Competition of Lactobacillus paracasei with Salmonella enterica for Adhesion to Caco-2 Cells

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Competition of commensal and probiotic bacteria with pathogens for adhesion and colonization is one of the important protective mechanisms of gastrointestinal tract. In this study, we examined the ability of Lactobacillus paracasei to inhibit the adhesion of pathogenic Salmonella enterica to human colon adenocarcinoma Caco-2 cells. Caco-2 cells were grown for 6 or 21 days to obtain nondifferentiated or well-differentiated cells, respectively. In adhesion experiments, bacteria were added to the cells for 2 or 4 hours. The number of attached bacteria was expressed as colony-forming units (CFUs), Caco-2 cells were counted in hemocytometer. Both bacterial strains used adhered better to well-differentiated than to nondifferentiated Caco-2 cells, however, the amount of Salmonella adhered to Caco-2 after 2 hours of contact was 12-fold higher in comparison to L. paracasei and almost 27-fold higher after 4 hours of contact. Two types of experiments were done: coincubation (both bacteria were added to Caco-2 cells simultaneously), and preincubation (L. paracasei was incubated with Caco-2 cells first, and then S. enterica was added). In coincubation experiment, the presence of L. paracasei decreased S. enterica adhesion by 4-fold and in preincubation experiment even 7-fold. Generally, Lactobacillus spent culture supernatants (SCSs) acted weaker as inhibitors of Salmonella adhesion in comparison to the whole L. paracasei culture in coincubation experiment. In conclusion, the displacement of pathogens by lactic acid bacteria and its secretions showed here depends on the time of bacteria-epithelial cell contact, and also on the stage of Caco-2 differentiation.

1. INTRODUCTION

Adhesion to mammal’s epithelial cells is a key process for bacteria to survive and colonize the gastrointestinal tract. For pathogenic bacteria, the adhesion to epithelium is a critical step, since it allows the release of enzymes and toxins initiating necrotic processes directly into the target cell, thereby facilitating the invasion.

The epithelial cells of gastrointestinal tract (GIT) are protected from pathogenic bacteria by a number of mechanisms. One of them is a reduction in pathogenic infections through competition of microbiota for adhesion sites with microbial pathogens and production of components with antimicrobial activity [1, 2]. To cause infection, pathogenic bacteria, after penetrating intestinal mucus, must adhere to enterocytes [3]. The initial step of adhesion in the case of Salmonella species is mediated by bacterial fimbriae which recognize certain receptors on eukaryotic cells [4]. Several studies indicate that lactic acid bacteria (LAB) could prevent the attachment of pathogens, in this way reducing colonization, and prevent infection [5–8].

Bacterial adhesion to intestinal epithelium has been studied in different experimental in vitro models involving polymer surfaces [9], intestinal mucus [10–12], or intestinal cell lines, for example, producing mucus HT29-MTX. In the present studies, we used human colon adenocarcinoma
epithelial Caco-2 cell monolayer [13] to investigate bacterial adhesion. The Caco-2 cells differentiate similarly to normal small intestinal epithelial cells expressing characteristic for immature as well as mature enterocytes with functional brush border microvilli and apical hydrolases [14–18]. Several studies have described adhesion to cultured cells of many different lactic acid bacteria [15, 19], *Salmonella* and other bacteria, as well as competition between the microbial species [20]. Chauvière et al. have shown that heat-killed *Lactobacillus* inhibits adhesion of diarrheagenic *Escherichia coli* (ETEC) to Caco-2 cells [14].

The aim of present in vitro study was to investigate the adhesion potency of gram-positive LAB, *Lactobacillus paracasei*, and gram-negative pathogen *Salmonella enterica* to nondifferentiated and well-differentiated Caco-2 cells monolayer and competitive exclusion of pathogenic bacteria by *Lactobacillus* or its secretions under different experimental conditions. The isolated-from contamination food, *Salmonella* is an adequate example of common microbial pathogen causing GIT infection. *L. paracasei* was selected among three *Lactobacillus* and two *Lactococcus* strains tested as the only strain adhering better to well-differentiated than to nondifferentiated Caco-2 cells. This finding allowed to presume that *L. paracasei* better than other LAB will compete with *Salmonella* for adhesion to Caco-2 cells.

