Research article

In vitro anti-adherence effect of probiotic *Lactobacillus* strains on human enteropathogens

Adriana Nowak,[*] Ilona Motyl

Institute of Fermentation Technology and Microbiology,
Lodz University of Technology, Wólczańska 171/173, 90-924 Lodz

[*] adriana.nowak@p.lodz.pl

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Abstract: *Probiotic bacteria possess great potential for producing antimicrobial substances that inhibit and control pathogenic bacteria in the human gastrointestinal tract. The aim of this study was to determine the anti-adherence properties of the probiotic Lactobacillus strains Lb. rhamnosus ŁOCK 0900, Lb. rhamnosus ŁOCK 0908, and Lb. casei ŁOCK 0919 (individually and in a 1:1:2 mixture) against the reference pathogens Clostridium difficile (ATCC 9689), Enterococcus faecalis (ATCC 29212), Listeria monocytogenes (ATCC 19115), and Staphylococcus aureus (ATCC 6538) using the Caco-2 human colon adenocarcinoma cell line. The mixture of probiotic strains inhibited the adherence of all pathogens, from 10.2% for E. faecalis ATCC 29212 to 97.2% for L. monocytogenes ATCC 19115. Of all the tested probiotic strains, Lb. casei ŁOCK 0919 reduced the adherence of S. aureus ATCC 6538 to the greatest extent (by 45.9%). These results suggest that adherence inhibition may involve competition for eukaryotic cell receptors and probiotic bacteria could protect the host cells from pathogen colonization and disease.*

Keywords: *adherence, intestinal microbiota, pathogens, inhibition, Caco-2 cells.*

Introduction

Probiotics are live microorganisms (mostly *Lactobacillus* and *Bifidobacterium* species) which, if administered in adequate amounts, confer a health benefit on the host [1, 2]. Probiotics are considered allochthonous microbiota which reside in the human colon temporarily. They can colonize the intestines for a prolonged period of time or even permanently, providing benefits for the host, which decline if probiotics are not administered continually. Some bacteria have developed special mechanisms to prevent removal from the human gastrointestinal tract and survive in that unfavourable environment, one of such adaptations being the ability to bind to host cells. Some probiotics can prevent gastrointestinal infections by inhibiting the adherence of pathogens to the colon epithelium. The effect depends on the specificity of the probiotic strain and the pathogen [3]. Bacteria with the highest adhesive ability have the greatest effect on host health; those with a lower

ability tend to be less virulent [4]. Probiotics adhering to epithelial cells in the gastrointestinal tract can prevent infections of enteric pathogens at an early stage by competition for nutrients and attachment sites, or by the secretion of antagonistic substances, such as lactic acid, bacteriocins, hydrogen peroxide, exopeptides, or exopolysaccharides [5-7]. The adherence of probiotics to epithelial cells is an important prerequisite for colonization of the gastrointestinal tract.

While there are a great number of cell lines available for *in vitro* investigation of adherence ability, it is recommended to use cultures of human intestinal epithelial cell lines, e.g., Caco-2 or HT-29 (human derived adenocarcinoma) because they are the most representative and comparable to in vivo models. The main benefit of using Caco-2 cells is that they grow in culture, forming a homogeneous and polarized cell monolayer that resembles mature human enterocytes in the small intestine [8].

The objective of the study was to determine the inhibition of pathogenic adherence to the human colon adenocarcinoma cell line Caco-2 by three strains of probiotic bacteria (individually and in a mixture). Four strains of gram-positive human enteric pathogens, both cocci and rods, were chosen for a competition assay.

Experimental

Probiotic strains and pathogens

The probiotic *Lactobacillus* strains used in the experiments were *Lactobacillus rhamnosus* ŁOCK 0900, *Lb. rhamnosus* ŁOCK 0908, and *Lb. casei* ŁOCK 0919. They are of human origin and have been documented and licensed as probiotics [9-13]. They are deposited at the Institute of Immunology and Experimental Therapy, Polish Academy of Sciences in Wroclaw, Poland. The strains are recommended for children with atopic dermatitis (they were used in double-blind placebo control studies with 200 atopic dermatitis children – grant no. 12010110 from the Centre for Research and Development, Poland – unpublished data). Before application, the bacteria were activated in liquid MRS broth (Merck) and incubated at 37°C for 24 h. Stock cultures were stored at 4-5°C for 24 h. Inocula (3%, v/v) consisted of 24 h cultures of bacteria in MRS. In the adherence assay, lactic acid bacteria (LAB) were plated on MRS agar (Merck). The probiotic mixture consisted of *Lb. rhamnosus* 0900, *Lb. rhamnosus* 0908, and *Lb. casei* 0919 at a ratio of 1:1:2.

