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Antagonistic effects of probiotic *Escherichia coli* Nissle 1917 on EHEC strains of serotype O104:H4 and O157:H7

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ABSTRACT

The largest EHEC outbreak up to now in Germany occurred in 2011. It was caused by the non-O157:H7 Shiga-toxinogenic enterohemorrhagic *E. coli* strain O104:H4. This strain encodes in addition to the Shiga toxin 2 (Stx2), responsible for the hemolytic uremic syndrome (HUS), several adhesins such as aggregative adherence fimbriae. Currently, there is no effective prophylaxis and treatment available for EHEC infections in humans. Especially antibiotics are not indicated for treatment as they may induce Stx production, thus worsening the symptoms. Alternative therapeutics are therefore desperately needed. We tested the probiotic *Escherichia coli* strain Nissle 1917 (EcN) for antagonistic effects on two O104:H4 EHEC isolates from the 2011 outbreak and on the classical O157:H7 EHEC strain EDL933. These tests included effects on adherence, growth, and Stx production in monoculture and co-culture together with EcN. The inoculum of each co-culture contained EcN and the respective EHEC strain either at a ratio of 1:1 or 10:1 (EcN:EHEC). Adhesion of EHEC strains to Caco-2 cells and to the mucin-producing LS-174T cells was reduced significantly in co-culture with EcN at the 1:1 ratio and very dramatically at the 10:1 ratio. This inhibitory effect of EcN on EHEC adherence was most likely not due to occupation of adhesion sites on the epithelial cells, because in monocultures EcN adhered with much lower bacterial numbers than the EHEC strains. Both EHEC strains of serotype O104:H4 showed reduced growth in the presence of EcN (10:1 ratio). EHEC strain EDL933 grew in co-culture with EcN only during the first 2 h of incubation. Thereafter, EHEC counts declined. At 24 h, the numbers of viable EDL933 was at or slightly below the numbers at the time of inoculation. The amount of Stx2 after 24 h co-incubation with EcN (EcN:EHEC ratio 10:1) was for all 3 EHEC strains tested significantly reduced in comparison to EHEC monocultures.

Obviously, EcN shows very efficient antagonistic activity on the EHEC strains of serotype O104:H4 and O157:H7 tested here regarding adherence to human gut epithelial cells, bacterial growth, and Stx2 production in vitro.

Introduction

In 2007, a review with the title "The non-O157 Shiga-toxigenic (verocytotoxigenic) *Escherichia coli*; under-rated pathogens" (Bettelheim, 2007) nicely outlined the problem of underestimated infections by non-O157 *E. coli*. In 2011, the non-O157 Shiga toxin-producing enterohemorrhagic *E. coli* strain O104:H4 caused the largest outbreak of EHEC recorded so far. A total of 2987 cases of gastroenteritis (18 deaths) and 855 cases of HUS (35 deaths) were reported (RKI, 2011). A single lot of Fenugreek seeds from Egypt was identified as the most likely source of the O104:H4 outbreak strain (Buchholz et al., 2011). However, contaminations with EHEC O104:H4 could not be detected in any analyzed sample (EFSA, 2011). Other studies found no signs of *E. coli* O104:H4 in the feces of cattle from northern Germany or France, indicating that ruminants are not the primary reservoir of this strain (Auvray et al., 2012; Wieler et al., 2011). So far, the most likely reservoir of *E. coli* O104:H4 is the human population (Harrington et al., 2006; Karch et al., 2012; Okeke et al., 2010).