2. MATERIALS AND METHODS

2.1. Bacterial strains and adhesion to Caco-2 cells

Two bacterial strains were used, isolated from human stool *Lactobacillus paracasei* IBB2588 (IBB PAS, Warsaw, Poland) and isolated from instant soup pathogenic *Salmonella enterica* subsp. *enteritidis* sv *Enteritidis* KOS 1663 (purchased from The National Salmonella Centre, Poland). *L. paracasei* was cultured in MRS broth (de Man, Rogosa, Sharpe) or on MRS plates (MRS broth supplemented with 1.5% agar, Biocorp Ltd., Poland) under anaerobic conditions (in anaerobic jar, OXOID Ltd., UK) at 37°C for 18–20 hours. The pathogenic *S. enterica* was cultured in Luria-Bertani broth (Biocorp Ltd., Poland) or on LB plates (LB supplemented with 1.5% agar, Biocorp Ltd., Poland) at 37°C for 18–20 hours under aerobic conditions. For the experiments, the overnight culture was 100-fold diluted in medium for Caco-2 cells but devoid of antibiotics and antimycotics. Then bacteria were incubated with Caco-2 cells for 2 or 4 hours, washed 3 times with sterile PBS (pH 7.4) and, after trypsinization, number of adhered bacteria was quantified as well as number of Caco-2 cells as described below. The number of bacteria adhering to the Caco-2 cells was expressed as colony-forming units (CFU). The CFUs were determined by plating of diluted bacterial suspensions on MRS or LB plates depending on bacterial strain, see above.

2.2. Caco-2 cell cultures

For the adhesion assay, a 3-week-old (well-differentiated) Caco-2 cell (ECACC 86010202) culture was used. Caco-2 cells were cultured in Dulbecco’s modified Eagle’s minimal essential medium DMEM (Sigma, USA) supplemented with 10% heat inactivated foetal bovine serum (Gibco, Invitrogen Corporation, USA), and 1% nonessential aminoacids solution (Sigma, USA) and antibiotics antimycotics solution (10 IU/mL penicillin G, 100 μg/mL streptomycin sulphate, and 250 ng/mL amphotericin B; all antibiotics and antimycotics were from Sigma, USA). The medium was replaced by a new one every two days. The Caco-2 cells were grown at standard conditions (37°C, 5% CO2, 95% humidity) on cover slides. After three weeks, cells were washed in PBS buffer and transferred into the culture medium without antibiotic and antimycotic solution, then used for adhesion experiments. After the experiment, cells were detached from cover glass and dispersed using trypsin-EDTA solution (0.5% porcine trypsin and 0.2% EDTA in PBS, Sigma, USA), and then counted in Bürker hemocytometer chamber (Merck, USA).

2.3. Competition studies

These studies were performed on *L. paracasei* IBB2588 and *S. enterica* KOS1663 submitted together to the Caco-2 cell culture. Overnight bacteria cultures were 100-fold diluted, up to about 1–2 × 10^7 bacteria/mL, mixed and added to Caco-2 cells for 2- or 4-hour incubation at 37°C. After trypsinization, the mixture of bacterial cells was diluted and plated on MRS plates (for *Lactobacillus*) and LB plates (for *Salmonella*) to estimate their CFUs. The following variants of the competition study were performed.

(i) Coincubation of *L. paracasei* IBB2588 and *S. enterica* KOS1663 strains with Caco-2 cells.

(ii) Coincubation of *L. paracasei* devoided of MRS broth and *S. enterica* with Caco-2 cells. In this experiment, *L. paracasei* overnight culture was centrifuged (5000 rpm/min), the spent culture supernatant (SCS) was removed, bacterial pellet resuspended in isotonic salt solution, and, together with *S. enterica*, incubated with Caco-2 cells.

(iii) Coincubation of *L. paracasei* total SCS (obtained in above described manner) and *S. enterica* with Caco-2 cells.

(iv) Coincubation of different fractions of *L. paracasei* SCS and *S. enterica* with Caco-2 cells. The total SCS of *L. paracasei* was distributed by centrifugation in test-tube filters (Millipore, USA) onto 4 fractions according to the size of molecules: fraction I- >30 kDa, fraction II- 30–10 kDa, fraction III- 10–5 kDa, and fraction IV- <5 kDa. The *S. enterica* was incubated at 37°C for 2 and 4 hours with 100-fold diluted supplementation of each fraction.

(v) Preincubation of Caco-2 cells with fresh MRS broth and subsequent addition of *S. enterica*.