The following reference pathogens [14-16] were used: *Clostridium difficile* (ATCC 9689), *Enterococcus faecalis* (ATCC 29212), *Listeria monocytogenes* (ATCC 19115), and *Staphylococcus aureus* (ATCC 6538). The strains were maintained as 20% glycerol stock preparations at -20°C. They were activated twice in liquid Nutrient broth with glucose (Merck) and incubated at 37°C for 24 h. Stock cultures were stored for 24 h at 4-5°C. In the competition adherence assay, the pathogens were plated on appropriate media: *Cl. difficile* ATCC 9689 on TSC agar (Merck) with D-cycloserine (Merck), *E. faecalis* ATCC 29212 on

Agar D-Coccosel (bioMèrieux), *L. monocytogenes* ATCC 19115 on Palcam Agar Base (Oxoid), and *S.aureus* ATCC 6538 on Baird-Parker RFP agar (Oxoid).

Cell culture

The procedure was described previously [17]. Caco-2 cells (ATCC HTB-37, lot no. 58844056) were cultured in Roux flasks as monolayers in Dulbecco's Modified Eagle's Medium (DMEM, Sigma) supplemented with 10% fetal bovine serum (FBS, Gibco), 4 mM glutaMAX^{TM} (Gibco, Invitrogen), 25 mM HEPES (Sigma), 100 μg/ml streptomycin and 100 IU/ml penicillin (Sigma). Cells were cultured at 37°C in 5% CO_2 for 7-10 days until the monolayer was formed. The adherent cells were washed every 3 days with 0.1 M PBS and a fresh medium was added. Cells were detached by trypsinization with 1% trypsin-EDTA (Sigma) for 2 min at 37°C. The cell suspension was centrifuged (187 × g, 5 min). The pellet was re-suspended in fresh DMEM. Subsequently, a cell count was performed with the use of hemocytometer and cell viability was determined by trypan blue exclusion.

Competition assay

The procedure was conducted as previously described [18], with some modifications. Caco-2 cells were seeded with 1 mL culture medium containing 2.5×10^6 cells/well in a 24-well tissue culture plate and incubated at 37°C in 5% CO_2. All bacteria were cultured at 37°C for 18 h, collected by centrifugation (10,700 × g, 10 min), washed with sterile PBS (pH 7.4) and suspended in non-supplemented DMEM (Sigma) at 1.0-2.0 × 10^8 CFU/mL, as measured earlier using a DEN-1 McFarland densitometer (Biosan, Riga, Latvia). Additionally, the initial number of bacteria was counted by the pour plate method. After reaching confluence, Caco-2 cells were washed with PBS. Bacterial suspensions were added to a monolayer of Caco-2 cells in triplicate, each time at a concentration of $1.0 – 2.0 \times 10^8$ CFU/mL. The pathogenic bacteria were added as single strains for individual adherence assays or in conjunction with probiotic strains (1:1) and incubated at 37°C in 5% CO_2 (Galaxy, New Brunswick) for 2 h. Unattached microorganisms were removed by washing with PBS three times. Caco-2 cells were detached with 1% trypsin-EDTA for 10 min and lysed with 0.1% (v/v) Triton X-100 for 5 min at room temperature. Adherent bacteria were enumerated by plate counting with an appropriate selective agar medium for each strain and incubated in aerobic or anaerobic conditions (as appropriate) at 37°C for 24 to 72 h.

For individual strains, adherence (the number of bacteria per 100 Caco-2 cells) was calculated as follows: [adherent bacteria divided by the number of Caco-2 cells in the well] × 100%.

In the competition test, the inhibition rate [%] was calculated according to the formula: 100 – [adherence of the tested sample × 100/adherence of the control]. This represents a percentage reduction in adherent pathogens in the presence of a probiotic (as compared to the results for the pathogen alone).

Statistical analysis

Two-way analysis of variance (ANOVA) was employed, using OriginPro 6.1 software to evaluate the experimental data. Significant differences were accepted at $P < 0.05$ as evaluated with Student's *t*-test (Statistica 10, StatSoft).

