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Reduction of *Escherichia coli* O157:H7 excretion in sheep by oral lactoferrin administration

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23 Abstract

24 Ruminants are an important reservoir of *E. coli* O157:H7, therefore reducing *E. coli*
 25 O157:H7 excretion by these animals could play a key role in reducing human infections.
 26 The present study investigates the potential of bovine lactoferrin, a natural antimicrobial-
 27 immunomodulatory protein of milk, to prevent colonization and excretion of *E. coli*
 28 O157:H7 in sheep. The effect of two different doses of lactoferrin (1.5 g or 0.15 g per 12
 29 hours) was evaluated on colonization of sheep intestine and faecal excretion of the
 30 NCTC12900 strain. Hereto, lactoferrin was orally administered to sheep during 30
 31 consecutive days and sheep were experimentally infected with *E. coli* O157:H7 on the
 32 second day of the lactoferrin administration. Interestingly, both lactoferrin dosages
 33 significantly reduced the number of *E. coli* O157:H7 in faeces as well as the duration of
 34 faecal excretion. The high dose group showed a significantly higher antibody response
 35 against EspA and EspB, two structural proteins of the bacterial type III secretion system
 36 (TTSS), than the *colonization control group*. The results suggest that oral lactoferrin
 37 administration could be used to prevent persistent colonization of sheep with *E. coli*
 38 O157:H7.

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43 Key words

44 Lactoferrin, *E. coli* O157:H7, sheep

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1. Introduction

Enterohemorrhagic *Escherichia coli* (EHEC) are zoonotic pathogens associated with haemorrhagic colitis (HC) and haemolytic uremic syndrome (HUS) in humans (Mead and Griffin, 1998). The main EHEC serotype responsible for these clinical signs is *E. coli* O157:H7 (Nataro and Kaper, 1998). Ruminants are the main reservoir of this microorganism which normally resides in their gut without causing apparent illness (Besser *et al.*, 1999). Many human *E. coli* O157:H7 infections originate, either directly or indirectly via contaminated food or water, from exposure to ruminant's feces. Therefore a key step towards protecting humans from *E. coli* O157:H7 infection could be the control and/or prevention of *E. coli* O157:H7 colonization of the ruminant's intestine. Several approaches have been suggested with variable success including vaccination, probiotic and antibiotic treatment and even diet management (Molbak *et al.*, 2002; Potter *et al.*, 2004; Callaway *et al.*, 2004, 2009). Although some of these approaches seem promising, none of them stop bacterial excretion for a 100 %.

Milk feeding protects young mammals from intestinal infections. This protection is attributed to the multiple anti-microbial, anti-inflammatory and immunomodulatory factors present in milk of which lactoferrin (LF) is one of the most important (Gallois *et al.*, 2007; Walker 2010). This molecule which is also present in mucosal secretions like tears, saliva and vaginal and airway surface fluid (Travis *et al.*, 1999; Gonzalez-Chavez *et al.*, 2009), is involved in host defense against pathogenic bacteria, fungi and protozoa, both directly and through regulation of the inflammatory response (Vorland, 1999; Tian *et al.*, 2010). Previously we showed that lactoferrin reduces *E. coli* O157:H7 growth and its attachment to epithelial cells *in vitro* (Atef Yekta *et al.*, 2010). This reduction is at

least partly due to a proteolytic effect of lactoferrin on the bacterial type III secretion system (TTSS) proteins such as EspA and EspB. This effect has also been shown on other bacteria (EPEP)(Ochoa *et al.*, 2003; Ashida *et al.*, 2004). However we could not show proteolytic effect of lactoferrin on bacterial protein intimin. The potential for using oral lactoferrin in animals has been validated with studies that showed no toxic effect attributed to oral delivery of lactoferrin in rats (Appel *et al.*, 2006).

Many studies on the control of *E. coli* O157:H7 focus on cattle, but sheep are also an important well-established model (Vande Walle *et al.*, 2010, a b, Woodward *et al.*, 2003). The aim of the present study was to investigate the potential of lactoferrin to prevent *E. coli* O157:H7 excretion in a sheep model.

2. Materials and methods

2.1. Bacterial inoculum

NCTC12900, a well characterized Shiga toxin (Stx) negative, nalidixic acid (Nal) resistant *E. coli* O157:H7 strain was kindly provided by Prof. M. Woodward (Woodward *et al.*, 2003). A Stx negative strain was used since this allowed us to perform experiments in A2 isolation units. Bacteria were grown overnight in Luria Bertani broth (LB) at 37°C while shaking (200 rpm), centrifuged (550 g, 10 min, 4°C) and subsequently re-suspended in sterile phosphate-buffered saline (PBS) to a concentration of 10^{10} CFU.

