Lactoferrin inhibits *E. coli* O157:H7 growth and attachment to intestinal epithelial cells

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**ABSTRACT:** Enterohemorrhagic *Escherichia coli* (EHEC) serotype O157:H7 strains are associated with haemorrhagic colitis and haemolytic uremic syndrome (HUS) in humans. Cattle are a reservoir of *E. coli* O157:H7. We studied the ability of bovine and human lactoferrin, two natural antimicrobial proteins present in milk, to inhibit *E. coli* O157:H7 growth and attachment to a human epithelial colorectal adenocarcinoma cell line (Caco-2). The direct antibacterial effect of bLF on *E. coli* O157:H7 was stronger than that of hLF. Nevertheless, both lactoferrins had bacteriostatic effects even at high concentrations (10 mg/ml), suggesting blocking of LF activity by a yet undefined bacterial defence mechanism. Additionally, both lactoferrins significantly inhibited *E. coli* O157:H7 attachment to Caco-2 cells. However, hLF was more effective than bLF, probably due to more efficient binding of bLF to intelectin present on human enterocytes leading to uptake and thus removal of bLF from the extracellular environment. Inhibition of bacterial attachment to Caco-2 cells was at least partly due to the catalytic effect of lactoferrins on the type III secreted proteins EspA and EspB.

**Keywords:** transferring; type III secretion system; EspA; EspB

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The enterohemorrhagic *Escherichia coli* (EHEC) strain O157:H7 is a major food-borne pathogen causing severe disease in humans worldwide. Healthy cattle are a reservoir of *E. coli* O157:H7. Bovine food products and fresh products contaminated with bovine waste are the most common sources for haemorrhagic colitis (HC) and the haemolytic uremic syndrome (HUS) (reviewed by Callaway et al., 2009). Three major virulence factors of *E. coli* O157:H7 have been identified including a pathogenicity island called the Locus of Enterocyte Effacement (LEE), Shiga toxins (Stx) and the plasmid (pO157) encoded enterohaemolysin gene (E-hlyA) that codes for a pore-forming cytolysin. *E. coli* O157:H7 colonization of the intestinal mucosa induces a histopathologic lesion defined as an “attaching and effacing” (A/E) lesion characterized by localized destruction of brush border microvilli and intimate attachment of the bacteria to host cell plasma membranes (Frankel et al., 1998; Karpman et al., 2002). The Locus of Enterocyte Effacement (LEE), genetically governs adhesion and subsequent pathology (Nataro and Kaper, 1998). It contains the eae gene, encoding the outer membrane protein intimin and its receptor Tir (Translocated intimin receptor) (Jerse et al., 1990). In addition, LEE encodes proteins of the type III secretion system (TTSS), which is made up of an EspA multifilament needle complex, used for insertion of the bacterial effector proteins EspB, EspD and Tir into the host cell. Injection of bacterial virulence factors via the TTSS and binding of intimin to Tir leads to a strong interaction between bacteria and host cells (Cookson...
and Woodward, 2003; Vilte et al., 2008). Virulence arises also from Shiga toxin production, encoded by Shiga toxin genes ($stx1$ and $stx2$), which are the primary factors responsible for the hemorrhagic aspect of diarrhoea and systemic complications (HUS). Shiga toxins act as N-glycosidases, cleaving ribosomal RNA leading to the inhibition of host cell protein synthesis (Endo et al., 1988).

Most adults recover from $E. coli$ O157:H7 infections without sequelae. Children and the elderly however, are more likely to experience complications such as HUS and even death. The use of antibiotics in treatment for $E. coli$ O157:H7 infections in humans is highly controversial as antibiotics might increase the risk of HUS (Safdar et al., 2002; Dundas et al., 2005; Panos et al., 2006). Thus, treatment is largely supportive. Nonetheless, innovative therapies such as the use of probiotics, monoclonal antibodies or recombinant bacteria to neutralize or bind toxins, are currently being explored (reviewed by Bavaro, 2009).

