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The probiotic *Escherichia coli* strain Nissle 1917 interferes with invasion of human intestinal epithelial cells by different enteroinvasive bacterial pathogens

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Abstract

The probiotic *Escherichia coli* strain Nissle 1917 (Mutaflor®) of serotype O6:K5:H1 was reported to protect gnotobiotic piglets from infection with *Salmonella enterica* serovar Typhimurium. An important virulence property of *Salmonella* is invasion of host epithelial cells. Therefore, we tested for interference of *E. coli* strain Nissle 1917 with *Salmonella* invasion of INT407 cells. Simultaneous administration of *E. coli* strain Nissle 1917 and *Salmonella* resulted in up to 70% reduction of *Salmonella* invasion efficiency. Furthermore, invasion of *Yersinia enterocolitica*, *Shigella flexneri*, *Legionella pneumophila* and even of *Listeria monocytogenes* were inhibited by the probiotic *E. coli* strain Nissle 1917 without affecting the viability of the invasive bacteria. The observed inhibition of invasion was not due to the production of microcins by the Nissle 1917 strain because its isogenic microcin-negative mutant SK22D was as effective as the parent strain. Reduced invasion rates were also achieved if strain Nissle 1917 was separated from the invasive bacteria as well as from the INT407 monolayer by a membrane non-permeable for bacteria. We conclude *E. coli* Nissle 1917 to interfere with bacterial invasion of INT407 cells via a secreted component and not relying on direct physical contact with either the invasive bacteria or the epithelial cells.
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1. Introduction

Probiotics have been defined as viable non-pathogenic microorganisms that, when administered to man or animals, confer health benefits to the host by improving the microbial balance of the indigenous microflora [1]. They are either used as feed or dietary supplements [2] or as pharmaceutical products for therapeutic purposes, mainly for gastrointestinal or genito-urinary indications [3]. Probiotic microorganisms currently in use are either bacteria, most commonly lactic acid bacteria (LAB) of the genera *Lactobacillus* and *Bifidobacterium*, or yeasts, such as the *Saccharomyces cerevisiae* strain CBS 5926, also known as '*Saccharomyces boulardii*' [4]. Besides LAB and yeasts, non-pathogenic *Escherichia coli* strains have also been used for the treatment of gastrointestinal diseases [5,6] as well as for colonization and infection prophylaxis in neonates [7–10]. One of the best-studied examples of probiotic *E. coli* strains is represented by strain Nissle 1917 (EcN) of serotype O6:K5:H1 [11]. This strain lacks typical virulence genes and is serum sensitive in contrast to probiotic lactobacilli. The serum sensitivity is based on a mutation in the *wzy* gene encoding the O6 antigen polymerase. The lack of O6 antigen polymerase results in the semi-rough O6 phenotype, which in turn is responsible for the serum sensitivity [12]. The stable presence of two cryptic plasmids is another typical property of this strain and is employed in strain-specific PCRs for the detection of EcN [13].

One well-known mode of action of probiotics is their antagonistic activity against intestinal pathogens, also called bacterial interference. This activity may be due to

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the production of specific antimicrobial substances, such as bacteriocins [14,15] or microcins [16].

EcN was originally isolated by Alfred Nissle in 1917 in course of a search for *E. coli* wild-type strains with antagonistic activity against enteric pathogens. Later it was shown that this strain produced some novel kind of colicin(s) of low-molecular mass [17,18], nowadays called microcins [16].

Recently, it has been reported that continuous communication between commensal microorganisms and host epithelia does occur which may be the basis for the development of stable microbial relationships in the gut [19–21]. Furthermore, this finding raises the possibility that microbe–host cell signalling might be another mode of action by which probiotic bacteria could stabilize intestinal microecology and effectively prevent colonization by enteric pathogens [22]. This might especially be true in the case of probiotic support of intestinal defense mechanisms against invasive pathogens.

Therefore, in the present study we have examined interference of EcN with invasion of intestinal epithelial cells by several enteroinvasive bacterial pathogens and by *Salmonella* in more detail.