2.2. Lactoferrin

Non-iron-saturated bovine lactoferrin (Ingredia nutritional, France) with 90% purity was used.

2.3. Animals

Seventeen 3-month-old male sheep (Belgian cross-breed, Zootechnical Centre, Leuven, Belgium) were used in this study. The faeces of these sheep were free of *E. coli* O157:H7 as demonstrated by immunomagnetic separation (IMS) and culturing (procedure described further). Animals were seronegative for antibodies against EspA, EspB and intimin, as determined by ELISA (described further). Selected animals were allowed to acclimatise for one week after arrival in our animal facility. Sheep were housed in groups of 8, 7 and 2 animals per pen and received grain-based pellets and water ad libitum.

2.4. Experimental procedures

Five animals received lactoferrin at high dose (1.5 g per 12 hours) (*high LF group*) and three animals at a 10 times lower dose (0.15 g per 12 hours) (*low LF group*) for 30 days. Animals were housed in the same pen as group 1 (n = 8). Lactoferrin was given orally to group 1 in a volume of 10 ml sodium bicarbonate buffer (10%) via a syringe allowing the sheep to drink the solution. The *colonization control* group 2 (n = 7) and the *LF control group* 3 (n = 2) received the sodium bicarbonate buffer or the high lactoferrin dose, respectively (Table 1). The *LF control group* allowed monitoring for visual side effects of the lactoferrin administration. After 1 day of lactoferrin administration, groups 1 and 2 received 10^{10} *E. coli* O157:H7 in 10 ml PBS orally for 2 consecutive days, whereas the *LF control group* 3 received PBS only.

Excretion of *E. coli* O157:H7 was monitored twice a week. Blood was collected weekly from the *vena jugularis* to test for serum antibodies against intimin, EspA and

EspB. All animal experiments were approved by the ethical committee of the Faculty of Veterinary Medicine (approval 2009/074).

2.5. Monitoring of *E. coli* O157:H7 excretion

Faecal excretion of *E. coli* O157:H7 was monitored as described by Vande Walle *et al.*, (2010 a), who formerly demonstrated the reproducibility of the *E. coli* O157:H7 excretion pattern in this sheep model. Briefly, 10 g faeces were diluted in modified tryptone soy broth (Oxoid Ltd, Hanst, UK) supplemented with 20 mg/ml novobiocin and subsequently homogenized using a stomacher. Ten-fold serial dilutions were spread-plated onto MacConkey agar plates supplemented with sorbitol, cefixime and tellurite and Nal (NalCT-SMAC) (MERCK, Darmstadt, Germany). Remaining broth was enriched for 6h at 42°C and subjected to immuno-magnetic separation (IMS) with O157 Dynabeads® (Invitrogen, Merelbeke, Belgium) according to the manufacturer's instructions. Finally, 100 µl was plated onto NalCT-SMAC. Colonies were confirmed to be *E. coli* O157 by a latex agglutination test (Oxoid Ltd, Basingstoke, UK). Colony counts were log10 transformed for data analysis. If *E. coli* O157 was not detected by direct plating, but only detected by enrichment, a concentration of 10 CFU/g was assigned (Vande Walle *et al.*, 2010 a). Excretion results were considered negative after 2 successive negative IMS results.

2.6. Serum antibody response against virulence factors of *E. coli* O157:H7

As described by Vande Walle *et al.*, (2010 a), sera were tested for the presence of antibodies against the following *E. coli* O157:H7 virulence factors: intimin, EspA and

EspB. Briefly, sera were heat-inactivated (30 min. at 56°C) and kaolin-treated. Polysorb 96-well plates (NUNC, Polysorb Immuno Plates, Roskilde, Denmark) were coated with 200 ng/well of recombinant intimin, EspA or EspB in PBS for 2h at 37°C and subsequently blocked overnight at 4°C using PBS + 0.2% Tween[®]80. After washing with PBS + 0.2% Tween[®]20, plates were incubated with two-fold serial dilutions of serum in PBS + 0.05% Tween[®]20 and with HRP-conjugated anti-sheep IgG-specific donkey antibodies (AbD Serotec, UK). Sera of sheep intramuscularly immunized with intimin, EspA and EspB during a former study (Vande Walle *et al.*, 2010 a), served as positive control. The cut-off values were calculated as the mean OD₄₀₅-values of all sera (dilution 1/10) obtained at day 0 increased with three times the standard deviation (cut-off values for intimin, EspA and EspB were 0.387, 0.412 and 0.312, respectively).