Natural anti-microbial proteins, such as lactoferrin might assist in the treatment of O157:H7 infections. Therefore, we examined the effect of human and bovine lactoferrin on $E. coli$ O157:H7. Lactoferrin (LF) is abundantly present in colostrum and milk and belongs to the transferrin family. Human colostrum contains $5.3 \pm 1.9$ mg/ml LF, while human milk contains $1$ mg/ml LF after the first month of lactation. Bovine colostrum contains $1.5$ mg/ml LF and the LF concentration in milk ranges from $0.02$ mg/ml to $0.20$ mg/ml (Shimazaki et al., 2000; Ochoa and Cleary, 2009). However, large-scale production of bovine LF is relatively easy meaning that it is financially feasible, especially for developing countries.

Lactoferrin exhibits anti-oxidant, antiviral, anti-inflammatory, immunomodulatory as well as anti-cancer activities, and interestingly can promote the growth of probiotic bacteria such as $Bifidobacterium$ (Aguila et al., 2001; Al-Nabulsi and Holley, 2007; Jenny et al., 2010; Tsuda et al., 2010; Xu et al., 2010). Lactoferrin's bacteriostatic effect is due to its ability to bind iron and limit its availability in the growth environment (Orsi, 2004). Binding of LF to the surface of Gram-negative bacteria initiates bactericidal effects by releasing lipopolysaccharide (LPS) from the membrane (Ellison et al., 1988; Orsi, 2004). Additional antimicrobial functions ascribed to LF are selective permeation of ions and due to its serine protease activity, disruption of the bacterial TTSS, thereby blocking bacterial adhesion (Ochoa et al., 2003).

**MATERIAL AND METHODS**

**Organisms and cell culture**

The $E. coli$ O157:H7 strain NCTC 12900, a well-characterized Shiga-toxin negative EHEC strain of human origin (Dibb-Fuller et al., 2001) was used in both bacterial growth and host cell attachment studies. We used this verocytotoxin negative strain for biosafety reasons, as in future experiments this strain was also going to be used in vivo in ruminants. The non-attaching, $E. coli$ strain DH5α, extensively used in recombinant DNA technology, served as a negative control.

Host cell attachment in the presence and absence of LF was evaluated using the Caco-2 human epithelial colorectal adenocarcinoma cell line, a well-established in vitro model for studying EHEC attachment (Izumikawa et al., 1998). Caco-2 cells were seeded into 24-well flat-bottom plates (Corning Inc., Corning, NY) at a density of $1 \times 10^5$ cells/well in Dulbecco’s modified Eagle’s medium (Gibco, Grand Island, NY) containing $1\%$ l-glutamine and $5\%$ heat-inactivated fetal bovine serum (Gibco), without antibiotics. Cells were grown to confluence at $37°C$ in a humidified atmosphere of $5\%$ CO$_2$ (approximately $72$ h).

**Recombinant intimin, EspA and EspB**

The plasmids pCVD468, pCVD469 and pMW103 were grown in LB supplemented with Kanamycin (25 µg/ml) and Ampicillin (100 µg/ml). Expression of EspA, EspB and the C-terminal 380 amino acids of intimin-γ (referred to as intimin) was induced by adding 1mM isopropyl-β-d-thiogalactopyranoside (IPTG). Recombinant His-tagged proteins were purified by nickel-affinity chromatography (Novagen®) and protein concentrations were determined using the bicinchoninic acid (BCA) method (Thermo Scientific, Rockford, USA).

**Lactoferrins**

Iron saturated bovine lactoferrin (bLF; Sigma, Bornem, Belgium), with 90% purity (SDS-PAGE) and > 85% iron saturation purified from bovine colostrum, and iron saturated human lactoferrin (hLF; Sigma, Bornem, Belgium), with the same purity and level of iron saturation, purified from human milk were used in this study.
**Effect of lactoferrins on *E. coli* O157:H7 growth**

*E. coli* O157:H7 overnight cultures were prepared by inoculating a colony isolated in a single well into a 10-ml tube containing Luria Bertani broth (LB; Becton Dickinson, Clax, France) and incubating the tube at 37°C for 12 to 18 h with shaking (200 rpm). Overnight *E. coli* O157:H7 cultures (1 ml) were pelleted by centrifugation (11,337 × g, 5 min) and reconstituted in 1 ml of LB medium.