(vi) Preincubation of *L. paracasei* with the Caco-2 cells for 2 hours, washing out the nonadhered lactobacilli and administration of *S. enterica* for further 2 or 4 hours.

2.4. Calculations and statistical analysis

Bacterial adhesion was expressed as the number of bacteria attached to one Caco-2 cell. Data were analyzed by one-way
ANOVA followed by Tukey posthoc test, and unpaired t-test (GraphPad Prism v.3.0, GraphPad Software, San Diego, CA, USA) and shown as mean and SEM. In all statistical analyses, $P<.05$ was taken as the level of significance.

3. RESULTS

3.1. Adhesion of L. paracasei and S. enterica to Caco-2 cells

We studied the adhesion of five LAB strains (three Lactobacillus and two Lactococcus). These bacteria adhered to Caco-2 cells in the range of 0.5 to 5 bacteria per one cell. The adherence of L. paracasei to Caco-2 cells was low (Figure 1(a)). Only 0.6 bacteria adhered to one nondifferentiated (growing for 6 days) Caco-2 cells, and about 1.5 bacteria adhered to well-differentiated (growing for 21 days) cells. There was no significant difference between 2- and 4-hour incubation of L. praca-casei with Caco-2. For competition experiments, the L. paracasei was selected since among all LAB strains tested L. paracasei was the only one adhering better to well-differentiated than to nondifferentiated Caco-2 cells exactly as pathogenic S. enterica. The adherence of S. enterica (Figure 1(b)) was high as compared to that of L. paracasei ($P<.01$). On the average, there were 6 and 25 bacteria per nondifferentiated Caco-2 cells after 2- and 4-hour incubation, respectively, and 18 and 40 bacteria per differentiat-ed Caco-2 cells after 2- and 4-hour incubation, respectively (Figure 1(b)). Thus, the rule, the longer the contact the higher adherence rate, was true for S. enterica but not for L. paracasei.

3.2. Competition between L. paracasei and S. enterica for adhesion to Caco-2 cells coincubation experiments

Coincubation of L. paracasei and S. enterica with Caco-2 cells for 2 and 4 hours resulted in about 4- and 2-fold decrease of S. enterica adhesion to Caco-2 cells, respectively, as compared to the experiment with incubation of S. enterica alone (Figures 2(b) versus 2(a) and Figures 3(b) versus 3(a)). A significant decrease ($P<.05$) in the number of S. enterica adhering to Caco-2 cells was also observed in a coincuba-

tion study when L. paracasei devoid of its growing medium and resuspended in salt solution was used (Figures 3(c) versus 3(a)). However, the decrease was statistically significant only after 4-hour incubation, and the effect was significantly weaker than that when L. paracasei was used with its incubation medium (Figures 2(c) versus 2(b) and Figures 3(c) versus 3(b)). The later indicated that not only L. paracasei by itself but also the substances secreted by L. paracasei to the medium might counteract the adhesion of S. enterica to Caco-2 cells. This was confirmed in the studies with the use of supernatant obtained after centrifugation of L. paracasei overnight culture (SCS). Four-hour incubation of S. enterica with SCS led to a significant decrease of adhesion of S. enterica to Caco-2 cells (Figures 3(d) versus 3(a)) while the 2-hour incubation did not show a statically significant effect (Figures 2(d) versus 2(a)). The results of incubation of S. enterica with four separate L. paracasei SCS fractions are shown on Figures 2(e)–2(h). Besides fraction IV (<5 kDa), the presence of remaining fractions resulted in significant reduction of S. enterica adhesion to Caco-2 cells (Figures 3(e) versus 3(a), 3(f) versus 3(a) and 3(g) versus 3(a)). Among these fractions, the strongest inhibition was observed for fraction II (30–10 kDa). Fresh MRS-broth medium did not influence the adhesion of S. enterica to Caco-2 cells (data not shown).

3.3. Competition between L. paracasei and S. enterica for adhesion to Caco-2 cells pre-incubation experiments

Preincubation of L. paracasei with Caco-2 cells for 2 hours and subsequent addition of S. enterica resulted in over 2-fold stronger inhibition of Salmonella adherence than in coincuba-tion experiment. This means that Lactobacillus inhibited Salmonella adherence 8- and 4-fold in pre- and coincubation experiment, respectively (Figures 2 and 4). The effect of preincubation was, however, statistically significant only in the 2-hour ($P<.05$) but not in the 4-hour coincubation experiment (Figure 4).