2.7. Statistical analysis

Statistical analysis was performed using SAS software version 1999. The Proc MIXED test was used to analyze bacterial excretion. The statistical model included the fixed effect of LF dose (low, high), the random effect of sheep nested within treatment and the residual errors. The duration of the excretion was compared in three groups of infected animals by using the t- test. Serum antibody titres were statistically analyzed by use of a General linear Model (repeated measures analysis of variance). Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Effect of lactoferrin on faecal *E. coli* O157:H7 excretion in sheep

In the present study, eight sheep receiving lactoferrin (five the high dose and three the low dose) in sodium bicarbonate buffer during 30 days and seven sheep receiving only the buffer, were inoculated on the second and third day of the lactoferrin administration with *E. coli* O157:H7. Four days after the first inoculation, animals in the *colonization control* group shed between 10^5 and 10^8 CFU *E. coli* O157:H7/g faeces with an average of 7×10^7 CFU/g (Fig 1). Subsequently, the number of excreted bacteria declined gradually and five of seven animals stopped shedding between 18 and 28 days post inoculation (PI). The two remaining animals excreted *E. coli* O157:H7 till the end of the experiment (day 28 PI).

In the *high LF* group, reduced faecal shedding was observed from day four onwards. On day seven, two animals already ceased excreting *E. coli* O157:H7. One week later, the faeces of two additional animals became negative (day 14 PI), while bacterial excretion in the single remaining animal ceased at day 21 PI. The shedding period for the *high LF* group (12.6 ± 1.17 days (mean $\pm$ SEM)) was significantly shorter than for the *colonization control* group (24.71 ± 0.68 days (mean $\pm$ SEM)) ($p < 0.05$).

Reduced faecal shedding was observed in the *LF low* group from day four PI onwards, however this reduction was only significant on day 4 and 7 PI. Day 14, one of the animals stopped excreting and day 17 the two remaining animals became negative. Nevertheless, during the second week of the experiment the number of bacteria excreted by the *low LF* group was higher than for the *high LF* group. Differences between the *high LF* and *low LF* groups were not significant (Fig 1). However, again the duration of

excretion (16.66 ± 0.76 days (mean $\pm$ SEM)) was significantly shortened in comparison with the *colonization control* group ($p < 0.05$), but not in comparison with the *high LF* group. The *LF control* group remained negative throughout the experiment.

3.2. Effect of lactoferrin on the IgG response against *E. coli* O157:H7 antigens

Since lactoferrin has been described to modulate the adaptive immunity (Legrand and Mazurier, 2010), the effect of oral lactoferrin treatment on the serum antibody response against virulence factors of *E. coli* O157:H7 including EspA, EspB and intimin was determined (Fig. 2). Two weeks after the experimental inoculation, the *colonization control* group, receiving *E. coli* O157:H7 only, showed a very low serum IgG response against intimin, EspA and EspB with maximal $\log_2$ titres of 4.54, 4.05 and 4.62, respectively. A similar response was observed in the *low LF* group. However, for the *high LF* group, the IgG response against EspA and EspB significantly ($p < 0.05$) increased with a peak at 2 and 3 weeks post infection for EspB and EspA, respectively. The IgG response against intimin did not significantly raised, as compared to the *LF control* and the *colonization control* group.

4. Discussion

Escherichia coli O157:H7 colonizes the intestinal tract of ruminants, making them the main reservoir for this human pathogen (Dean-Nystrom *et al.*, 1999; La Ragione *et al.*, 2009). Therefore, reducing or eliminating *E. coli* O157:H7 excretion by ruminants is important to decrease the rate of human infection.

Several strategies have been described to reduce excretion such as isolating animals which excrete more than 10^4 CFU/g *E. coli* O157:H7, the so called "super shedders" (Stephens *et al.*, 2008; Stephens *et al.*, 2009), vaccination (Potter *et al.*, 2004; Peterson *et al.*, 2007), antibiotic treatment (Molbak *et al.*, 2002), probiotics (Callaway *et al.*, 2004), bacteriophages (Callaway *et al.*, 2008) and dietary changes (Callaway *et al.*, 2009). However, continued research for effective pre-harvest interventions is needed.

Lactoferrin has a direct antimicrobial effect on *E. coli* O157:H7 (Atef Yekta *et al.*, 2010) and is responsible for the proteolytic degradation of EspA and EspB (Atef Yekta *et al.*, 2010). In addition, lactoferrin is an immunomodulatory protein (Legrand and Mazurier, 2010). These findings resulted in the following hypothesis: lactoferrin might be used to reduce *E. coli* O157:H7 colonization of the intestinal tract and consequently, faecal shedding by sheep.

