



Protective effects of Lactobacilli, Bifidobacteria and Staphylococci on the infection of cultured HT29 cells with different enterohemorrhagic *Escherichia coli* serotypes are strain-specific

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ABSTRACT

In this study, we investigated the interaction of 19 benign strains of lactic acid bacteria (LAB), bifidobacteria and staphylococci with enterohemorrhagic *Escherichia coli* (EHEC) strains of different serotypes and virulence gene spectrum in a HT29 cell culture infection model. As markers of infection, the secretion of interleukin 8 (IL-8) and the activation of the transcription factor NF-κB by the infected cells were determined. With 12 of 19 tested strains, a weak reduction <30% of IL-8 secretion of HT29 cells after co-infection with EHEC O157:H7 strain EDL933 was observed. Six strains reduced the IL-8 secretion up to 60% and the strain *B. adolescentis* DSMZ 20086 decreased the IL-8 production about 73%. In further co-infection assays with EHEC strains of the serotypes O103:H2, O26:H⁺, O157:H⁺ and O113:H21, different abilities of the LAB strains to influence the infection with the different EHEC strains were noted. Therefore, the protective anti-inflammatory effect is strain specific for LAB and also depends on the application of EHEC strains with different sero- and virulence types. The differences in efficacy of protective bacteria against certain EHEC strains were unexpected and have not been shown so far.

Furthermore, we could show that the inhibitory effects were not attributed to lower adhesion abilities of EHEC to the production of organic acids by the benign bacteria. In addition, viable bacteria are needed to inhibit the IL-8 secretion.

Moreover, the NF-κB activation was reduced significantly by all tested LAB strains in co-infection trials, but was not strain-specific.

The model described here is useful to screen for basic effects of protective bacteria that are able to counteract EHEC-mediated effects on human cells, and to study the molecular interaction between bacteria as well as between bacteria and human cultured cells.

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1. Introduction

Infection with enterohemorrhagic *Escherichia coli* (EHEC) can cause hemorrhagic colitis and hemolytic uremic syndrome (HUS) (Nataro and Kaper, 1998). Typical HUS is characterized by the triad of hemolytic anemia, low platelet count (thrombocytopenia) and acute renal failure (Noris and Remuzzi, 2005; Taylor, 2008). Main reservoirs of EHEC are cattle, transmission occurs mainly by raw and undercooked food, especially minced meat, other meat products and dairy products (Rangel et al., 2005). High virulence of this foodborne pathogen is related to the production of phage-encoded Shiga toxins (Stx1 and Stx2) as well as the ability to develop attaching-effacing lesions by direct bacteria-cell contact (Kaper, 1998).

In North America, Japan and parts of Europe, most outbreaks are due to EHEC serotype O157:H7, whereas other serotypes are important health concerns in other developed countries (Croxen and Finlay, 2010). After binding to the host cell membrane, EHEC O157:H7 use a type three secretion system encoded on a pathogenicity island termed locus of enterocyte effacement (LEE) to inject effector proteins into the cytosol of the infected eukaryotic cell. The translocated intimin receptor (Tir) targets to the plasma membrane where it then binds to the *eae*-encoded bacterial outer membrane protein intimin, ultimately leading to rearrangements of the host cell cytoskeleton, formation of pedestals underneath intimately adherent bacteria, and to attaching/effacing lesions. Among other signaling processes, EHEC induces the activation of mitogen-activated-protein-kinases and nuclear transcription factors as NF-κB in the infected epithelial cell, which leads to the production and secretion of chemokines such as interleukin-8 (IL-8) (Ceponis et al., 2005). IL-8 serves as a potent chemoattractant that delivers neutrophils to the site of bacterial infections.

Shiga toxin binds to globotriaosylceramide (Gb3), a receptor which occurs in cholesterol-enriched microdomains in the plasma membrane

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of the eukaryotic cells to induce – among others – programmed cell death of the host cell by the release of cytochrome c from the mitochondria, activation of caspases and degradation of the DNA repair enzyme, PARP (O'Loughlin and Robins-Browne, 2001). Another phage-coded toxin is the cytolethal distending toxin V (CDT-V), which causes an arrest in the G2/M phase in human brain microvascular endothelial cells, and death of endothelial cells (Bielaszewska et al., 2005; Janka et al., 2003).

