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

M. Atef Yekta[1], F. Verdonck[1], W. Van Den Broeck[1], B.M. Goddeeris[1,2], E. Cox[1], D. Vanrompay[3]

[1]Faculty of Veterinary Medicine, Ghent University, Ghent, Belgium
[2]Faculty of Bioscience Engineering, Catholic University, Leuven, Belgium
[3]Faculty of Bioscience Engineering, Ghent University, Ghent, Belgium

ABSTRACT: Enterohemorrhagic *Escherichia coli* (EHEC) serotype O157:H7 strains are associated with haemorraghic 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

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 ± 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 anticancer 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 1×10^5 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, Claix, 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 (10^7 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 (OD_{600nm}) 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 CO_2 (CDP 030, Balzers, Sercolab), mounted on metal stubs, platinum- coated (JFC-1300 autofine coater, Jeol) and finally examined by a Jeal 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 $5 × 10^3$ 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 (10^7 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% CO_2. After infection for 4 h at 37 °C, non-adherent bacteria were removed by washing prepa-

rations three times with PBS. 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 phenylmethyl sulfonyl 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-
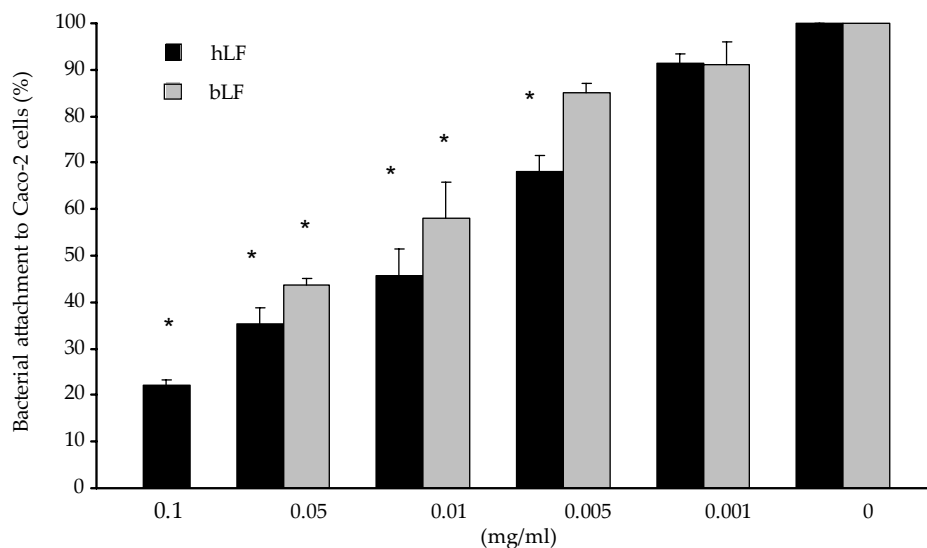

Figure 1. Lactoferrin significantly reduced *E. coli* O157:H7 attachment to Caco-2 cells. Results are represented as the mean values ± S.E.M (*n = 3*). Asterisks indicate statistically significant different between lactoferrins treated groups and the control (0 mg/ml lactoferrin) (*P* < 0.05)
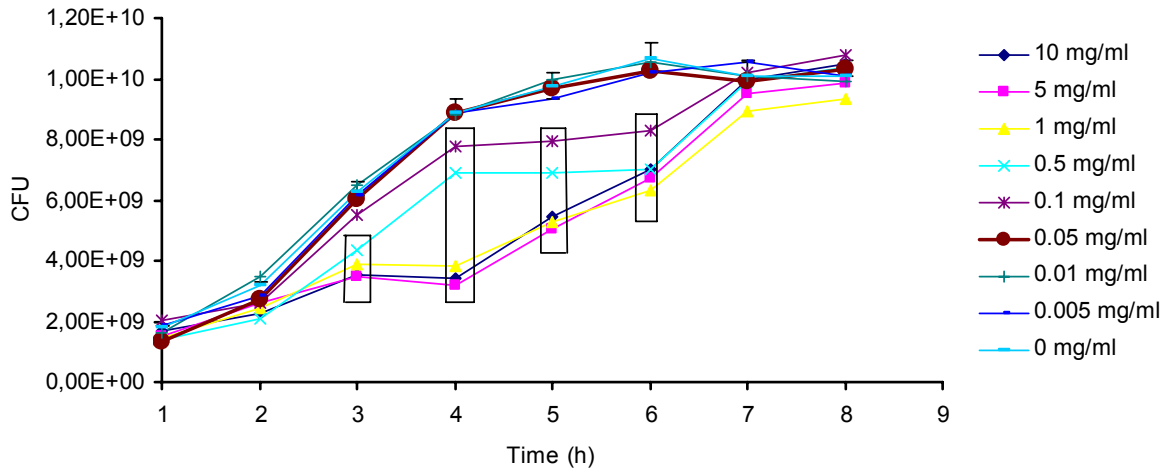
Figure 2. Inhibitory effect of bovine lactoferrin on the growth of *E. coli* O157:H7. The results are represented as the mean CFU ± S.E.M. (*n* = 3). Error bars are only shown for the 0.05 mg/ml bovine lactoferrin. The data in the rectangles are significantly different from the control (0 mg/ml bovine lactoferrin)