2. Materials and methods

2.1. Bacterial strains, media and growth conditions

The probiotic EcN was obtained from Ardeypharm GmbH (Herdecke, Germany). Its isogenic microcin-negative mutant (SK22D) was constructed by deletion of the genes *mchD*, *mchE*, and *mchF* [23]. The isogenic Fim- and Foc-negative EcN mutant [24] was constructed by the method of Datsenko and Wanner [25]. First the *foc* gen-cluster was replaced by a *cat* antibiotic resistance cassette, which was part of a PCR product generated with plasmid pKD3 as the template and the primer pair: p1 (5′-GTTGTGATGACAGATACGGTGTGCGTAGTTCAA-TTAAAACAGGAATTAAATATGGTGTAGGCTGGA-GCTGCTT-3′) and p2 (5′-ATATAAAGAGCAGTAA-TATCATTACCGCCACAACTGCATTCTACATATGA-ATATCCTCCTTAGTTCCTA-3′). From the resulting chloramphenicol resistant EcN derivative the *cat* cassette was deleted after transformation with plasmid pKD20. The pKD20-harboring strain was subsequently cured from this plasmid by growth at 42°C. In a second round of mutagenesis the whole *fim* gene cluster was replaced by a *cat* antibiotic resistance cassette as before and with the primer pair: p3 (5′-TATTGCTAACCCAGCACAGCTA-GTGCGCGTCTGTAATTATAAGGGAAAAACGATG-GTGTAGGCTGGAGCTGCTT-3′) and p4 (5′-TTTA-GCTTCAGGTAATATTGCGTACCAGCATTAGCAA-TGTCCTGTGATTTCTTTACATATGAATATCCTCC-TTAGTTCCTA-3′).

EcN was cured from both plasmids (pMUT1 and pMUT2) [13] after (i) transformation with pMUT1 labelled with the tetA and the *sacB* gene replacing the unlabeled pMUT1 and (ii) transformation with pMUT2 labelled with the *kan* and the *sacB* genes replacing the unlabeled pMUT2 [24]. EcN derivatives, which had lost both plasmids, were selected for on sucrose containing plates [26]. One such derivative was employed in invasion assays (see below).

Salmonella enterica serovar Typhimurium strain C17 is a clinical isolate obtained from the Institut für Hygiene und Medizinische Mikrobiologie, Universität Würzburg. *E. coli* K-12 strain DH5α, *S. enterica* serovar Typhimurium strain SL1344, *Yersinia enterocolitica* strains WA-C and WA314, *Shigella flexneri* strain M90T, *Legionella pneumophila* strain Corby and *Listeria monocytogenes* strain EGD were from the strain collection of the Institut für Molekulare Infektionsbiologie, Universität Würzburg, Würzburg, Germany. Bacteria were cultivated in complete MEM cell culture medium (C.C.pro GmbH, Neustadt/W., Germany) containing 10% fetal calf serum (PAA, Cölbe, Germany) and 0.1 mM non-essential amino acids (C.C.pro GmbH, Neustadt/W., Germany) overnight at 37°C in a shaking incubator. For the differentiation between the spontaneous rifampicin resistant EcN and its derivatives and the other bacterial strains employed, bacteria were seeded in parallel on Luria–Bertani (LB) plates containing 10 μg rifampicin or on LB plates without any antibiotics (*Listeria*, *Salmonella*, *Shigella*, *Yersinia*). The solid medium for the cultivation of *L. pneumophila* was BCYE agar [27]. The difference in colony numbers between the rifampicin-containing and rifampicin-free plates represented the number of non-EcN colonies.

2.2. Cell culture conditions

Human embryonic intestinal epithelial INT407 cells were maintained in complete MEM cell culture medium (C.C.pro GmbH, Neustadt/W., Germany) containing 10% fetal calf serum (PAA, Cölbe, Germany) and 0.1 mM non-essential amino acids (C.C.pro GmbH, Neustadt/W., Germany) at 37°C in a cell culture incubator. They were split twice a week at a ratio of 1:5.

2.3. Invasion assay

Invasion assays were performed essentially as described earlier with minor alterations [28]. After addition of the bacteria (about 2×10^6, i.e. beginning of the exponential growth phase) to the confluent INT407 monolayer in wells of a 24-well plate the centrifugation step was omitted and the invasion period was extended to 3 h. Extracellular bacteria were subsequently killed by addition of 100 μg ml^{-1} gentamicin during a 1-h incubation period. Intracellular bacteria were enumerated after Triton X-100 lysis of the INT407 cells and by plate count. Invasion efficiency was expressed as the percentage of the inoculum surviving

the gentamicin treatment. Relative invasion of invasive bacteria in the presence of an *E. coli* strain was expressed as percentage of the relative invasion in the absence of any *E. coli* strain, which was by definition 100%. Results presented are the mean of at least four independent duplicate experiments.