There seems to be a correlation between inflammatory serum parameters and a high risk of developing typical HUS during the prodromal phase of diarrhea caused by EHEC; low neopterin and interleukin 10 levels and high IL-8 levels are indicators of a high risk for developing HUS in EHEC-infected children (Westerholt et al., 2000). The therapy of HUS is supportive, because it is not specifically targeted to the pathology, and the benefits of antibiotic therapy are uncertain (Goldwater, 2007; Scheiring et al., 2010).

The World Health Organization defines probiotics as live microorganisms that, when administered in adequate amounts, confer a beneficial effect on the health of the host (FAO/WHO, 2001). The health-promoting effects of probiotic bacteria are thought to be mediated by three main mechanisms, (I) the microbe-microbe interactions including the production of antimicrobial compounds like lactic acid production as well as the ability to adhere to the surface of epithelial cells and reduce the adhesion-ability of pathogens; (II) the enhancement of epithelial barrier function by various mechanisms and (III) the immunomodulatory effects via interactions with immune cells (Lebeer et al., 2008). It is already known, that certain probiotic microorganisms have the ability to antagonize EHEC infections. Johnson-Henry et al. (2007) could show that surface-layer protein extracts from *L. helveticus* inhibit the adhesion of EHEC O157:H7 to human intestinal cells; *L. acidophilus* A4 inhibited significantly the attachment of EHEC O157:H7 to HT29 intestinal epithelial cells and simultaneously increased the expression of MUC2 (Kim et al., 2008). Asahara et al. (2004) fed mice with *B. breve* and thus, inhibited the Shiga toxin production of the administered EHEC strain. The probiotic strain *L. rhamnosus* GG suppresses the internalization of EHEC O157:H7 into a human epithelial cell line (Hirano et al., 2003), reduced morphological changes in epithelial monolayer, protected them against EHEC-induced redistribution of tight junction proteins (Johnson-Henry et al., 2008) and also significantly suppress the EHEC induced chemokine expression of infected Caco-2 cells (Toki et al., 2009). The molecular mechanisms responsible for the so called probiotic effect are not fully understood.

The aim of this study was to set up a model for screening the effects of benign and probiotic bacteria on infection parameters of EHEC bacteria. Therefore we tested different strains of the benign genera *Bifidobacterium*, *Lactobacillus* and coagulase-negative *Staphylococcus* as well as probiotic bacteria for co-infection assays with certain EHEC strains in a HT29 cell culture model.

2. Materials and methods

2.1. Cell culture and bacterial culture conditions

Human colon adenocarcinoma cells (HT29 cells; DSMZ no. ACC 299) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 1% glutamine and 1% penicillin/streptomycin (Invitrogen, Germany) at 37 °C and with 5% CO₂. For infection assays, DMEM without antibiotics was used. Enterohemorrhagic *E. coli* strains used in this study are listed in Table 1. Further bacterial strains were *Bifidobacterium adolescentis* DSMZ 20083, *B. adolescentis* DSMZ 20086, *B. breve* DSMZ 20213, *B. lactis* NCC 362, *Lactobacillus acidophilus* BFE 704, *L. delbrueckii* ssp. *bulgaricus* DSMZ 20081, *L. fermentum* DSMZ 20052, *L. helveticus* DSMZ 20075, *L. intestinalis* DSMZ 6629, *L. johnsonii* DSMZ 10533, *L. johnsonii* BFE 663, *L. paracasei* BFE 688, *L. paracasei* BFE 675, *L. paralimentarius* DSMZ 13238, *L. plantarum* (Sterr et al., 2009), *L. reuteri* ATCC 55730, *L.*

Table 1

Virulence factors and disease association of EHEC strains used in this study.