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 4×10^4
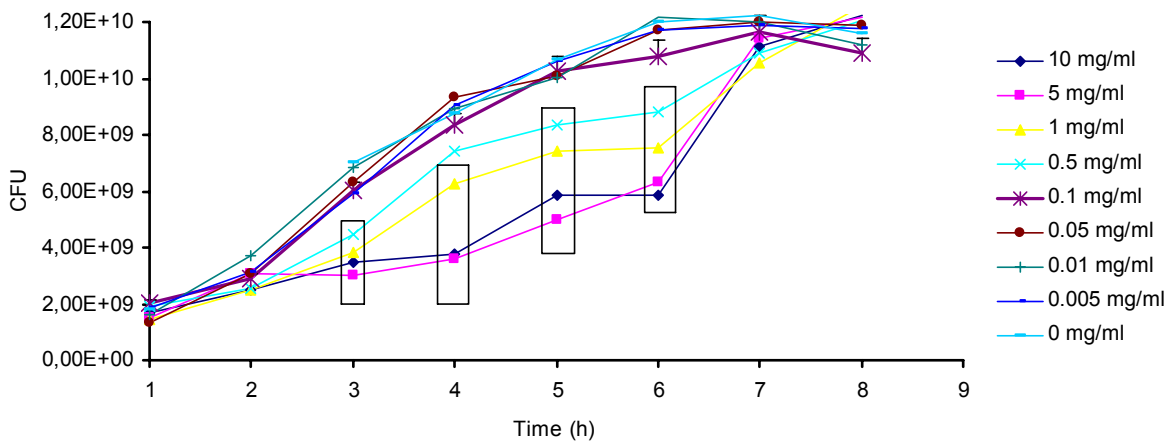

Figure 3. Inhibitory effect of human lactoferrin on the growth of *E. coli* O157:H7. The results are represented as the mean CFU ± S.E.M. (*n* = 3). Error bars are only shown for the 0.1 mg/ml human lactoferrin. The data in the rectangles are significantly different from the control (0 mg/ml human lactoferrin)
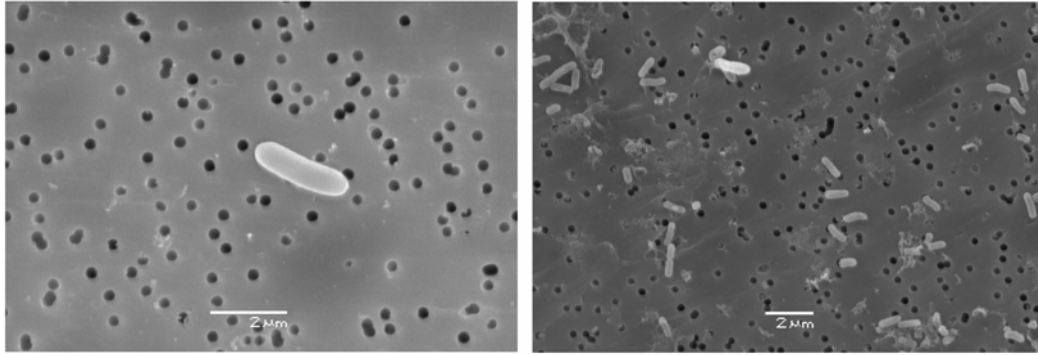
Figure 4. Scanning electron microscopy of *E. coli* O157:H7 after eight hours incubation with 10 mg/ml bovine lactoferrin (left) and no lactoferrin (right)

