 vials in order to establish several cultures from each LAB strain (*L. rhamnosus* 1010, *L. acidophilus* 11, *L. paracasei* 8458). Six cultures were treated with different prebiotics and a control culture in prebiotic-free DMEM was established for all *Lactobacillus* strains used in the experiments. Prebiotic solution was added to the lactobacilli cultures to a final concentration of 15 mg/ml. The cultures were grown for 18h at 37ºC. At the end of the incubation period, the bacterial concentration in the treated and control cultures was determined by measurement of optical density at 600 nm and the calculated concentration was verified by cell counting using Bürker chamber. Prior inoculation of post confluent Caco-2, HT-29 and LS 180 monolayers, the LAB cultures were diluted in order to reach approximate concentration of $1x10^8$ bacteria/ml.

Adhesion assay

Caco-2, HT-29 and LS 180 cells were expanded in 75 cm^2 culture flasks. When the cultures reached 70-80% confluence the cells were trypsinized and counted in Bürker camber. Cell viability was evaluated using trypan blue staining. $5x10^4$ cells/well were seeded in 12-well plates (TPP, Switzerland). When the cultures reached 70% confluency the medium was replenished every day consecutively for a period of 7 to 10 days. Cultivation was performed under standard conditions as described in the subsection "Cell lines and culture conditions". 48h before the adhesion assay 2 mM sodium butyrate was added to the HT-29 cultures in order to induce cell differentiation.

The adhesion assay was performed according to the method described by Duary *et al.*, (2011) with slight modifications (Duary *et al.*, 2011). One hour prior addition of LAB the culture medium was removed; the cell monolayers were washed with D-PBS (Sigma-Aldrich, Germany) and then fed with 1 ml DMEM devoid of antibiotics and FBS. $4x10^6$ Lactobacilli (treated with prebiotics or control untreated LAB)/ml were added to different wells with post confluent Caco-2, HT-29 and LS 180 cells. The plates were incubated for 1h at 37ºC, 5% CO_2 and high humidity. At the end of the incubation period, the cell monolayers were washed five times with D-PBS to release unbound bacteria. Then, the cells were detached by incubation with trypsin/EDTA solution at room temperature for 10-15 min. To release attached lactobacilli the cells were lysed with 0.9% Triton X100 dissolved in PBS. The resulting lysates containing cell-associated bacteria were serially diluted in D-PBS and plated on MRS agar (2%). The plates were incubated for 24-48 h at 37ºC and the colonies formed (B_1 CFU/ml) were enumerated. Adhesion was expressed as the percentage of recovered viable bacteria compared to the initial population (B_0 CFU/ml) added to the cell monolayers. Percent adhesion was calculated using the following formula: $(B_1/B_0) \times 100$. All samples were assayed in triplicates.

Statistics

Results were presented as ± standard error of the mean (±SEM). Statistical significance of the data was determined using the Mann-Whitney test (Stat View software, USA). *P*-values less than 0.05 were considered statistically significant.

Results and Discussion

Adhesion to the intestinal surface is considered to be one of the main characteristics of bacteria with probiotic potential (Piatek *et al.*, 2012). It has been determined as a mechanism that protect the

host from pathogen invasion and it is effective even when the bacterial binding is transient (Candela *et al.,* 2008). This supports the need to study the factors that affect the adhesion properties of probiotic bacteria. One of these factors could be prebiotics, which are commonly applied in mixture with probiotics (synbiotics) to stimulate their growth and activity in the intestines. Therefore, we have chosen to study the potential influence of six commercial prebiotic substances on the adhesion properties of three strains lactobacilli belonging to species with industrial application as probiotics.