<i>E. coli</i> strain	Serotype	<i>stx</i>	<i>eae</i>	<i>ehx</i>	<i>cdt-V</i>	Disease	Reference
EDL933	O157:H7	1/2	+	+	–	–	(O'Brien et al., 1984)
PMK5	O103:H2	1	+	+	–	HUS	(Mariani-Kurkdjian et al., 1993)
2331/01	O26:H–	1/2	+	+	–	HUS	(Creuzburg and Schmidt, 2007)
5791/99	O157:H–	2	+	+	+	HUS	(Friedrich et al., 2006)
TS 18/08	O113:H21	2	–	+	+	–	(Slanec et al., 2009)

+, present; –, absent.

rhamnosus GG, *Staphylococcus carnosus* DSMZ 20501, *S. pasteurii* LTH 5211. *E. coli* strains were grown under aerobic conditions at 37 °C in Luria-Bertani (LB) broth containing 10.0 g/L Bacto tryptone; 5.0 g/L Bacto yeast extract and 10.0 g/L NaCl (pH 7.0). *Bifidobacteria* strains were grown under anaerobic conditions at 37 °C in bifidobacterium medium (M58, DSMZ) containing 10.0 g/L Bacto tryptone; 5.0 g/L Bacto yeast extract; 5.0 g/L Difco beef extract; 10.0 g/L glucose; 3.0 g/L K₂HPO₄; 1.0 g/L Tween 80; 0.5 g/L cystein-HCl×H₂O and 10.0 g/L sodium ascorbate (pH 6.8). *Lactobacilli* were grown under anaerobic conditions at 37 °C in modified MRS (mMRS) medium containing 10.0 g/L Bacto tryptone; 8.0 g/L Difco beef extract; 4.0 g/L Bacto yeast extract; 10.0 g/L dextrose; 1.0 g/L Tween 80; 5.0 g/L sodium acetate; 2.0 g/L K₂HPO₄×3H₂O; 2.0 g/L diammonium hydrogen citrate; 0.2 g/L MgSO₄×7H₂O and 0.05 g/L MnSO₄×H₂O (pH 6.2). *Staphylococcus* strains were grown at 37 °C in Brain Heart Infusion (Merck, Germany).

2.2. Infection assays

Totals of 4×10⁵ HT29 cells were seeded in 24 well plates 24 h prior to infection. An overnight culture of the respective EHEC strain was used to inoculate a fresh culture in LB broth and was incubated under aerobic conditions at 37 °C for 90 min. *Bifidobacteria*, *Lactobacilli* and *Staphylococcus* strains were used as overnight cultures. The bacteria were harvested by centrifugation and washed with PBS. HT29 cells were infected either with *E. coli* (multiplicity of infection (MOI)=2) or with one of the other bacteria (MOI=200) alone, or simultaneously in a co-infection assay using the same MOIs.

Tumor necrosis factor alpha (TNF-α; 50 ng/mL; Sigma) was added to unstimulated cells (positive control) and to co-infected cells (viability control). The viability of the bacteria prior to infection was controlled by culture techniques. After 1 h incubation, the cells were washed with PBS and new cell culture media was added. Five hours later, the cell culture supernatants were harvested and the IL-8 levels were determined by using an enzyme-linked immunosorbent assay. All infection assays were performed as triplicates and repeated twice.

Additionally to the infection assays with viable bacteria, infection assays with the culture supernatants and with non-viable bacteria were performed for the strains *S. pasteurii*, *L. rhamnosus* GG and *L. johnsonii*. The supernatants were harvested by centrifugation of the overnight cultures, sterile filtered (0.2 μm) and used directly (100 μl) in the infection assay as described above. For the preparation of non-viable cells, the overnight cultures were sedimented and resuspended in PBS, the MOI was determined and the culture was frozen. The defrosted cultures were treated by ultrasound (10 min, Sonifier B-12, Branson Sonic Power Co, Danbury, USA) and disrupted using a Ball Mill (10 min, 30 Hz, ball-size 0.25–0.5 mm diameter, Retsch MM301, Haan, Germany). The inactivation was controlled by culture techniques. In the infection assays, the amount of non-viable cells was calculated to the MOI 200.

2.3. Determination of IL-8 production

In order to determine the amount of IL-8 secreted by HT29 cells in the culture supernatant, an enzyme-linked immunosorbent assay (ELISA) was used as described with minor modifications (Schulte and

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