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 in-

timin. 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.
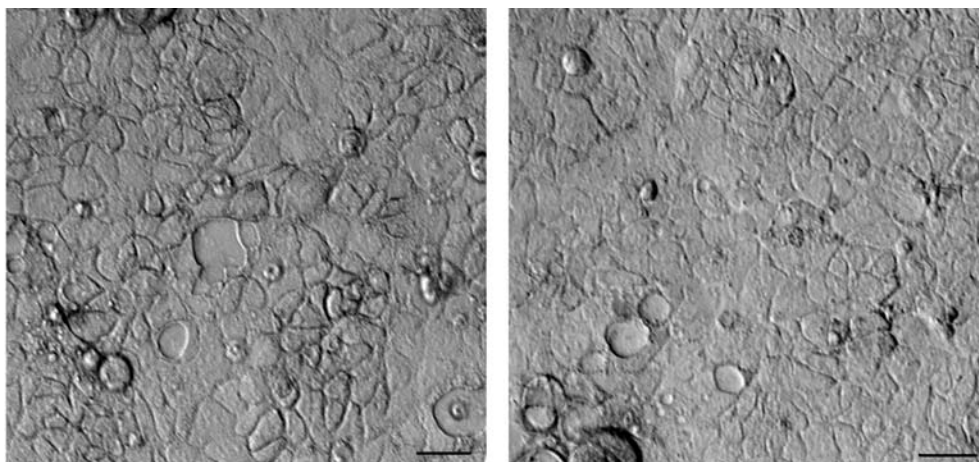

Figure 5. Light microscopic view of Caco-2 cells after four hours incubation with 0.1 mg/ml human lactoferrin (left) and no lactoferrin (right); bars represent 20 µm

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 lactoferrampin 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 (Chapple 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. Chapple et al. (2004) observed the same while studying the association of human lactoferricin 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. Ovotransferrin was more effective than human and bovine lactoferrin in preventing attachment and entry of *Chlamydophila 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 bLF 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

REFERENCES

Aguila A, Herrera AG, Morrison D, Cosgrove B, Perojo A, Montesinos I, Perez J, Sierra G, Gemmell CG, Brock JH (2001): Bacteriostatic activity of human lactoferrin against *Staphylococcus aureus* is a function of its iron-binding properties and is not influenced by antibiotic resistance. Fems Immunology and Medical Microbiology 31, 145–152.

Al-Nabulsi AA, Holley RA (2007): Effects on *Escherichia coli* O157:H7 and meat starter cultures of bovine lactoferrin in broth and microencapsulated lactoferrin in dry sausage batters. International Journal of Food Microbiology 113, 84–91.

Baker EN, Baker HM (2005): Molecular structure, binding properties and dynamics of lactoferrin. Cellular and Molecular Life Sciences 62, 2531–2539.

Baker EN, Baker HM (2009): A structural framework for understanding the multifunctional character of lactoferrin. Biochimie 91, 3–10.

Bavaro MF (2009): *Escherichia coli* O157: what every internist and gastroenterologist should know. Current Gastroenterology Reports 11, 301–306.

Beeckman DSA, Van Droogenbroeck C, De Cock BJA, Van Oostveldt P, Vanrompay DCG (2007): Effect of ovotransferrin and lactoferrins on *Chlamydophila psittaci* adhesion and invasion in HD11 chicken macrophages. Veterinary Research 38, 729–739.

Callaway TR, Carr MA, Edrington TS, Anderson RC, Nisbet DJ (2009): Diet, *Escherichia coli* O157:H7, and cattle: a review after 10 years. Current Issues in Molecular Biology 11, 67–79.

Chapple DS, Hussain R, Joannou CL, Hancock REW, Odell E, Evans RW, Siligardi G (2004): Structure and association of human lactoferrin peptides with *Escherichia coli* lipopolysaccharide. Antimicrobial Agents and Chemotherapy 48, 2190–2198.

Cookson AL, Woodward MJ (2003): The role of intimin in the adherence of enterohaemorrhagic *Escherichia coli* (EHEC) O157:H7 to HEp-2 tissue culture cells and to bovine gut explant tissues. International Journal of Medical Microbiology 292, 547–553.