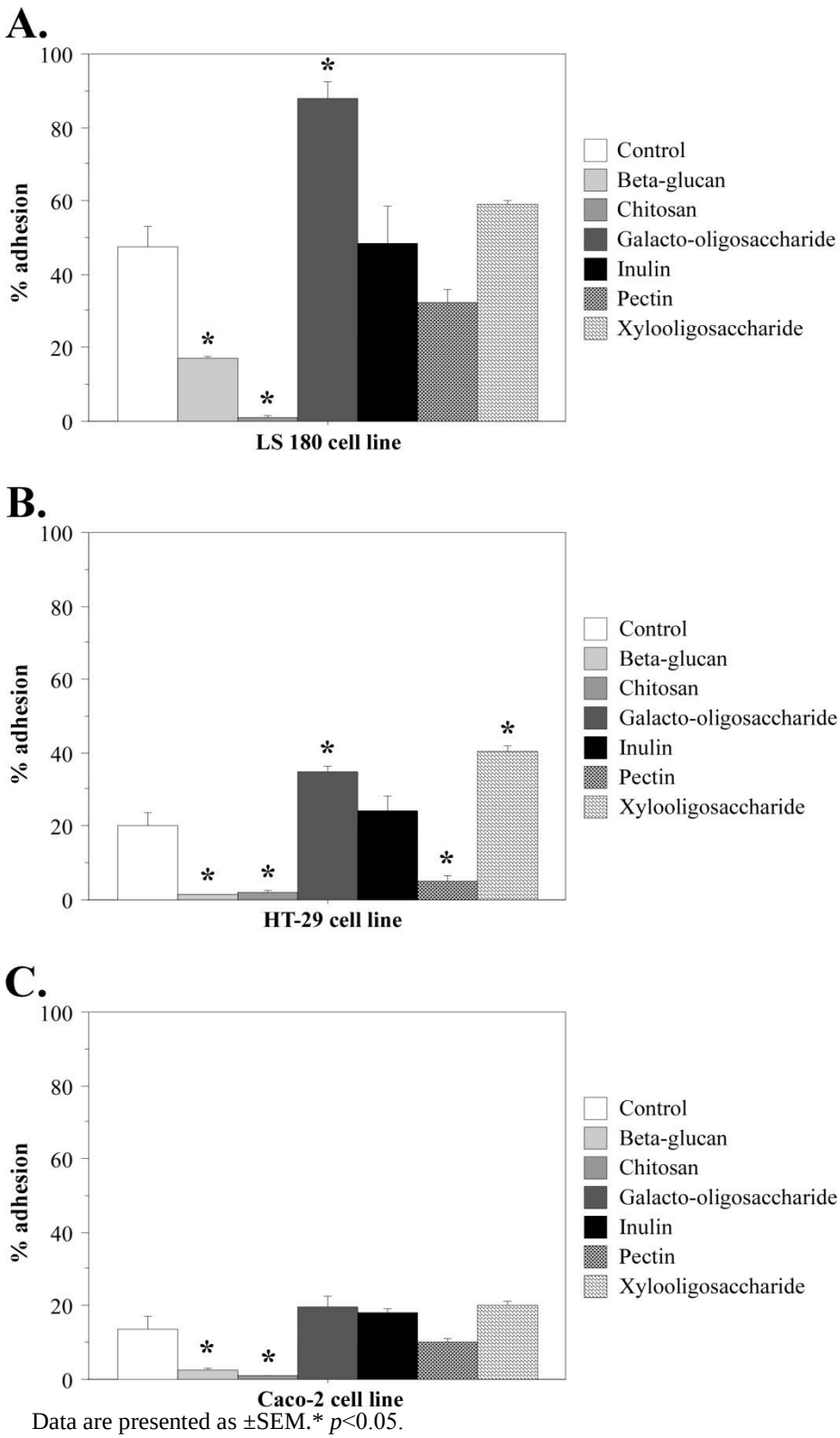
L. rhamnosus 1010, *L. paracasei* 8458 and *L. acidophilus* 11 cells were cultured in growth medium containing particular prebiotic (inulin, beta-glucan, galacto-oligosaccharide, xylooligosaccharide, pectin, chitosan) for 18 h at 37ºC, 5% CO_2, 95% atmospheric air in a humidified incubator. Control cultures from the three LAB strains were grown in medium without prebiotic under the same conditions and time period. Adhesion assays were performed with three different human colon derived cell lines – Caco-2, HT-29 and LS 180. The determined adhesion percentages for *Lactobacillus rhamnosus* 1010 are shown on figure 1.