2.4. Tests for anti-invasive activity of EcN and its derivatives

The anti-invasive activity of EcN and its derivatives was quantified in invasion assays by determining the invasion efficiency of the respective invasive strain alone, together with the negative *E. coli* control strain DH5α, or EcN or one of its derivatives. The invasion assays were started by addition of the invasive strain alone or together with an equal amount of one of the *E. coli* strains. Invasion efficiency of each of the invasive strains alone was defined as 100% relative invasion. Invasion ability in the presence of *E. coli* DH5α or EcN was expressed as the percentage of invasion in the absence of any *E. coli* strain. In addition, the pH of the medium was determined at the beginning and at the end of the invasion period using a piece of pH-indicator paper (Hartenstein, Würzburg, Germany).

In some assays EcN was not added together with the invasive strain into the cell culture medium on top of the INT407 monolayer. Instead EcN was added into a trans-well unit (Millipore, Eschborn, Germany) with a porous membrane of pore size 0.4 μm containing cell culture medium. In control experiments aliquots from the medium below the trans-well unit were plated on LB agar at 0, 1, 2 and 3 h after addition of EcN into the unit.

Results presented are the mean of at least three independent duplicate experiments.

2.5. Statistical analysis

Percent invasion values of bacterial strains were compared using two-tailed *t*-tests. Values were considered significantly different if $P < 0.01$.

3. Results

3.1. Bacterial invasion of INT407 cells

Invasion efficiency of each strain into the human embryonic intestinal epithelial INT407 cells was quantified by employing the gentamicin protection assay. The selected invasive strains were able to invade INT407 cells (Fig. 1). Therefore, the INT407 cells were chosen as a model for bacterial invasion of human intestinal epithelial cells. In contrast, the probiotic EcN (0.02% invasion) was not invasive, just as little as the negative control strain *E. coli* DH5α (0.01% invasion).

3.2. EcN interferes with bacterial invasion

It has been reported, that EcN protects gnotobiotic piglets from an infection by *Salmonella* [29]. Because an important virulence factor of pathogenic *Salmonella* strains is the ability to invade host epithelial cells, we tested the hypothesis whether EcN might interfere with invasiveness of *Salmonella*. For that purpose the invasion efficiency of *Salmonella* alone and in the presence of either the control strain *E. coli* DH5α or of EcN was determined in invasion assays. Results of such experiments clearly demonstrated an inhibitory effect of EcN on the invasiveness of *S. enterica* serovar Typhimurium strains C17 as well as SL1344 (Fig. 1A). The inhibition efficiency depended on the ratio of EcN per *Salmonella* (Fig. 2). However, *Salmonella* invasiveness was not inhibited by EcN killed by heat or UV-irradiation. In a second set of gentamicin protection assays, we tried to find out, if the observed anti-invasive activity of EcN might not be limited to *S. enterica* serovar Typhimurium but might affect other invasive bacterial species as well. Therefore, the invasion efficiency of the Gram-negative *Y. enterocolitica* strains WA314 and WA-C, the *S. flexneri* strain M90T and the *L. pneumophila* strain Corby and of the Gram-positive *L. monocytogenes* strain EGD were determined in the presence and absence of EcN. The invasion efficiency was reduced by 40% (*L. pneumophila*) to 65% (*L. monocytogenes*) in the presence of EcN (Fig. 1B–E). The inhibition of invasion by EcN of all invasive strains was highly significant (*P*-values between 0.003 for *L. pneumophila* and 0.000002 for *S. enterica* serovar Typhimurium strain SL1344). The viability of any invasive strains was not affected by the presence of EcN during the invasion assays (data not shown). No inhibition of invasion was observed with the *E. coli* K-12 strain DH5α (Fig. 1). These results demonstrated the ability of EcN to interfere with the invasiveness of strains from several Gram-negative and one Gram-positive bacterial species. To further characterize the anti-invasive activity of EcN additional invasion experiments with *Salmonella* were performed.