Regardless of the origin, the severity of the disease caused by EHEC O104:H4 with respect to HUS development (Frank et al., 2011) and neurological symptoms (Jansen and Kielstein, 2011) was alarming. This new *E. coli* strain, a combination of an enteroaggregative *E. coli* (EAEC) and an enterohemorrhagic *E. coli* (EHEC) strain (Mellmann et al., 2011; Rohde et al., 2011), exhibited a unique set of virulence factors, which contributed to its hazardousness. One of the most important virulence factors of *E. coli* O104:H4 is Shiga toxin 2 (Stx2) (Pacheco and Sperandio, 2012), which is closely

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related to Stx2 from *E. coli* O111:H− strain JB1-95 (Laing et al., 2012). The two-component Shiga toxin (AB_5) is released from dying bacteria, binds to globotriaosylceramide (Gb3) on eukaryotic cells via subunit B, and ultimately inhibits protein synthesis (subunit A), which leads to cell death by apoptosis (Karch et al., 2012; Pacheco and Sperandio, 2012). Small blood vessels, which are found in the lungs, digestive tract or kidneys, are the main targets for Shiga toxin. Bloody diarrhea, renal dysfunction and kidney failure, damage of lungs, and severe neurological symptoms can be the result (Jansen and Kielstein, 2011; Karch et al., 2012).

Another important virulence factor of EHEC O104:H4 is a plasmid encoding an extended-spectrum class A beta lactamase (CTX-M-15), which is responsible for resistance against a wide range of antibiotics (Bielaszewska et al., 2011). Whilst some antibiotics might be useful in the treatment of patients with EHEC O104:H4 (Bielaszewska et al., 2012), antibiotic treatment of EHEC is not generally recommended due to apprehension of increased Shiga toxin production (Smith et al., 2012; Wong et al., 2000). Furthermore, *E. coli* O104:H4 shows an aggregative adherence pattern (stacked-brick) like enteroaggregative *E. coli* (EAEC) (Bielaszewska et al., 2011). In contrast to its close relative EAEC 55989, EHEC O104:H4 harbors genes for aggregative adherence fimbriae I (AAF/1) and not AAF/III (Mellmann et al., 2011).

The most fearsome aspect of the EHEC outbreak in 2011 is that it might happen again. Different 'under-rated pathogens' (Bettelheim, 2007) may arise and prove current treatment options as insufficient. Highly antibiotic-resistant pathogens are on the rise while development of new antibiotics is stagnating. Treatment of gastrointestinal diseases with probiotics as an alternative to antibiotics has been widely discussed over the last few years (Carey et al., 2008; Eaton et al., 2011; Fukuda et al., 2012; Muniesa et al., 2012; Oelschlaeger, 2010; Sonnenborn and Schulze, 2009). Probiotic bacterial strains are mostly members of the Gram-positive genera *Lactobacillus* and *Bifidobacterium*, but also Gram-negative bacteria such as the probiotic *E. coli* strain Nissle 1917 (EcN) are used.

EcN has been in use in medicine as a probiotic drug (Mutaflor®) since 1917 (Nissle, 1918) and can be applied for the treatment of various dysfunctions and diseases of the intestinal tract (Montrose and Floch, 2005; Schultz, 2008; Sonnenborn and Schulze, 2009). EcN is an effective alternative to the 'gold standard' mesalazin (5-aminosalicylic acid) in maintaining remission in patients with ulcerative colitis (Kruis, 2004; Rembacken et al., 1999). Other indications are chronic habitual constipation (Bär et al., 2009; Möllenbrink and Bruckschen, 1994) and diarrhea in young children (Henker et al., 2007, 2008). Means and molecular mechanisms responsible for EcN's clinical efficacy are only partially understood.

It has been shown that EcN inhibits invasion of intestinal epithelial cells by enteroinvasive bacteria (Altenhoefer et al., 2004). Moreover, EcN induces human beta-defensin 2 in epithelial cells via its flagellin (Schlee et al., 2007; Wehkamp et al., 2004). Antagonistic effects of EcN on colonization of the mouse intestine by EHEC O157:H7 strain EDL933 (Leatham et al., 2009) and inhibition of Shiga toxin production of several STEC strains (Reissbrodt et al., 2009) have been reported.