The results demonstrate significantly reduced binding ability of *L. rhamnosus* 1010 to all three cell lines following treatment with beta-glucan and chitosan. Incubation of the lactobacilli with xylo- and galacto-oligosaccharides enhanced their adhesion properties and statistically significant difference compared to the untreated control was found with HT-29 cells and LS 180 cells (only for galacto-oligosaccharides treatment). A moderate tendency for increased adhesion of inulin-treated *L. rhamnosus* 1010 was observed (Fig. 1B and C). Incubation with pectin reduced the adhesion properties of *L. rhamnosus* 1010. Similar results were obtained from the adhesion assay with *Lactobacillus acidophilus* 11 (Fig. 2). Again, beta-glucan, chitosan and pectin treatment reduced the LAB binding abilities to human enterocyte-like cell lines. Xylooligosaccharides and galacto-oligosaccharides enhanced the adhesion of *L. acidophilus* 11 – significant difference compared to the untreated control was found with HT-29 cells. Treatment with inulin however did not affect markedly *L. acidophilus*11 adhesion.

The effects of incubation with different prebiotics on the adhesion properties of *Lactobacillus paracasei* 8458 are shown on figure 3. The binding abilities of this strain LAB were significantly reduced after treatment with beta-glucan and chitosan. Tendency for elevated adhesion of *L. paracasei* 8458 was found as a result of treatment with galacto-oligosaccharides and xylooligosaccharides, while inulin treatment did not show a pronounced effect. Interestingly, treatment of *L. paracasei* with apple pectin led to increased adhesion to LS 180 cells and a trend for stronger effect than the treatment with inulin was found (Fig. 3A, B and C). These results differ from the data obtained with the other two type's lactobacilli, which demonstrates a strain-specific effect of pectin treatment.