3.3. Microcins are not responsible for anti-invasive activity of strain Nissle 1917

The probiotic EcN contains two microcin determinants and produces at least one microcin [11]. Therefore, it was possible that the anti-invasive activity of EcN might be the result of the bacteriocidal action of the microcin(s). To test this hypothesis we employed the isogenic microcin-negative EcN mutant, strain SK22D, in invasion assays with *Salmonella* and one *Yersinia* strain. Strain SK22D was as effective in inhibiting the invasiveness of *S. enterica* serovar Typhimurium strains C17 and SL1344 and of *Y. enterocolitica* strain WA-C as the parent strain EcN (Fig. 3). The inhibition of invasion by EcN was obviously not de-

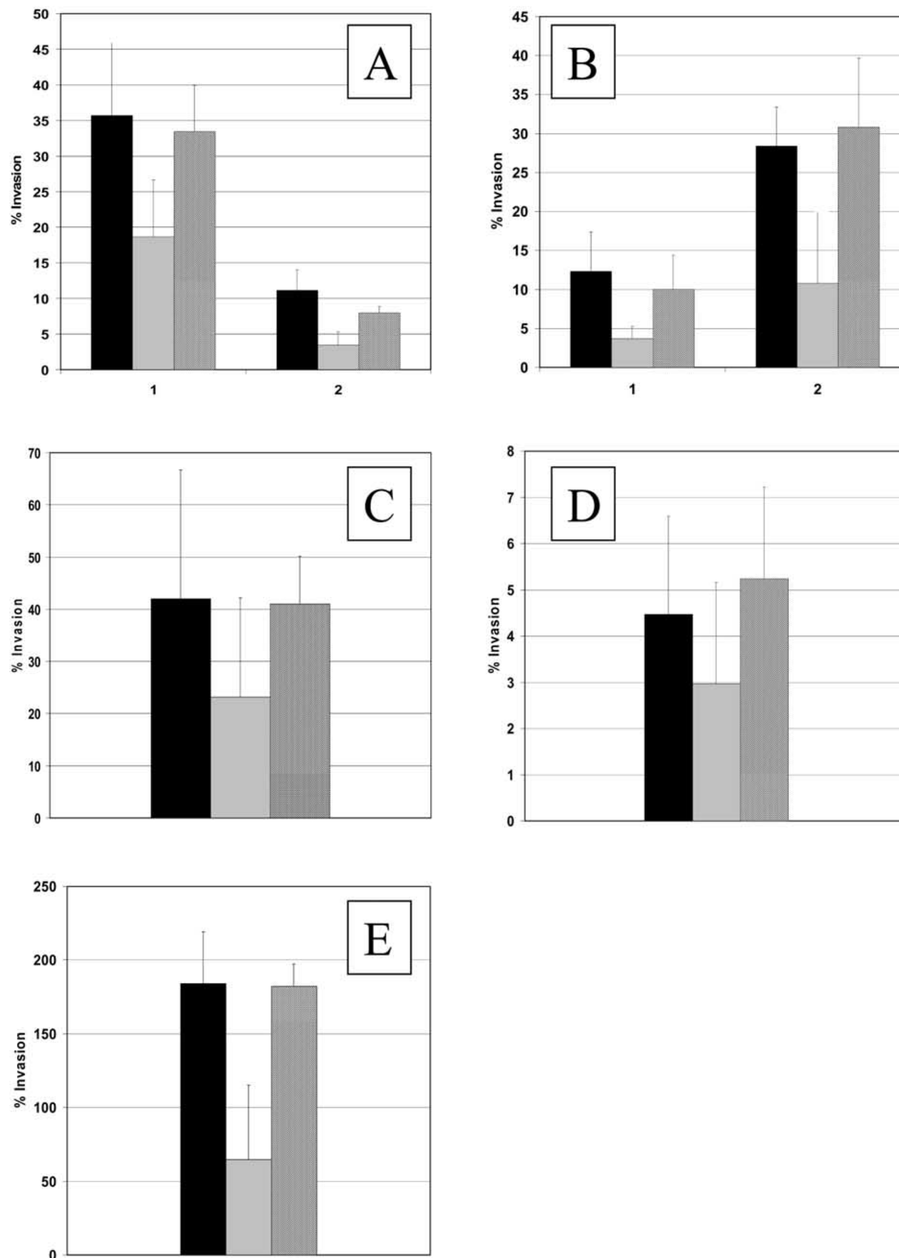

Fig. 1. Invasion efficiencies of the invasive strains alone (black bars), of the invasive strains in the presence of the probiotic EcN (gray bars), or of the invasive strains in the presence of *E. coli* DH5α (striped bars) obtained with the human intestinal epithelial cell line INT407. Invasion was determined by the invasion assay (see Section 2). The invasion efficiency (% invasion) of *S. enterica* serovar Typhimurium strain C17 (A, panel 1) and SL1344 (A, panel 2), *Y. enterocolitica* strains WA314 (B, panel 1) and WA-C (B, panel 2), *S. flexneri* strain M90T (C), *L. pneumophila* strain Corby (D) and *L. monocytogenes* strain EGD (E) in the presence and absence of the probiotic EcN or DH5α is expressed as the percentage of the inoculum surviving the gentamicin treatment.

pendent on the microcin(s) produced by this probiotic strain.