Bacteria (10⁷ CFU/ml) were incubated at 37°C for 8 h in LB broth supplemented with different concentrations (zero, 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1.0, 5.0 and 10 mg/ml) of human or bovine LF. Selected concentrations were within the physiological range. Bacterial growth was monitored spectrophotometrically (OD₆₀₀nm) on the hour for 8 h subsequently. At the same time, viable bacteria were counted by spread plating appropriate bacterial serial dilutions onto LB medium plates. After 8 h, bacteria were washed three times with LB medium, inoculated into a 10-ml tube containing LB broth and incubated at 37°C for 5 h with shaking (200 rpm). In addition, we also examined the surface of LF treated bacteria, 1 and 8 h after adding LF using scanning electron microscopy (SEM) as described by Vandekerckhove et al., (2009). Briefly, the bacterial pellets were fixed in a HEPES-buffered 2% paraformaldehyde-2.5% glutaraldehyde solution for 24 h and were critical point dried using CO₂ (CDP 030, Balzers, Sercolab), mounted on metal stubs, platinum coated (JFC-1300 autofine coater, Jeol) and finally examined by a Jeol JSM 5600 LV SC. El. Microscope (Jeol, Germany). Thus, we studied the effect of lactoferrins on bacterial growth but at the same time we also defined the maximum human and bovine lactoferrin concentrations, which did not inhibit bacterial growth. These were subsequently used in cell attachment assays.

**Lactoferrin cytotoxicity assay**

The cell attachment assay was performed using Caco-2 cells. To check the putative cytotoxic effect of lactoferrins, Caco-2 cells were first seeded in 96-well plates at a concentration of 5 × 10⁴ cells/ml and exposed for 4 h to concentrations of zero, 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1.0, 5.0 or 10 mg/ml human or bovine lactoferrins in culture medium. Incubations were performed in duplicate. Cytotoxicity was assessed in a dose dependent manner by the 3-(4,5-dimethylthiazol-2-yl-2,5- diphenyltetrazolium bromide) MTT assay measuring mitochondrial activity (Mosmann, 1983). Viable cells reduce the tetrazolium salt MTT to a colored water-insoluble formazan salt. After it is solubilized, formazan can be quantified spectrophotometrically at 585 nm. The MTT assay was performed as follows. Ten μl MTT (5 mg/ml, Sigma) in Hanks balanced salt solution (Invitrogen) was added to each well and after 3.5 h of incubation at 37°C, the MTT solution was replaced by 200 μl DMSO in ethanol (1/1 v/v). The plates were agitated for 15 min on a platform shaker (450 RPM) to dissolve the formazan crystals and subsequently analyzed spectrophotometrically at both 585 nm (OD1) and 620 nm (OD2). The latter wavelength was used to correct for cell debris and well imperfections. Final optical densities obtained from formazan formation were presented as OD1 minus OD2.

**Effect of lactoferrins on *E. coli* O157:H7 attachment to Caco-2 cells**

The attachment efficiencies of *E. coli* O157:H7 in the presence and absence of lactoferrins were determined by performing attachments assays using the Caco-2 human intestinal cell line. Lactoferrins were used at the highest concentration which did not decrease *E. coli* O157:H7 growth in LB broth. Thus, maximum concentrations of 0.1 mg/ml and 0.05 mg/ml of human and bovine LF were used, respectively. For each LF, 3 additional lower concentrations (0.01, 0.005 and 0.001 mg/ml) were used to study concentration-dependent effects. The effect of lactoferrins on Caco-2 cells was monitored using an Olympus IX81 microscope equipped with a cell*M Imaging system (Olympus). *E. coli* O157:H7 overnight cultures were prepared by inoculating a colony isolated in a single well into a 10-ml tube containing LB broth and incubating the tube at 37°C for 12 to 18 h with shaking (200 rpm). Overnight *E. coli* O157:H7 cultures (1 ml) were pelleted by centrifugation (11,337 × g, 5 min) and reconstituted in 1 ml of DMEM.

Confluent Caco-2 monolayers were infected with *E. coli* O157:H7 (10⁷ CFU/ml) in the presence or absence of different concentrations of bovine or human LF and further incubated for 4 h at 37°C and 5% CO₂. After infection for 4 h at 37 °C, non-adherent bacteria were removed by washing prepa-
Caco-2 cells were lysed by adding 0.25% trypsin for 15 min (37°C) and vigorous pipetting, followed by vortexing of the cell suspension. Adherent *E. coli* O157:H7 cells were enumerated by spread plating appropriate serial dilutions onto LB medium plates, in duplicate. The LB medium plates were incubated at 37°C for 24 h, and the resultant CFU were enumerated. The attachment efficiency of *E. coli* O157:H7 was expressed as a percentage based on the initial inoculum that was recovered as adherent *E. coli* O157:H7 cells. The attachment efficiency of each isolate was measured in duplicate wells in at least three independent experiments (Figure 1).