Dibb-Fuller MP, Best A, Stagg DA, Cooley WA, Woodward MJ (2001): An *in-vitro* model for studying the

interaction of *Escherichia coli* O157: H7 and other enteropathogens with bovine primary cell cultures. Journal of Medical Microbiology 50, 759–769.

Dundas S, Todd WTA, Neill MA, Tarr PI (2005): Using antibiotics in suspected haemolytic-uraemic syndrome – Antibiotics should not be used in *Escherichia coli* O157:H7 infection. British Medical Journal 330, 1209–1209.

Ellison RT, Giehl TJ (1991): Killing of Gram negative bacteria by lactoferrin and lysozyme. Journal of Clinical Investigation 88, 1080–1091.

Ellison RT, Giehl TJ, Laforce FM (1988): Damage of the-outer membrane of enteric Gram negative bacteria by lactoferrin and transferrin. Infection and Immunity 56, 2774–2781.

Endo Y, Tsurugi K, Yutsudo T, Takeda Y, Ogasawara T, Igarashi K (1988): Site of action of a vero toxin (VT2) from *Escherichia coli* O157:H7 and of shiga toxin on eukaryotic ribosomes RNA N-glycosidase activity of the toxins.European Journal of Biochemistry 171, 45–50.

Fincher LM, Parker CD, Chauret CP (2009): Occurrence and Antibiotic Resistance of *Escherichia coli* O157:H7 in a Watershed in North-Central Indiana. Journal of Environmental Quality 38, 997–1004.

Frankel G, Phillips AD, Rosenshine I, Dougan G, Kaper JB, Knutton S (1998): Enteropathogenic and enterohaemorrhagic *Escherichia coli*: more subversive elements. Molecular Microbiology 30, 911–921.

Gomez HF, Ochoa TJ, Carlin LG, Cleary TG (2003): Human lactoferrin impairs virulence of *Shigella flexneri*. Journal of Infectious Diseases 187, 87–95.

Groenink J, Walgreen-Weterings E, van't Hof W, Veerman ECI, Amerongen AVN (1999): Cationic amphipathic peptides, derived from bovine and human lactoferrins, with antimicrobial activity against oral pathogens. FEMS Microbiology Letters 179, 217–222.

Haney EF, Nazmi K, Lau F, Bolscher JG , Vogel H J (2009): Novel lactoferrampin antimicrobial peptides derived from human lactoferrin. Biochimie 91, 141–154.

Izumikawa K, Hirakata Y, Yamaguchi T, Takemura H, Maesaki S, Tomono K, Igimi S, Kaku M, Yamada Y, Kohno S, Kamihira S (1998): *Escherichia coli* O157 interactions with human intestinal Caco-2 cells and the influence of fosfomycin. Journal of Antimicrobial Chemotherapy 42, 341–347.

Jenny M, Pedersen N , Hidayat BJ, Schennach H, Fuchs D (2010): Bovine colostrum modulates immune activation cascades in human peripheral blood mononuclear cells *in vitro*. New Microbiologica 33, 129–135.

Jerse AE, Yu J, Tall BD, Kaper JB (1990): A genetic locus of eneropathogenic *Escherichia coli* necessary for the production of attaching and effacing lesions on tissue culture cells. Proceedings of the National Academy of Sciences of the United States of America 87, 7839–7843.

Karpman D, Bekassy ZD, Sjogren A C, Dubois MS, Karmali MA, Mascarenhas M, Jarvis KG, Gansheroff LJ, O'Brien AD, Arbus GS, Kaper JB (2002): Antibodies to intimin and *Escherichia coli* secreted proteins A and B in patients with enterohemorrhagic *Escherichia coli* infections. Pediatric Nephrology 17, 201–211.

Kawasaki Y, Tazume S, Shimizu K, Matsuzawa H, Dosako S, Isoda H, Tsukiji M, Fujimura R, Muranaka Y, Isihida H (2000): Inhibitory effects of bovine lactoferrin on the adherence of enterotoxigenic *Escherichia coli* to host cells. Bioscience Biotechnology and Biochemistry 64, 348–354.

Mosmann T (1983): Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. Journal of Immunological Methods 16, 55–63.

Naidu SS, Erdei J, Czirok E, Kalfas S, Gado I, Thoren A, Forsgren A, Naidu AS (1991): Specific binding of lactoferrin to *Escherichia coli* isolated from human intestinal infections. Apmis 99, 1142–1150.