Some of the fitness factors thought to contribute to the probiotic properties of *E. coli* Nissle 1917 are multiple iron acquisition systems (*ent*, *iro*, *iuc*/*aer*, *ybt*, *chu*, *cit*), different adhesins (F1C fimbriae, F1A fimbriae, Curli fimbriae, H1 flagella), and 2 different microcins (M, H47) (Patzer et al., 2003; Schlee et al., 2007; Valdebenito et al., 2006; Vassiliadis et al., 2010). An additional safety aspect in comparison to probiotic lactobacilli is EcN's serum sensitivity, due to a mutation in the *wzy* gene encoding the O6 antigen polymerase (Grozdanov et al., 2002). This results in a truncated O6 carbohydrate side chain of its lipopolysaccharide, leading to the remarkable feature that the EcN strain is immunogenic without being immunotoxic.

The objective of the present study was to investigate potential antagonistic effects of EcN on adhesion, growth, and Shiga toxin production of EHEC strains, such as the O157:H7 strain EDL933 or EHEC O104:H4 isolates from the 2011 outbreak in Germany.

Materials and methods

Bacterial strains

Bacterial strains used in this study are listed in Table 1. Bacteria were grown in TY medium (10 g/l tryptone, 5 g/l yeast extract, and 5 g/l NaCl) or DMEM medium containing 10% fetal bovine serum (PAA, Cölbe, Germany) at 37 °C.

Caco-2 and LS-174T cell cultures

The human colonic epithelial cell lines Caco-2 (mucin-negative) and LS-174T (mucin-positive) were obtained from CLS (Eppelheim, Germany). The cell lines were cultured in DMEM medium containing 10% fetal bovine serum (FBS) and maintained at 37 °C and 5% CO_2. Passages of Caco-2 cells were performed every 3–4 days with a split ratio of 1:4. LS-174T cells were cultured for 2–3 days and then subcultured with a split ratio of 1:2.5. All cell culture media and supplements were purchased from PAA.

Adhesion to Caco-2 and LS-174T cells

The cells were grown until confluency in cell culture flasks and seeded in 24-well microtiter plates. Caco-2 and LS-174T cells were seeded at 4.0×10^5 and 1.0×10^6 cells/well, respectively, and incubated for 24 h at 37 °C and 5% CO_2 to reach confluency (6.0×10^5 and 1.4×10^6 for Caco-2 and LS-174T, respectively). The DMEM medium was replaced with fresh medium 30 min before incubation with the bacterial inoculum. Bacterial overnight cultures in TY medium were used to inoculate cell cultures in DMEM medium, which were incubated for 2 h at 37 °C under shaking conditions. Monocultures with EcN ($\sim 1.5 \times 10^7$ CFUs/well) or pathogenic *E. coli* (pEc) ($\sim 1.5 \times 10^7$ CFUs/well) and co-cultures at a ratio of 1:1 (EcN:pEc; $\sim 1.5 \times 10^7$:1.5×10^7 CFUs/well) or 10:1 (EcN:pEc; $\sim 1.5 \times 10^8$:1.5×10^7 CFUs/well) were made with these 2-h cultures. The CFUs/well of the inocula were confirmed plating serial dilutions on agar plates. 24-well microtiter plates with Caco-2 or LS-174T cells and the bacteria were incubated for 2 h at 37 °C and 5% CO_2. This was followed by removal of the supernatant containing non-adhering bacteria and 3 times washing of the epithelial cell layer with 1 ml PBS. Epithelial cells were resuspended in 1 ml Trypsin-EDTA (1:250, PAA) and lysed during 15 min of extensive shaking. Serial dilutions of the resulting bacterial suspension were placed on agar plates. To differentiate between the bacterial strains, the CFUs/ml on LB-agar, and LB-agar + antibiotic (TY3730, TY3456: 50 μg/ml Ampicillin; 55989: 6 μg/ml doxycyclin) were determined. Chromogenic ECC agar plates (Medco, München, Germany) were used to distinguish between EDL933 (pink color) and EcN (mauve color).

Determination of bacterial growth in monoculture and co-culture

The CFUs/ml of inocula ($t = 0$ h) and supernatants ($t = 2$ h) from the adhesion experiments were determined, and the supernatants were further incubated at 37 °C and 0% CO_2 to assess the CFUs at $t = 5$ h and $t = 24$ h. CFUs/ml of individual strains in co-culture were determined as described in the paragraph above.