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The effect of calcium ions on adhesion and competitive exclusion of *Lactobacillus* ssp. and *E. coli* O138

Nadja Larsen [a,*], Peter Nissen [a], William G.T. Willats [b]

[a] *Food Microbiology, Department of Food Science (IFV), The Royal Veterinary and Agricultural University (KVL), Rolighedsvej 30, 1958 Frederiksberg C, Denmark*
[b] *Institute of Molecular Biology, Copenhagen University, Øster Farimagsgade 2A, 1353, Copenhagen, Denmark*

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Abstract

The adhesion abilities of 11 strains of *Lactobacillus* were determined *in vitro* using the IPEC-J2 cell line as a model system. Bacteria cultures included the probiotic strains *L. rhamnosus* GG, *L. reuteri* ATCC 55730, *L. johnsonii* NCC 533 and *L. reuteri* DSM 12246, and new isolates of *Lactobacillus* ssp. Adhesion was quantified by scintillation counting of radiolabelled bound bacteria. The highest adhesion of 38%, was determined for *L. reuteri* DSM 12246 followed by *L. plantarum* Q47 with an adhesion level of 24%. Other strains showed moderate to low binding of less than 16%. Competitive adhesion experiments on IPEC-J2 cells demonstrated that strongly adhesive strains, as *L. reuteri* DSM 12246 and *L. plantarum* Q47, significantly reduced the attachment of the less adhesive strains, such as *L. rhamnosus* GG and *L. johnsonii* NCC 533, both under condition of co-incubation and in displacement assays, indicating that bacteria may share the same binding sites for attachment to intestinal cells. Furthermore, it was revealed that calcium ions significantly increased the binding of tested lactobacilli to IPEC-J2 cells; and therefore, added calcium may be useful in enhancing the adhesion of normally weakly adhesive probiotic cultures. In contrast, no significant change in adhesion of lactobacilli was observed in the presence of Mg and Zn ions. Displacement assays performed with pathogenic *E. coli* O138 showed that all tested *Lactobacillus* strains reduced the attachment of *E. coli* O138 to IPEC-J2 by more than 2-fold both in the presence and the absence of calcium ions. The strains of *Lactobacillus* did not differ significantly in the extent of their inhibition of *E. coli* O138 adhesion, indicating that the reduced adhesion of *E. coli* O138 was due to steric hindrance of the binding sites rather than to specific interactions.

Keywords: Adhesion; *Lactobacillus* species; Divalent ions; Probiotic

1. Introduction

Lactobacillus species are desirable members of intestinal microbiota and act as probiotic bacteria in the gut. The beneficial effects of *Lactobacillus* on health are well-documented and include stabilization of the indigenous microflora, prevention and treatment of diarrhoea, alleviation of lactose intolerance, increased nutritional value of foods, stimulation of immune system, and reduction of serum cholesterol levels (Isolauri et al., 1994; Hooper et al., 1999; Isolauri, 2001).

An important step in the successful colonization and execution of probiotic effects is the ability of bacterial strains to adhere to intestinal epithelium. High adherence is commonly used as a selection criterion for probiotic strains (Bernet et al., 1994; Hudault et al., 1997; Blum et al., 1999). Several *in vitro* models for assessing the adhesive abilities of bacteria, based on tissue-cultured cells and intestinal mucus preparations, have been developed. For example, the epithelial-like tumor cell lines, HT-29 and Caco-2, have been used extensively to select for adhesive strains. The porcine intestinal epithelial cell line IPEC-J2 is a more recently developed and so far less extensively used cell line which has been reported as a relevant *in vitro* model system for intestinal cell-to-cell interactions (Schierack et al., 2005). IPEC-J2 cells can differentiate in culture and exhibit enterocytic features, like microvilli, tight junctions and glycocalyx-bound mucin. As shown by numerous studies, adhesion in different *in vitro* models varies even within the same strain, indicating that bacterial structures involved in binding to epithelial cells and to mucus may be different.

* Corresponding author. Tel.: +45 35 28 32 35; fax: +45 35 28 32 14.
E-mail address: nf@kvl.dk (N. Larsen).

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Furthermore, the experimental evidence shows that adhesion may be favoured by such factors as a particular medium, temperature and pH (Jones et al., 1976; Tuomola et al., 2001). It has also been suggested that divalent ions, as e.g., calcium, have an influence on bacterial attachment (Zarate et al., 2002). Calcium is known to promote non-specific interactions such as neutralization of the electrical double layer between the cells as well as specific adhesive interactions with protein and polysaccharide adhesin molecules at the cell surface (Deman et al., 1974; Geesey et al., 2000). However, the effects of calcium on adhesion of probiotic bacteria have not been thoroughly investigated. A few reported experiments have shown that adherence of lactobacilli to Caco-2 and intestine mucus might be either calcium promoted or not influenced by calcium, depending on the strain (Kleeman and Klaenhammer, 1982; Chauviere et al., 1992; Bernet et al., 1994; Gusils et al., 2003).