3.4. *Physical contact neither between invasive bacteria nor between INT407 cells and EcN is necessary for inhibition of invasion*

Besides microcin(s) EcN expresses three different adhesins. Type 1 and F1C fimbriae together with the curli-type adhesin might be important colonization factors. These adhesins might also be responsible for the observed adherence of EcN to INT407 cells (data not shown). Effective adherence of EcN to the epithelial cells might block receptors necessary for interaction with invasive bacteria to induce their internalization. To address this question, gentamicin protection experiments were performed with EcN separated by a membrane from both, the invasive bacteria and the epithelial cell layer. Control experiments with EcN

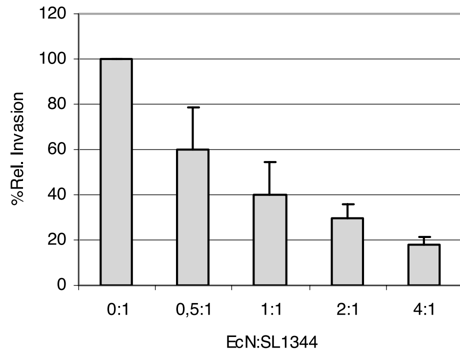

Fig. 2. Dose-dependent inhibition of *S. enterica* serovar Typhimurium strain SL1344 invasion of INT407 cells by the probiotic EcN. The invasion efficiency of *S. enterica* serovar Typhimurium SL1344 in the absence of EcN was defined as 100% relative invasion.

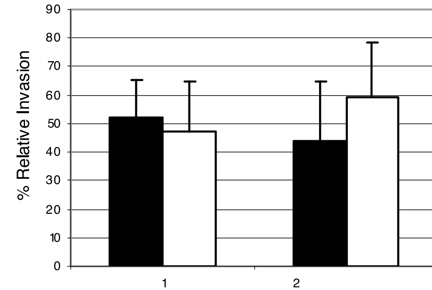

Fig. 4. Comparison of the invasion efficiencies of *S. enterica* serovar Typhimurium strain C17 in the presence of either EcN [1] or its isogenic microcin-negative mutant SK22D [2]. In parallel experiments, the strain Nissle 1917 or SK22D was added into the same compartment as the *S. enterica* serovar Typhimurium strain C17 and the INT407 cells (black bars) or in a trans-well unit (white bars) separated from both, the *Salmonella* strain and the INT407 cells by a membrane non-permeable for bacteria.

proved the membrane of the trans-well unit to be non-permeable for EcN (data not shown). Even in this experimental setting EcN was as effective in inhibiting invasion of INT407 cells by *S. enterica* serovar Typhimurium as in assays without separation of EcN (Fig. 4). Therefore we concluded, interference of EcN with bacterial invasion does not rely on direct physical contact between EcN and INT407 cells or EcN and the invasive bacteria.

3.5. Neither Fim nor Foc nor plasmid-encoded gene products are involved in the anti-invasive activity

To further support the finding that adhesins of EcN are not a prerequisite for inhibition of invasion, invasion assays were performed with a Fim- and Foc-negative isogenic deletion mutant of EcN. The relative invasion of *S. enterica* serovar Typhimurium strain SL1344 of 11.1% in the absence of EcN was reduced to 4,6% in the presence of the Fim, Foc EcN deletion mutant ($P < 0.0004$). This inhibition is similarly effective as that of the parent strain. To test for a potential role of gene products encoded by one or both of the two plasmids typically harbored by EcN we also included an isogenic but plasmid-cured EcN derivative in invasion assays. Even the cured EcN strain was able to interfere effectively with the invasiveness of *S. enterica* serovar Typhimurium strain SL1344 (5,1% invasion of *Salmonella* in the presence of the cured EcN; $P < 0.000017$).