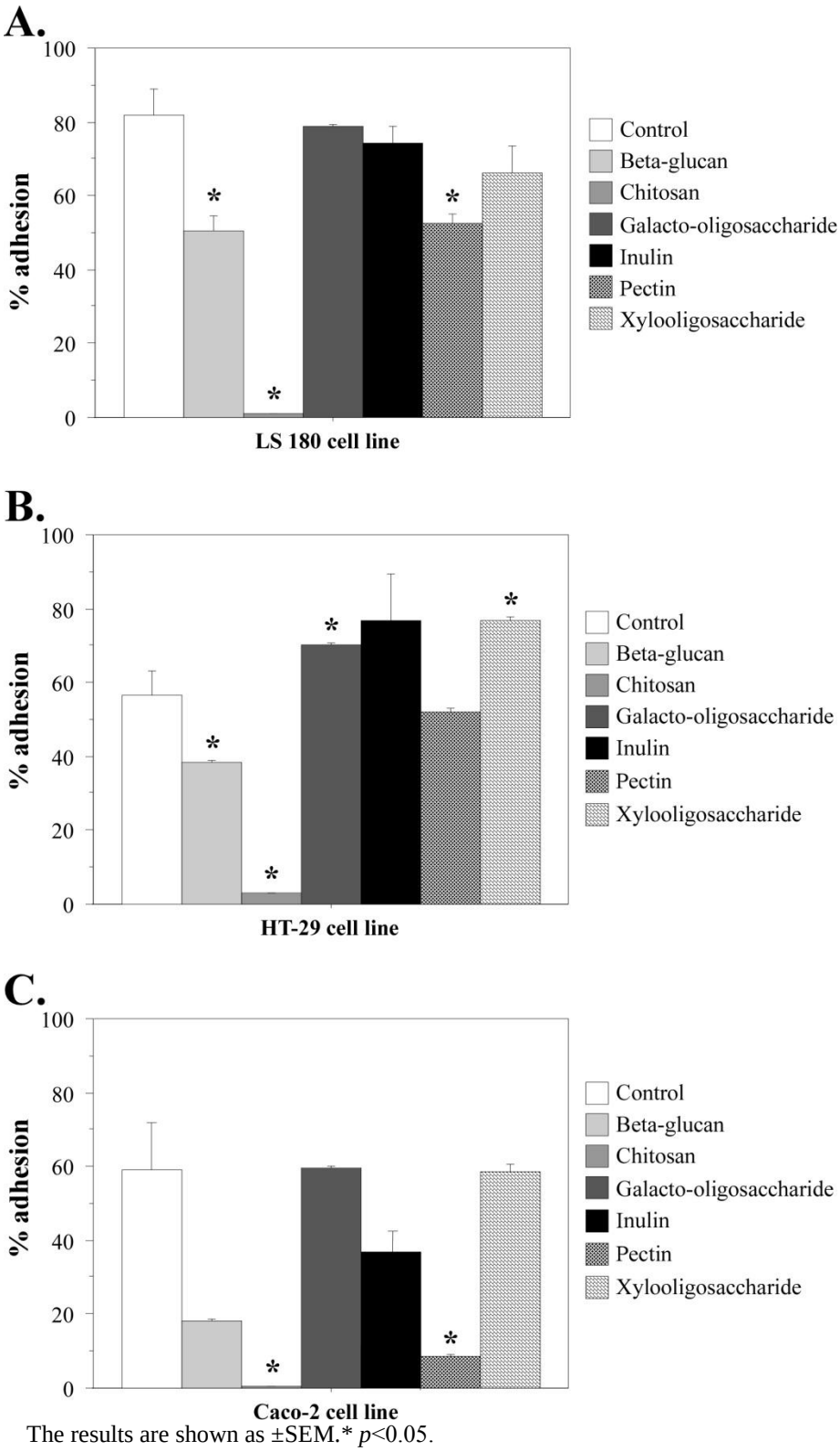
Binding properties of lactobacilli vary considerably among strains and species (Jacobsen *et al.,* 1999, Piatek *et al.,* 2012). For example, there are reports indicating quite diverse range of adhesion properties of *Lactobacillus rhamnosus* – from adhesion percentage below 1 to up to 20% adhesion and more(Gopal *et al.,* 2001, Markowicz *et al.,* 2014, Mousavi *et al.,* 2016, Tuomola and Salminen, 1998). Various adhesion percentages have been determined also for different strains of *Lactobacillus acidophilus* and *Lactobacillus paracasei* (Candela *et al.,* 2008, Xu *et al.,* 2009, Zivkovic *et al.,* 2016).

Fig.1 Adheson of *Lactobacillus rhamnosus* 1010 following treatment with different prebiotics. Graphs represent percent adhesion of the lactobacilli to LS 180 (A.), HT-29 (B.) and Caco-2 (C.) cells