Adhesive probiotics are generally considered to be more effective in competitively excluding pathogenic bacteria when compared to non-adhesive strains (Vesterlund et al., 2004). The activity of probiotics in exclusion of pathogens may be due to the stimulation of the immune system or to direct competition with pathogens for the same binding sites on epithelial cells. One widespread pathogen, enteropathogenic *Escherichia coli* (EPEC), belongs to the coliform faecal group, restricted to organisms that grow in the gastrointestinal tract of humans and warm-blood animals (Giammanco et al, 1996; Go and Cunha, 2004). EPEC causes gastroenteritis in human and domestic animals, traveller's diarrhoea and sporadic cases of hemorrhagic colitis characterized by bloody diarrhoea. Several probiotic strains of *Lactobacillus* and *Bifidobacteria* were reported to inhibit adhesion of enteropathogenic *E. coli* to intestinal mucosa (Collado et al., 2005) and to Caco-2 cell line (Lee et al., 2000; Forestier et al., 2001; Gopal et al., 2001; Lee and Puong, 2002; Gagnon et al., 2004). However, according to the other publications, no competitive exclusion of pathogenic *E. coli* by lactobacilli was observed in similar assays (Ouwehand et al., 2001; Parassol et al., 2005).

The aim of the present study was to investigate the adhesion of both known strains and new isolates of probiotic *Lactobacillus,* incubated separately and in competition using IPEC-J2 cell line model. Possible inhibition of attachment of enteropathogenic *E. coli* O138 to IPEC-J2 by lactobacilli was also studied. In addition, we examined the effect of divalent ions on the adhesion of *Lactobacillus* ssp. and *E. coli* incubated alone and in competitive assays on IPEC-J2 cells.

2. Materials and methods

2.1. Chemicals

Chemicals were purchased from Sigma-Aldrich A/S (Denmark) or Merck (Damstadt, Germany) unless indicated otherwise.

2.2. Bacterial strains and growth media

The *Lactobacillus* strains examined in this study are listed in Table 1. The strains were maintained in de Man, Rogosa and Sharpe broth (MRS; Difco Laboratories; Becton, Dickinson and Co, USA) containing 15% (vol/vol) glycerol at −80 °C.

Table 1
Origin and source of investigated *Lactobacillus* strains

Strain	Origin	Source [a]
L. reuteri DSM 12246	Pig faeces	DSM, Germany
L. reuteri ATCC 55730	Human intestine	BIOGAIA, Sweden
L. reuteri DC 20	Human isolate	Danisco A/S, Denmark
L. plantarum Q47	Biopsy of human adults	KVL, Denmark
L. plantarum DC 13	Raw ham	Danisco A/S, Denmark
L. plantarum M.1.1	Faeces of human babies	KVL, Denmark
L. rhamnosus GG	Human adult faeces	LGG® products, Valio Ltd., Finland
L. johnsonii NCC 533	Human intestine	NESTEC LTD, Switzerland
L. paraplantarum D14	Human adult faeces	KVL, Denmark
L. acidophilus X37	Biopsy of human adults	KVL, Denmark
L. crispatus LMG 18191	Chicken faeces	LMG, Belgium

[a] DSM — Deutsche Sammlung von mikroorganismen und Zellkulturen Gmb H, Braunschweig, Germany; LMG — Laboratorium voor Microbiologie (Co-ordinated Collections of Micro-organisms), Universiteit Gent, Belgium; ATCC — The American Type Culture Collection, Manassas, Virginia, USA.

E. coli O138 (strain no. 99–10502–1) is a Shiga-like toxins producing strain and was isolated from pigs with post-weaning diarrhoea. The strain was kindly donated by the Danish Institute for Food and Veterinary Research (Copenhagen, Denmark) and maintained in Luria–Bertani (LB) broth in 15% (vol/vol) glycerol.

2.3. IPEC-J2 cell line

The piglet jejunal epithelial cell line IPEC-J2 was kindly provided by Professor Anthony Blikslager, North Carolina State University, USA. The cells were cultivated in tissue culture flasks (TPP, Switzerland) in a 1:1 mixture of Dulbecco's Modified Eagle Medium (DMEM) and F12 solution supplemented with 100 mg/l streptomycin (Fluka Chemie GmbH, Steinheim, Switzerland), 100 mg/l penicillin, 2 mM L-glutamine, 1 mM pyruvate and 10% (vol/vol) fetal bovine serum (Cambrex Bio Science, Verviers, Belgium). The cells were routinely grown at 37 °C in a 95% air — 5% CO_2 atmosphere in humidified incubator. The culture medium was replaced every other day.

2.4. In vitro adhesion assays on IPEC-J2

Adhesion assays were performed essentially according to the method of Kühle et al. (2006). Briefly, 2 mL of IPEC-J2 cell suspension in DMEM were added in 12 wells tissue culture plates (Greiner Bio-One, Germany) at a concentration of approximately 5.0×10^5 cells/mL and grown as described above 4–5 days until confluence. Prior adhesion assays IPEC-J2 monolayers were washed twice with DMEM without antibiotics (streptomycin and penicillin). Bacterial strains were cultivated in MRS broth containing 2.5 μCi/mL of L-[methyl-^{3}H] methionine (1 mCi/mL; Amersham Bioscience, Sweden) under micro-aerophilic conditions at 37 °C. Cells were harvested at the stationary phase after 18 h of incubation at an