Nataro JP, Kaper JB (1998): Diarrheagenic *Escherichia coli*. Clinical Microbiology Reviews 11, 142–201.

Ochoa TJ, Cleary TG (2009): Effect of lactoferrin on enteric pathogens. Biochimie 91, 30–34.

Ochoa TJ, Noguera-Obenza M, Ebel F, Guzman CA, Gomez HF, Cleary TG (2003): Lactoferrin impairs type III secretory system function in enteropathogenic *Escherichia coli*. Infection and Immunity 71, 5149–5155.

Orsi N (2004): The antimicrobial activity of lactoferrin: Current status and perspectives. Biometals 17, 189–196.

Panos GZ, Betsi GI, Falagas ME (2006): Systematic review: are antibiotics detrimental or beneficial for the treatment of patients with *Escherichia coli* O157:H7 infection? Alimentary Pharmacology & Therapeutics 24, 731–742.

Safdar N, Said A, Gangnon RE, Maki DG (2002): Risk of hemolytic uremic syndrome after antibiotic treatment of *Escherichia coli* O157: H7 enteritis – A meta-analysis. Jama-Journal of the American Medical Association 288, 996–1001.

Schroeder CM, Zhao CW, DebRoy C, Torcolini J, Zhao SH, White DG, Wagner DD, McDermott PF, Walker RD, Meng JH (2002): Antimicrobial resistance of *Escherichia coli* O157 isolated from humans, cattle, swine, and food. Applied and Environmental Microbiology 68, 576–581.

Senkovich O, Cook WJ, Mirza S, Hollingshead SK, Protasevich II, Briles DE, Chattopadhyay D (2007): Structure of a complex of human lactoferrin N-lobe with pneumococcal surface protein A provides insight into microbial defense mechanism. Journal of Molecular Biology 370, 701–713.

Shimazaki K, Uji K, Tazume T, Kumura H, Shimo-Oka T (2000): Approach to identification and comparison of the heparin-interacting sites of lactoferrin using synthetic peptides. Lactoferrin: Structure, Function and Applications 1195, 37–46.

Shin K, Wakabayashi H, Yamauchi K, Yaeshima T, Iwatsuki K (2008): Recombinant human intelectin binds bovine lactoferrin and its peptides. Biological & Pharmaceutical Bulletin 31, 1605–1608.

Tsuda H, Kozu T, Iinuma G, Ohashi Y, Saito Y, Saito D, Akasu T, Alexander D B, Futakuchi M, Fukamachi K, Xu JG, Kakizoe T, Iigo M (2010): Cancer prevention by bovine lactoferrin: from animal studies to human trial. Biometals 23, 399–409.

Van der Kraan MIA, Van Marle J, Nazmi K, Groenink J, Van't Hof W, Veerman ECI, Bolscher J GM, Arnerongen AVN (2005): Ultrastructural effects of antimicrobial peptides from bovine lactoferrin on the membranes of *Candida albicans* and *Escherichia coli*. Peptides 26, 1537–1542.

Vandekerckhove A, Glorieux S, Van den Broeck W, Gryspeerdt A, Van der Meulen KM, Nauwynck HJ (2009): *In vitro* culture of equine respiratory mucosa explants. Veterinary Journal 181, 280–287.

Vilte DA, Larzabal M, Cataldi AA, Mercado EC (2008): Bovine colostrum contains immunoglobulin G antibodies against intimin, EspA, and EspB and inhibits hemolytic activity mediated by the type three secretion system of attaching and effacing *Escherichia coli*. Clinical and Vaccine Immunology 15, 1208–1213.

Vorland LH, Ulvatne H, Andersen J, Haukland HH, Rekdal O, Svendsen JS, Gutteberg TJ (1998): Lactoferricin of bovine origin is more active than lactoferricins of human, murine and caprine origin. Scandinavian Journal of Infectious Diseases 30, 513–517.

Xu XX, Jiang HR, Li HB, Zhang TN, Zhou Q, Liu N (2010): Apoptosis of stomach cancer cell SGC-7901 and regulation of Akt signaling way induced by bovine lactoferrin. Journal of Dairy Science 93, 2344–2350.

Corresponding Author:

Maryam Atef Yekta, Ghent University, Faculty of Veterinary Medicine, Laboratory of Immunology, Ghent, Belgium
Tel. +32 9 264 7339, E-mail: maryam.atefyekta@ugent.be