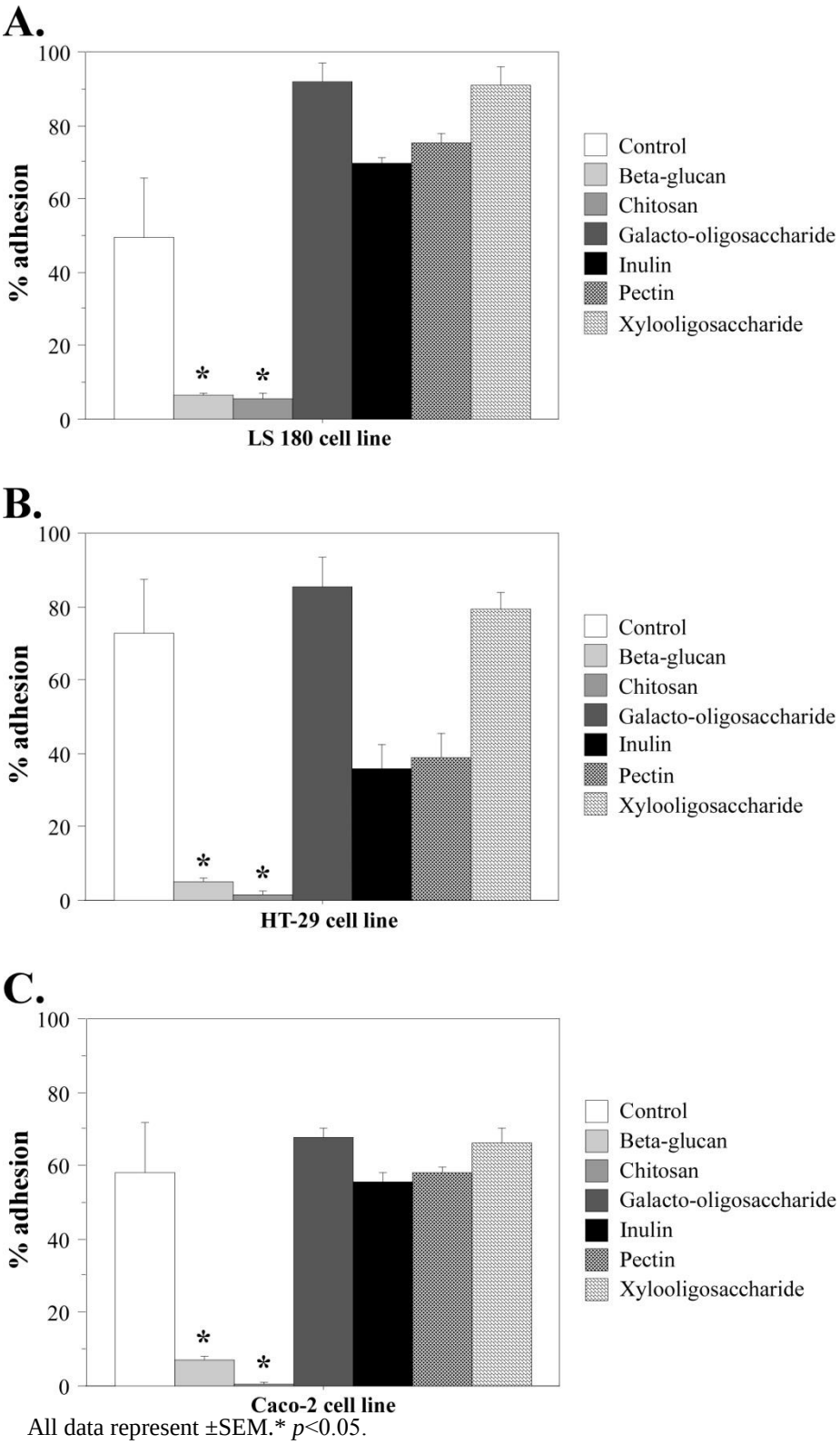
Data are presented as ±SEM.* $p<0.05$.

Fig.2 *Lactobacillus acidophilus* 11 binding properties to different enterocyte-like cell lines after treatment with prebiotics. A. Adhesion of *L. acidophilus* 11 to LS 180 cells; B. Results from adhesion assay performed with HT-29 cells; C. *L. acidophilus* 11 binding to Caco-2 cells



The results are shown as ±SEM.* $p<0.05$.

Fig.3 Adhesion properties of *Lactobacillusparacasei* 8458 following treatment with prebiotics. A. Binding of L. paracasei 8458 to LS 180 cells; B. Adhesion to HT-29 monolayers; C. Results from adhesion assay with Caco-2 cells



All data represent ±SEM.* $p<0.05$.