There were no statistically significant differences in Salmonella adherence to Caco-2 cells preincubated for 2 hours with Lactobacillus culture and to Caco-2 cells devoid of nonadhered L. paracasei cells (to get rid of nonadhered

**Figure 1:** Adhesion of (a) L. paracasei and (b) S. enterica to nondifferentiated and well-differentiated Caco-2 cells in 2- and 4-hour experiments. Mean and SEM, t-test. Asterisks indicate the statistical differences: $^*P<.05$, $^{**}P<.01$, $^{***}P<.001$. 

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**Figure 2:** Two-hour coincubation experiments. Adhesion of *S. enterica* to Caco-2 cells under different conditions: (a) *S. enterica* was incubated with Caco-2 cells; (b) *S. enterica* and *L. paracasei* were coincubated; (c) *L. paracasei* overnight culture was washed with PBS, resuspended in isotonic salt solution and incubated with *S. enterica*; (d) *L. paracasei* overnight culture was centrifuged and spent supernatant (SCS) was incubated with *S. enterica*; (e) *S. enterica* incubated with *L. paracasei* SCS fraction I (>30 kDa); (f) *S. enterica* incubated with *L. paracasei* SCS fraction II (30–10 KDa); (g) *S. enterica* incubated with *L. paracasei* SCS fraction III (10–5 KDa); (h) *S. enterica* incubated with *L. paracasei* SCS fraction IV (<5 KDa). Numbers represent the amount of adhered bacteria per 1 Caco-2 cell. Mean and SEM, t-test. Asterisks indicate the statistical differences: *P < .05, **P < .01, ***P < .001.

**Figure 3:** Four-hour coincubation experiments. Adhesion of *S. enterica* to Caco-2 cells under different conditions: (a) *S. enterica* was incubated with Caco-2 cells; (b) *S. enterica* and *L. paracasei* were coincubated; (c) *L. paracasei* overnight culture was washed with and resuspended in isotonic salt solution and coincubated with *S. enterica*; (d) *L. paracasei* SCS was incubated with *S. enterica*. Numbers represent the amount of adhered bacteria per 1 Caco-2 cell. Mean and SEM, t-test. Asterisks indicate the statistical differences: *P < .05, **P < .01, ***P < .001.

LAB after 2 hours of incubation, Caco-2 cells were washed with PBS.

In another type of preincubation experiment *L. paracasei* was incubated for 2 hours in DMEM (medium for Caco-2 cell culture), spoon down and the SCS was added to Caco-2 cell culture for 2 hours prior to *S. enterica* addition. Over 2-fold inhibition of *Salmonella* adhesion by this supernatant was observed (Figure 4).

### 4. DISCUSSION

Lactic acid bacteria of normal intestinal microbiota are known to counteract the pathogenic bacteria invasion. Such inhibitory effects of lactobacilli can be explained by a mechanism of nonspecific steric hindrance on the receptors for pathogens [21, 22]. Other inhibition mechanisms cannot be excluded, for example, some metabolic products from LAB, such as lactic acid, exopeptides, or exopolysaccharides, may inhibit the adherence of pathogenic bacteria.

In the present study, Caco-2 cell monolayer grown on the glass was used as a model for investigation of bacterial adhesion to the intestinal epithelium. Besides lack of mucus production, this model offers a number of interesting features ideal for investigation bacterial adherence. The nondifferentiated and well-differentiated Caco-2 cells were used as a model of nondifferentiated crypt and differentiated villi enterocytes, respectively [16–18]. The adhesion of pathogenic *S. enterica* KOS1663 to Caco-2 cells was examined in the presence of *L. paracasei* IBB2588 or secreted products of the LAB metabolism as a potential competitor or inhibitor. The examined salmonellae adhered to well-differentiated and to
This indicates that both, growing medium with side products of bacterial metabolism of complete Lactobacillus SCS and the bacteria devoid of SCS inhibit the adhesion of antimicrobial compound(s). We have shown that tors but also an action of produced or secreted by may involve not only competition for eukaryotic cell recep-

sion. These results are especially impressive if added to preincubated with S. enterica KOS1663 and L. paracasei coincubation S. enterica added to preincubated with L. paracasei Caco-2 cells S. enterica added to preincubated with L. paracasei supernatant Caco-2 cells