Results and discussion

As presented in Table 1, *S.aureus* ATCC 6538 was found to be the most adhesive (2440 bacteria per 100 Caco-2 cells), followed by *E. faecalis* ATCC 29212 (2360 bacteria per 100 Caco-2 cells). *Cl. difficile* ATCC 9689 was the least adhesive (880 bacteria per 100 Caco-2 cells).

Table 1. Adherence of pathogens per 100 Caco-2 cells in the presence of probiotic lactobacilli (in the competition test)

Pathogen	Control pathogen	In the presence of			
		Lb. rhamnosus 0900	*Lb. rhamnosus* 0908	*Lb. casei* 0919	Probiotic mixture
Enterococcus faecalis ATCC 29212	2360	2490	2525	1940	2119
Staphylococcus aureus ATCC 6538	2440	4400	2480	1320	1800
Listeria monocytogenes ATCC 19115	1560	116	1.92	920	44
Clostridium difficile ATCC 9689	880	88	10.4	840	88

In the competition test, *Lb. casei* ŁOCK 0919 resulted in a marked reduction in the adherence of *S.aureus* ATCC 6538 (45.9%) and *L. monocytogenes* ATCC 19115 (41.0%), a moderate reduction in that of *E. faecalis* ATCC 29212 (17.8%), and a slight reduction in that of *Cl. difficile* ATCC 9689 (4.5%) (Figure 1 and Table 1). *Lb. rhamnosus* 0900 and 0908 strongly reduced the attachment of *L. monocytogenes* ATCC 19115 and *Cl. difficile* ATCC 9689 (over 90%) (Figure 1, Table 1). The adherence of *E. faecalis* ATCC 29212 was the least inhibited (only by *Lb. casei* 0919), while it was slightly stimulated by *Lb. rhamnosus* 0900 and 0908. Only *Lb. casei* 0919 reduced the adherence of *S. aureus* ATCC 6538 (by 45.9%), while *Lb. rhamnosus* 0900 strongly stimulated this process (over 40%) (Figure 1).