### Effect of lactoferrins on TTSS proteins

Proteolysis of *E. coli* O157:H7 recombinant intimin, EspA and EspB by lactoferrins was determined as follows. Intimin, EspA and EspB (10 µg/ml) were incubated in DMEM in the presence or absence of 10 mg/ml LF for 4 h at 37°C. Subsequently, His-labelled fragments were identified by Western blotting using a mouse monoclonal antibody against histidine (Sigma, Bornem, Belgium). Lactoferrin is a member of the serine protease family. Therefore, as a control, recombinant proteins were also incubated with lactoferrins (10 mg/ml) in the presence of the serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF), (0.25mM) (Sigma, Bornem, Belgium), for 4 h at 37°C. Proteolysis was again analysed by Western blotting.

### Statistics

Statistical analysis was performed by the Proc MIXED test using SAS software S version 8.2 (SAS Institute Inc., Cary, NC, USA). Results were presented as mean OD ± SD and mean colony forming units (CFU) ± SD for the bacterial growth studies and as mean percentages of bacterial attachment ± SD for the cell attachment study.

### RESULTS

#### Effect of lactoferrins on *E. coli* O157:H7 growth

To determine the effect of LF on *E. coli* O157:H7 growth, bacteria were incubated with several concentrations of human and bovine LF. *Escherichia coli* O157:H7 growth was significantly inhibited from three to six hours post incubation (PI) using 0.5 to 10 mg/ml and 0.1 to 10 mg/ml of human or bovine LF, respectively (Figure 2 and 3). Thus, bLF had a stronger inhibitory effect on *E. coli* O157:H7 growth than hLF. However, at 8 hours PI, all growth curves of LF-treated bacteria and untreated controls reached the same OD value, even at the high-

![Figure 1. Lactoferrin significantly reduced *E. coli* O157:H7 attachment to Caco-2 cells. Results are represented as the mean values ± S.E.M (n = 3). Asterisks indicate statistically significant different between lactoferrins treated groups and the control (0 mg/ml lactoferrin) (P < 0.05)](image_url)
est LF concentration used. Human and bovine LF had no effect on E. coli O157:H7 growth at concentrations of 0.1 and 0.05 mg/ml, respectively. After 8 h, lactoferrins were removed and bacteria were allowed to grow again in fresh medium. Resulting growth curves were identical to the ones of untreated controls (data not shown).

The maximum non-growth-inhibitory concentrations to be used in subsequent cell attachment assays were 0.1 mg/ml and 0.05 mg/ml for human or bovine LF, respectively. Scanning electron microscopy of bacteria incubated with lactoferrins revealed no obvious findings except for the presence of significant fewer bacteria when using 10 mg/ml bLF (Figure 4).

**Lactoferrin cytotoxicity assay**

None of the lactoferrin concentrations tested was cytotoxic to Caco-2 cells, as compared to untreated control cells (Figure 5). Thus, maximum non-growth-inhibitory concentrations of lactoferrins could be used in a subsequent cell attachment assay.

**Effect of lactoferrins on E. coli O157:H7 attachment to Caco-2 cells**

Lactoferrins had no effect on Caco-2 cells (Figure 5). In the absence of LF, a mean of $4 \times 10^4$
CFU/well (100%) was recovered from Caco-2 cells. In the presence of lactoferrins, *E. coli* O157:H7 attachment to Caco-2 cells decreased in a concentration-dependent manner (Figure 1). Overall, hLF inhibited *E. coli* O157:H7 attachment more effectively, also at 0.05 mg/ml hLF. At the highest LF concentrations used, namely 0.1 mg/ml for hLF and 0.05 mg/ml for bLF, bacterial attachment was reduced by 78% and 57%, respectively, as compared to untreated bacteria (100% attachment; *P* < 0.05).