In our studies we have determined high percent adhesion for the control untreated with prebiotics samples from all three *Lactobacillus* strains, which again confirms the vast variation of adhesive properties of different strains lactobacilli. Another example that supports this fact is the species *Lactobacillus plantarum* – the reported percentages adhesion of different strains *L. plantarum* vary from 6.7 to 80(Moussavi and Adams, 2010, Tuomola and Salminen, 1998).

In general, prebiotics are considered to beneficially affect the growth and activity of probiotic bacteria. Interestingly, when investigating cell adhesion it turns that not all prebiotic ingredients have a positive effect on this property. Our results demonstrate a very interesting finding – the prebiotic polysaccharides beta-glucan and chitosan significantly reduce the adhesion properties of lactobacilli. All three treated *Lactobacillus* species exhibited reduced binding ability to human enterocyte-like cell lines. Concerning beta-glucan our results differ from previous findings. Ruso and colleagues (2012) demonstrated that beta-glucans increased the growth, viability and colonization ability of probiotic microorganisms (Russo *et al.*, 2012). We suggest that the reason for these different effects is the origin of the substance used for the studies. In our experiments we have used beta-glucan from barley, while Ruso and colleagues investigated the effect of bacterial beta-glucan. Arena *et al.,* (2014) reported stimulatory effect of barley beta-glucan containing food matrices on the probiotic performances of four *Lactobacillus* strains (Arena *et al.,* 2014). However, strong enhancement of the adhesion properties has not been detected and one of the strains showed reduced adhesion to Caco-2 cells in the presence of beta-glucan enriched pasta (Arena *et al.,* 2014).The mechanism leading to the negative effect of beta-glucan and chitosan on lactobacilli adhesion is unknown, but one could speculate that the prebiotic polysaccharides interact with surface determinants that mediate the bacterial adhesion to the intestinal surface. Beta-glucan and chitosan treatment could result in specific or non-specific binding to bacterial adhesins or in steric hindrance of adhesins. Further experiments are needed to confirm this hypothesis.

Comparing the adhesion percentages of each LAB strain to the three enterocyte-like cell lines it is evident that the highest binding levels of *L. rhamnosus* 1010 and *L. acidophilus* 11 were achieved with LS 180 cells. The adhesion of these LAB strains to HT-29 and Caco-2 cells was lower, especially for Caco-2 cells. On the other hand, *L. paracasei*8458control culture and the cultures treated with galacto-oligosaccharides, xylooligosaccharides, pectin and inulin showed high percent adhesion to Caco-2 cells. However, the highest adhesion levels of *L. paracasei* 8458 were detected with HT-29 cells (for the control and galacto-oligosaccharides- and xylooligosaccharides-treated cultures) and LS 180 cells (for the control, inulin-, pectin-, xylo- and galacto-oligosaccharides-treated cultures). The higher ability of LAB to adhere to HT-29 and LS 180 cells could be explained with the production of mucin by these cell lines, as determined by McCool *et al.,* (1994) for LS 180 cells (McCool *et al.,* 1994)and by Velchich *et al.,* (1995) for HT-29 cells treated with sodium butyrate (Velcich *et al.,* 1995).

In conclusion, the present report demonstrates specific effects on lactobacilli adhesion properties induced by treatment with different prebiotics: incubation of *Lactobacillus rhamnosus* 1010, *Lactobacillus paracasei* 8458 and *Lactobacillus acidophilus* 11 with beta-glucan or chitosan dramatically reduces bacterial adhesion to human colon-derived cell lines; xylooligosaccharides and galacto-

oligosaccharides treatment stimulates lactobacilli adhesion, while apple pectin exhibited strain-specific effects. These data provide basis for future investigations on the mechanisms that modulate bacterial adhesion in the presence of different prebiotics.

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