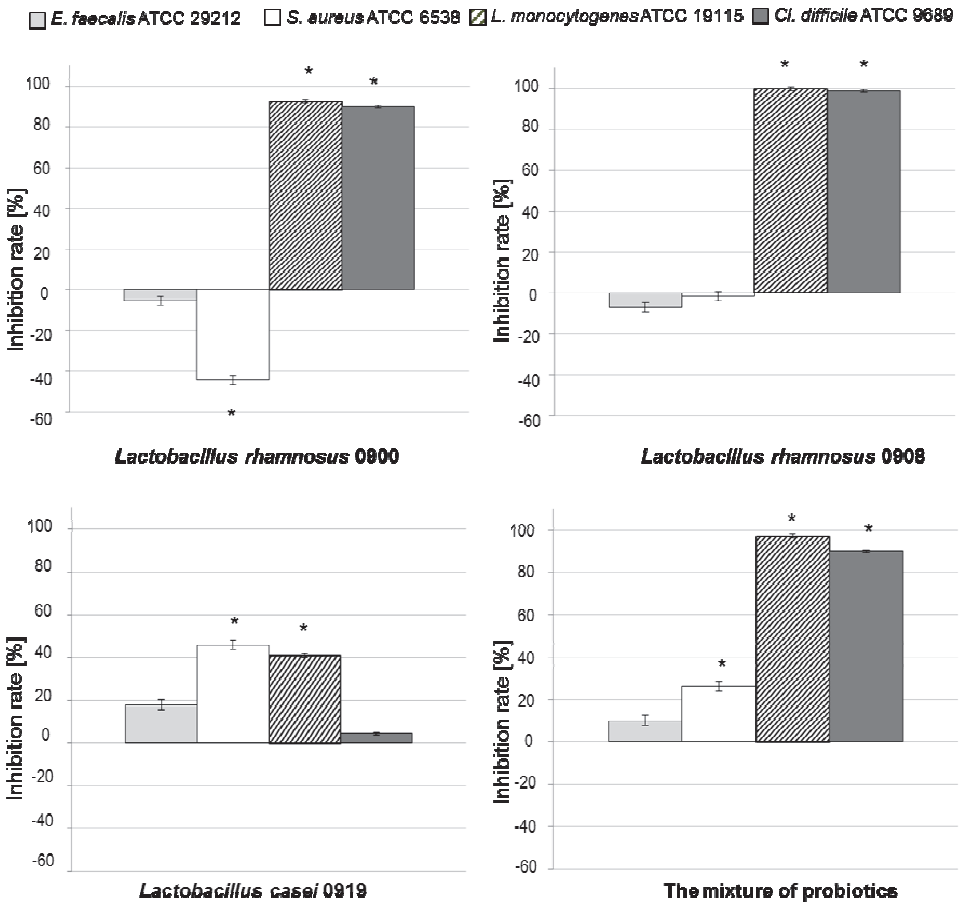

Figure 1. The effect of probiotic *Lactobacillus* strains and their mixture on the adhesion of pathogens to Caco-2 cells. Calculation of the inhibition rate (%) was explained in the text. The data shown are means from two replicates (± SD). *Results significantly different from the control (adherence of each pathogen alone), ANOVA ($P < 0.05$)

The adherence of all pathogens was lowered by the mixture of probiotic strains, and that of *L. monocytogenes* ATCC 19115 and *Cl. difficile* ATCC 9689 was inhibited to the greatest extent (over 90%). The inhibition rate for *E. faecalis* ATCC 29212 and *S. aureus* ATCC 6538 was 10.2% and 26.2%, respectively (Figure 1, Table 1).

Adherence is an essential step in bacterial pathogenesis or infection, required for colonizing a new host, since it allows for the release of enzymes and toxins initiating necrotic processes directly into the target cells, thereby facilitating invasion [5]. Our previous research showed the tested lactobacilli to have good anti-adherence activity against *Salmonella enterica* serovar Typhimurium ATCC

14028, *Escherichia coli* ATCC 10536, and *Candida albicans* ATCC 10231 [18]. In the present work, *Lb. casei* 0919 was the only strain to inhibit the adherence of all the tested pathogens. In our previous study, *Lb. casei* 0919 strongly adhered to the Caco-2 cell monolayer and strongly inhibited the adherence of *C. albicans* ATCC 10231 [18]. The strain has several genes in the pLOCK919 plasmid involved in adherence. According to Koryszewska-Bagińska et al. [12], the genome of the strain reveals the presence of factors relevant to the colonization of the human gastrointestinal tract, including proteins critical to adhesion to the host's structural factors. Those results were confirmed in the study of Aleksandrzak-Piekarczyk [19]. Among three strains, *Lb. casei* 0919 demonstrated the highest adhesion potential to biotic (collagen, gelatin, mucous) and abiotic (polystyrene, glass) surfaces, depending on the presence of the pLOCK919 plasmid. In the current study, *Lb. casei* 0919 only slightly inhibited the adherence of *Cl. difficile* ATCC 9689 (4.5%, not significant at $P < 0.05$), in contrast to *Lb. rhamnosus* 0900 and 0908 and the mixture of probiotic strains (over 90% inhibition).

Cl. difficile is a constituent element of the human colonic microbiota. If the physiological bacterial barrier in the colon is altered or damaged by antibiotics, this pathogenic species may proliferate and cause damage due to the production of several toxins (TcdA and TcdB) [20]. *Cl. difficile* is responsible for potentially deadly infections, being the etiologic agent of *Cl. difficile*-associated diarrhea (CDAD), also known as pseudomembranous colitis [20, 21]. In a study of Banerjee et al. [21], *Lb. delbrueckii* ssp. *bulgaricus* B-30892 reduced the adhesion of *Cl. difficile* ATCC 9689 by 81%, while Gueimonde et al. [22] reported that *Lb. casei* TMC 0409 increased its adhesion by 45%.

The next microorganism examined in our study was the human uropathogen *E. faecalis* ATCC 29212, which is the second most common pathogen isolated from hospitalised patients with urinary tract infections as well as from some community patients with such infections [16]. Lactobacilli can displace adherent uropathogenic *E. faecalis*, possibly through biosurfactant production. In our study, only *Lb. casei* ŁOCK 0919 inhibited the adherence of these bacteria by 17.8%, while Velraeds et al. [23] reported 77% inhibition for two strains of lactobacilli. In the presence of *Lb. rhamnosus* 0900 and 0908, slight stimulation of adherence of *E. faecalis* ATCC 29212 was observed, but it was not significant ($P < 0.05$).