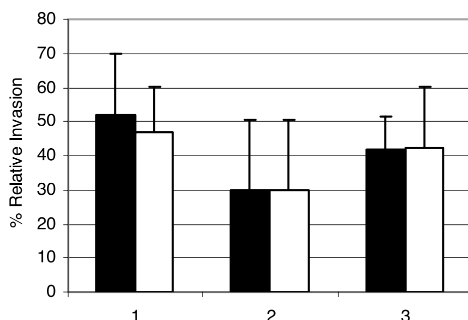

Fig. 3. Comparison of the invasion efficiencies of *S. enterica* serovar Typhimurium strains C17 [1] and SL1344 [2] and *Y. enterocolitica* strain WA-C [3] in the presence of EcN (black bars) or its isogenic microcin-negative mutant SK22D (white bars) with INT407 cells.

4. Discussion

The probiotic EcN is already in use for successful prevention and treatment of various diseases of the digestive tract of humans since several decades. Even though it is obviously safe its mode of action is still unknown. In the gnotobiotic piglet model EcN was shown to protect these animals from a *Salmonella* infection. Because invasion of enterocytes is an important virulence factor of *Salmonella*, we tested for inhibition of *Salmonella* invasion of INT407 cells by EcN. The results of invasion experiments clearly demonstrated EcN to inhibit invasion by *Salmonella*. Another non-pathogenic fecal *E. coli* strain, EM0, protects only germ-free mice but not conventional mice against infection of *S. enterica* serovar Typhimurium strain C5. In addition, this fecal *E. coli* strain shows no inhibitory effect against invasion by *Salmonella* strain C5 of Caco-2/TC7 cells [30]. Either the anti-invasive activity of EcN is specific for this strain or the ability of probiotic *E. coli* to interfere with bacterial invasion depends on the cell line employed.

EcN was demonstrated to inhibit not only *Salmonella* invasion but also invasion of INT407 cells by *Y. enterocolitica*, *S. flexneri*, *L. pneumophila* and *L. monocytogenes*. Similarly, *Lactobacillus acidophilus* strain LA1 inhibits cell invasion of Caco-2 cells by enteropathogenic *E. coli*, *S. enterica* serovar Typhimurium and *Yersinia pseudotuberculosis* [31]. This anti-invasive activity of LA1 seems however to be a secondary effect due to a bactericidal activity in the supernatant of LA1 [32]. Also EcN produces a bacteriocidal product, microcin(s). However, the microcin-negative isogenic mutant of EcN is as effective as the parent strain in interfering with bacterial invasion. In addition, growth rate of *Salmonella* during the 3 h

invasion period was not affected by the presence of EcN or its microcin-negative mutant SK22D (data not shown).

Another anti-invasion mechanism could be blocking of epithelial surface receptors by binding of EcN to these receptors or by binding of EcN to the respective ligands of the invasive bacteria. By separating EcN from the INT407 monolayer and from *Salmonella* with a porous membrane with pore size of 0.4 μm interaction of EcN with INT407 cells as well as with *Salmonella* was no longer possible. Nevertheless, invasion was inhibited as effective as in experiments without separation of EcN. These results indicated the anti-invasive mechanism relaying not on a direct physical contact between EcN and epithelial cells or the invasive bacteria.

For *L. casei* strain GG inhibition of *Salmonella* invasion of Caco-2 cells was the result of the acidification of the medium and subsequently the activation of a bacteriocidal substance, because after neutralization at pH7, no inhibition of the *Salmonella* invasion was observed [33]. In our invasion assays with *Salmonella* the pH of the cell culture medium was not different in wells with only *Salmonella* and wells with EcN plus *Salmonella* after the 3-h invasion period. Obviously, the anti-invasive mechanism of EcN does not involve/rely on acidification.

For other probiotics such as *Lactobacillus johnsonii* or *Lactobacillus casei* Shirota inhibition of *S. enterica* serovar Typhimurium adherence to human intestinal mucus was reported whereas *L. casei* GG and *L. rhamnosus* increased adhesion [34]. Adherence of enteropathogenic *E. coli* to HT29 cells can be inhibited by the induction of mucin expression by certain lactobacilli [35]. We cannot rule out that EcN also induces mucin expression resulting in interference with invasion, because we did not determine mucin expression by INT407 cells.

In conclusion, EcN is likely to interfere with bacterial invasion of INT407 cells by secreting a component that might act on the epithelial cells or/and the invasive bacteria. Because the invasion systems used by the bacterial strains included in this study are so diverse, we favor the hypothesis of an anti-invasive secreted component of EcN acting on a central process or structure of the INT407 cells involved in the internalization of all these invasive bacteria. Work is in progress to identify the EcN gene(s) responsible for the anti-invasive activity of EcN, which might be part of the probiotic nature of this *E. coli* strain.

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