**Effect of lactoferrins on TSSS proteins**

Both lactoferrins reduced *E. coli* O157:H7 attachment to Caco-2 cells significantly at non-growth-inhibitory concentrations indicating that mechanisms other than growth reduction are involved. We examined the effect of LF on the bacterial TTSS of *E. coli* O157:H7. As shown by Western blotting, LF degraded EspA and EspB, but not intimin. The proteolytic effect of LF was prevented by a serine protease inhibitor.

**DISCUSSION**

Even though the use of antibiotics for treating *E. coli* O157:H7 infections in humans is typically avoided and remains controversial, increasing antibiotic resistance in this bacterium is a concern. Several studies have already demonstrated that antibiotic resistant *E. coli* O157:H7 can be isolated from humans, cattle, feed and even from surface waters (Schroeder et al., 2002; Fincher et al., 2009). Thus, there are several reasons for developing new anti-microbial strategies for treatment of human infections and preventing *E. coli* O157:H7 infections in cattle or at least reduce faecal shedding significantly in these animals. Here, we examined the effect of human and bovine lactoferrin on *E. coli* O157:H7 growth and on attachment to human cells.
Growth inhibition was more pronounced when using bLF. Groenink et al. (1999) reported the same findings. Bovine LF inhibited the growth of S. aureus, S. mutans, S. sobrinus, S. salivarius as well as of E. coli, K. pneumoniae, P. intermedia, P. gingivalis, and F. nucleatum, while hLF only inhibited growth of S. mutans, S. salivarius and P. intermedia (Groenink et al., 1999). Different antimicrobial activities could be due to more efficient bLF binding to E. coli O157:H7. Naidu et al., (1991) studied the binding of hLF and bLF to 169 E. coli strains (ETEC and EHEC) isolated from human intestinal infections and found large variations in the range of 3.7 to 73.4% and 4.8 to 61.6% for hLF and bLF, respectively (Naidu et al., 1991). On the other hand, the results could also be attributed to structural and functional differences between bovine and human LF. The primary structure of bLF is 69% identical to hLF, (reviewed by Baker and Baker 2005). However, the anti-microbial activity resides mainly in the basic N1-domain of lactoferrins containing two stretches, designated lactoferricin and lactoferrampin (reviewed by Baker and Baker, 2009). Others have reported the same findings. Lactoferricin (25-residue cationic disulphide cross-linked peptide of lactoferrins) of bovine origin was more active on E. coli (ATCC 25922) and S. aureus (ATCC 25923) than lactoferricins of human, caprine and murine origin (Vorland et al., 1998). The anti-microbial properties of bovine lactoferricin are also stronger than those of their human counterparts (Haney et al., 2009).

Nevertheless, none of the lactoferrin concentrations used in our study resulted in 100% cell death. To our knowledge 100% cell death has only been observed when using lactoferricin or lactoferrampin, which are more potent bacterial killers than the larger protein. Bovine lactoferricin and lactoferrampin are normally both internalized within a few minutes in E. coli K12, concurrently with disruption of membrane integrity and killing of E. coli (Van der Kraan et al., 2005). However, in the present study, CFUs for controls and treated bacteria were statistically the same until 2 h and SEM revealed no obvious surface changes, which means that bacterial killing by lactoferricin or lactoferrampin is not important in our experiment.

Growth inhibition by lactoferrins was significant (at 0.1 to 10 mg/ml) from 3 to 6 h post incubation. Thus, it takes time to notice a significant anti-microbial effect, which was also observed by Ellison and Giehl (1991) and Kawasaki et al. (2000). This could be due to the relatively slow interaction of LF with the bacterial LPS, known to result in bacterial killing. Bacterial outer membranes are usually asymmetric membranes containing the polyanionic glycolipid lipopolysaccharide (LPS) in the outer leaflet and phospholipids in the inner leaflet. To stabilize the anionic surface of the outer membrane, the LPS is partially neutralized by divalent cations, such as Mg$^{2+}$ and Ca$^{2+}$. Cationic peptides, such as LF-derived anti-microbial peptides can interact with the divalent cation-binding sites of LPS, thereby distorting the integrity of the outer membrane (Chappell et al., 2004).