Figure 4: Adhesion of S. enterica to Caco-2 cells preincubated with L. paracasei or its culture spent supernatant (SCS) as indicated. Bars represent mean values with respective SEM. Asterisks indicate the statistical differences: ∗P < .05, ∗∗P < .01, ∗∗∗P < .001 (one-way ANOVA followed by Tukey posthoc test).

a lesser degree to nondifferentiated Caco-2 cells several times better than the lactobacilli (Figure 1). Ours [23] and others [24] studies have shown that the colonization of lactobacilli takes place mainly in the intestinal crypts, whereas the pathogenic bacteria encounter mostly the upper part of intestinal villi and thus adhere to well-differentiated enterocytes (i.e., expressing the brush border). Among 5 LAB strains tested, the L. paracasei IBB2588 was the only one that preferred to attach to well-differentiated Caco-2 cells (this strain attached to well-differentiated cells 2.5-fold better than to nondifferentiated). Most probably these bacteria, similarly to S. enterica, use receptor-type adhesion mechanism and receptors are better developed in well-differentiated cells. This feature of L. paracasei IBB2588 seemed to create conditions for stronger inhibitory effect, if present, on pathogen adhesion.

Our studies have shown that in the presence of L. paracasei, S. enterica adhered to Caco-2 cells with a 4-fold lower efficiency, and the adhesion was further reduced (8-fold) when L. paracasei was preincubated with Caco-2 cells prior to Salmonella addition. These results are especially impressive if one takes in consideration several-fold lower adhesion ability of L. paracasei in comparison to Salmonella. This finding also indicates that the phenomenon of adherence inhibition may involve not only competition for eukaryotic cell receptors but also an action of produced or secreted by L. paracasei antimicrobial compound(s). We have shown that Lactobacillus SCS and the bacteria devoid of SCS inhibit the adhesion of Salmonella, nevertheless, the effect was weaker than that of complete Lactobacillus culture containing bacteria in its growing medium with side products of bacterial metabolism. This indicates that both, L. paracasei cells and some bacte-

rial products not defined in this study may act as inhibitors of S. enterica adhesion. Substances produced by lactobacilli show also other futures. Coconnier-Polter et al. have found that cell-free culture supernatant of L. acidophilus LB decreases intracellular ATP in S. enterica SL1344. It also releases lipopolysaccharide, increases permeability of the bacterial membrane and the sensitivity of Salmonella to sodium dodecyl sulfate [25]. The same authors have shown that L. acidophilus culture supernatant inhibits adhesion-dependent Salmonella-induced interleukin-8 production [6].

Several studies have been done on the nature of the secreted by Lactobacillus antibacterial substances. There are data indicating that the inhibitory molecules are of low molecular weight (not exciding 3 kDa) [26] or even as small as acting through pH modification lactic acid [27]. In our experimental system, this seems not to be the case, since the pH of Caco-2 cell medium was monitored during each experiment and it was stable in the range 7.2–7.5 due to the buffering properties of the medium. Moreover, we found that the SCS fraction containing molecules within a range 30–10 kDa produced the strongest inhibition of S. enterica. This suggests the relevance of some substance(s) much larger than the lactic acid. This is in agreement with other group of data that indicate on peptides as antimicrobial factors [28] or synergistic action of lactic acid and proteinaceous substances [29]. Recapitulate, it seems that individual LAB (probiotic) strains produce different and of diverse mechanisms of action antibacterial substances characteristics for a particular strain.

The important feature of the described here competition phenomenon is its transitory character. The strongest adhesion inhibition was observed in 2-hour experiment. Longer, 4-hour coincubation of L. paracasei and S. enterica led to a partial restoration of the pathogen adhesion to Caco-2 cells (Figures 3 and 4).

The ability of selected strains of Lactobacillus (probiotics) to inhibit the adhesion of pathogenic bacteria is highly specific, and depends on both the probiotic and pathogen strain [30, 31]. This indicates the need of a case-by-case characterization of the probiotic strains. Except specific antibacterial substances produced by LAB, the inhibition of adhesion could be related to the presence of specific adhesion molecules and receptors for which probiotic and pathogen are competing. It has to be taken under consideration that observed in vitro inhibitory effect of of probiotics on pathogen adhesion has to be confirmed in vivo.

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