L. monocytogenes is the etiological agent of listeriosis. It causes severe infections, such as gastroenteritis, septicemia, and meningitis in humans [24]. The virulence of *L. monocytogenes* stems from its capacity to adhere, invade and multiply. Invasion of the intestinal barrier is the first step in the infection process. The pathogen has been detected on the surface of equipment used in the meat and dairy industry, with surface-adhered cells capable of contaminating food during processing [25]. In our study, all the tested *Lactobacillus* strains strongly inhibited the adherence of *L. monocytogenes* ATCC 19115 to Caco-2 cells, from

41.0% to 99.8%, depending on the strain. Bendali et al. [26] reported that *Lb. paracasei* prevented the adherence of *L. monocytogenes* EGDe to stainless steel, PTFE and Caco-2 cells, with the inhibition rate being higher than 90% in all cases. Guiemonde et al. [22] reported that *Lb. casei* TMC 0409, *Lb. acidophilus* TMC 0356, and *Lb. rhamnosus* LA-2 decreased the adhesion of *L. monocytogenes* ATCC 15313 to human intestinal mucosa by 21.3%, 19.1%, and 25.9%, respectively. Probiotic lactobacilli significantly inhibited listeria infections in an *in vitro* C2Bbe1 epithelial cell model due to a combination of acid production and the secretion of an unidentified protein [24].

S.aureus is responsible for a diverse spectrum of human diseases with at least 10 adhesins produced by that species being characterized in detail [27]. Those adhesins and surface proteins may be virulence factors involved in human infections. The gastrointestinal tract can be colonized by vancomycin-resistant *S.aureus* [28]. Its presence increases in the stools of inpatients and infants [27]. In particular, atopic dermatitis patients are more frequently colonized by *S.aureus* as compared to healthy children [29]. In our study, *Lb. casei* ŁOCK 0919 inhibited the adherence of *S. aureus* ATCC 6538 to the greatest extent (45.9%), followed by the mixture of probiotic strains (26.2%), while both *Lb. rhamnosus* strains stimulated the adherence of the pathogen (a 44.5% increase was observed for the strain 0900). Tuomola et al. [30] demonstrated that two strains of *Lb. rhamnosus* enhanced the adhesion of *Salmonella* Typhimurium to the human intestinal mucosa, suggesting that the pathogen interacts with probiotics. Adherence inhibition to Caco-2 cells of more than 60% was reported by Ren et al. [27] for two different lactobacillus strains. The inhibition rate depended on the condition of lactobacillus cells, their growth phase and cell density used in the experiment. High density (1×10^9 CFU/mL) live cells from the late logarithmic growth phase were the most effective.

Adherence is a very complex process. The inhibition of pathogen adhesion to the intestinal epithelium may prevent colonization and reduce the risk of a systemic infection [26]. Adherence inhibition appears to depend on both the lactobacillus and the pathogen tested, indicating very high specificity. Moreover, it is influenced by environmental and physiological conditions. *Lactobacillus* adherence is still poorly characterized and little is known about its mechanism. Several bacterial components, including cell wall proteins, carbohydrates, and teichoic and lipoteichoic acids, have been suggested to be involved in the adherence of probiotics to the intestinal mucosa. The most likely mechanisms involved in the inhibition of enteropathogen adherence by lactobacilli are competition for adherence receptors and the soluble factors (surface proteins) released in the gut lumen, which prevent pathogenic adhesion and colonization.

Although the adherence of *E. faecalis* ATCC 29212 and *S. aureus* ATCC 6538 was stimulated by *Lb. rhamnosus* (0900 and 0908), it was inhibited by the mixture of probiotic strains. This may be attributed to the fact that the mixture contains 50% *Lb. casei* 0919, which reduces the adhesion of both pathogens,

so the mixture of probiotic strains did inhibit the adherence of those pathogens (by 10.2% and 26.2%, respectively).

Conclusions

Our findings indicate that the inhibition of pathogen adherence by probiotic strains of the genus *Lactobacillus* is species-specific. The results suggest that the studied lactobacilli, both individually and as a mixture, successfully inhibit *L. monocytogenes* and *Cl. difficile* infections in the gut; however, more research needs to be done, including *in vivo* studies. Importantly, the ability to inhibit adherence depends on pathogen specificity.

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