However, only at high LF concentrations (1.0, 5.0 and 10 mg/ml) was bacterial growth completely arrested for one hour. Thus, at sublethal concentrations, human and bovine lactoferrins acted bacteriostatically on E. coli O157:H7. The bacteria recovered and started to grow again. Chappell et al. (2004) observed the same while studying the association of human lactoferrin peptides with E. coli NCTC 8007 serotype O111. Thus, other events are maybe required for lactoferrins to be highly effective and simply coating the bacterial surface is not adequate. On the other hand, E. coli O157:H7 might have also developed a bacterial defence system leading to blockage of lactoferrins. This may explain why low and high (1, 5 and 10 ml/ml) LF concentrations had no effect or only a temporary growth inhibitory effect, respectively. Blockage of LF could be due to LPS-mediated shielding of porins from LF interaction (Naidu et al., 1991) and/or to an interaction with a bacterial surface protein, as described by Senkovich et al., (2007) for the pneumococcal surface protein A (PspA). Two helices of PspA bind in grooves in the human lactoferrin bactericidal domain and make specific interactions with basic residues from helix 1 and the N-terminus, thereby blocking LF activity (Baker and Baker, 2009). However, further research is needed to explore this hypothesis.

Lactoferrin and the avian homologue ovotransferrin impair bacterial type III secretion system function in enteric Gram-negative pathogens (reviewed by Ochoa and Cleary, 2009) and the avian respiratory pathogen Chlamydophila psittaci (Beeckman et al., 2007), thereby decreasing their ability to adhere and invade host cells. Both human and bovine lactoferrin inhibited E. coli O157:H7 adherence to Caco-2 cells in a dose-dependent manner. Overall, the anti-adhesive effect of hLF was higher than that of bLF. This could be due to the fact that hLF was more effective in destroying E. coli O157:H7 virulence factors required for attachment to human cells.
Beeckman et al. (2007) described a similar finding. Ovo transferrin was more effective than human and bovine lactoferrin in preventing attachment and entry of Chlamydo phila psittaci to avian macrophages (Beeckman et al., 2007). On the other hand, uptake of bLF in Caco-2 cells might be more effective than hLF as demonstrated by Shin et al. (2008), who studied the interaction between human and bovine LF and intelectin, a lectin present on the brush border of intestinal cells. Thus, internalized bLF could no longer prevent bacterial attachment to host cells.

Ochoa et al. (2003) demonstrated the effect of human lactoferrin on enteropathogenic E. coli (EPEC). Lactoferrin blocked EPEC-mediated actin polymerization in HEP2 cells and blocked EPEC-induced hemolysis. The mechanism of these actions was lactoferrin-mediated degradation of Type III secreted proteins necessary for bacterial contact and pore formation, particularly EspB. Lactoferrin is also responsible for the degradation of the Shigella TTSS proteins IpaB and IpaC (Gomez et al., 2003). In our study, lactoferrin degraded recombinant EHEC EspA and EspB, which indeed could contribute to its anti-microbial activity.

In conclusion, the direct antibacterial effect of bLF on E. coli O157:H7 was stronger than for hLF. Nevertheless, both lactoferrins acted bacteriostatically even at high LF concentrations (10 mg/ml), suggesting blocking of LF activity by a yet unknown bacterial defence mechanism. Additionally, both lactoferrins significantly inhibited E. coli O157:H7 attachment to Caco-2 cells. However, hLF was more effective than bLF. This is maybe due to more efficient binding of LF to intelectin on human enterocytes and subsequent uptake and thus removal of bLF from the extracellular environment. Inhibition of attachment was at least partly due to the catalytic effect of lactoferrins on the type III secreted proteins EspA and EspB. Further research is needed into the use of LF for supporting human treatment and/or for preventing E. coli O157:H7 infections in ruminants.

Acknowledgments

The authors wish to thank Gent University for providing a PhD grant (No. 01W04407) to Maryam Atef Yekta. This study was funded by the Federal Public Service of Health, Food Chain Safety and Environment (Grant No. S6172) and the Research Foundation Flanders (FWO-Vlaanderen). The authors gratefully acknowledge M. J. Woodward for providing E. coli O157:H7 strain NCTC12900, G. Cleary for providing the plasmids encoding EspA and EspB, D. O’Brien for providing the intimin encoding plasmid and C. Cuvelier for providing the Caco-2 cell line. H. Favoreel is acknowledged for assistance during bio-imaging.

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Received: 2010–05–04
Accepted after corrections: 2010–08–10

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