Previously, we developed an oral infection model in sheep creating persistent *E. coli* O157:H7 shedders (Vande Walle *et al.*, 2010). At present, the model was used to study the effect of the natural antimicrobial-immunomodulatory milk protein lactoferrin on *E. coli* O157:H7 excretion. Moreover, the sheep infection model was used to examine the antibody responses against intimin and the *E. coli* O157:H7 type III secretion proteins EspA and EspB.

In our study, lactoferrin was administered in bicarbonate buffer. The buffer closes the esophageal groove, so that lactoferrin passes rumen, reticulum and omasum and directly reaches the abomasum (Rosenberger, 1979). Delivering lactoferrin in the abomasum will prevent its bacterial degradation in rumen and reticulum and will enhance its degradation by pepsin. Pepsin cuts lactoferrin in different peptides of which one peptide, lactoferricin,

has been demonstrated to exert a stronger bactericidal, immunomodulatory and inflammatory effect than lactoferrin (Gifford *et al.*, 2005). Once lactoferrin reaches the small intestine it probably is only slowly degraded, since it is strongly resistant to trypsin and trypsin-like enzymes (Brines and Brock, 1983). Whether lactoferricin shows a similar resistance, has not been tested yet.

Both lactoferrin dosages were capable to significantly reduce the concentration of *E. coli* O157:H7 in the faeces and to shorten the duration of faecal excretion, indicating that sufficient functional active lactoferrin reaches the intestine to interfere with bacterial colonization. Interestingly, sheep, which received the high dose of lactoferrin, showed a higher antibody response against EspA and EspB in comparison to the control group. The peak of the antibody response was 2 to 3 weeks post inoculation, when the excretion of *E. coli* O157:H7 had completely ceased. A similar increase in antibody response was observed in chickens orally fed lactoferrin from birth on and orally vaccinated with an infectious bursal disease (Gumboro) vaccine at 1 and 3 weeks of age (Hung *et al.*, 2010). As in our study, this effect was observed with the highest dose. Furthermore, a significant increase in total serum IgA and IgG and an increased mitogen-induced proliferation of peripheral blood lymphocytes was demonstrated. Studies in mice demonstrated increases in total antibody, B-, T- and NK-cells amounts (Teraguchi *et al.*, 2004; Varadhachary *et al.*, 2004; Wolf *et al.*, 2007). In humans, oral supplementation resulted in an enhanced T cell activation, but no effects on B-cells or antibody production have been described (Mulder *et al.*, 2008).

Lactoferrin has important immunomodulatory activities such as increased maturation of B- and T-lymphocytes and increased recruitment and maturation of antigen-presenting

cells which occur by influencing pattern recognition receptor-mediated cell signaling (Curran *et al.*, 2006; De la Rosa *et al.*, 2008; Legrand and Mazurier, 2010). Whether such effects are responsible for the increased antibody response in our study still has to be determined. In this study, we used an Stx-negative strain and there is evidence that infections with STEC can suppress the development of specific cellular immune responses in cattle (Hoffman *et al.*, 2007). Stx could counteract the immuno-modulatory effects of lactoferrin. This will be examined in a future experiment in biosafety level 3 large animal facilities.

5. Conclusion

This is the first study demonstrating the reduction of *E. coli* O157:H7 excretion by oral administration of the natural antimicrobial protein, lactoferrin. Moreover, the results suggest that lactoferrin could become an important tool to decrease colonization pressure on farms and to prevent contamination of food by *E. coli* O157:H7 and, consequently to decrease *E. coli* O157:H7 associated illness in humans. However, more convenient lactoferrin administration methods need to be evaluated.

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397 Figure 1. Effect of lactoferrin administration on faecal excretion of *E. coli* O157:H7 by
 398 sheep. Results are presented as the mean $\log_{10}$ values of colony forming units (CFU)/g
 399 faeces $\pm$ SEM. * Significant differences ($p < 0.05$) between the *colonization control*
 400 group and the *high LF* group are indicated by $\times$. Significant differences ($p < 0.05$)
 401 between the *colonization control* group and the *low LF* group are indicated by +.
 402 Differences between the *high LF* and *low LF* groups were not significant.

403

404 Figure 2. Mean serum antibody responses in the groups infected with *E. coli* O157:H7.
 405 Results are presented as the mean $\log_2$ values of antigen-specific IgG titers. DPI: days
 406 post inoculation. Significant differences ($p < 0.05$) between the *colonization control*
 407 group and the *high LF* group are marked with $\times$. For EspA, the observed difference
 408 between the *low LF* group and the *colonization control* group was not significant.

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Table 1: Experimental set-up

Group number	N	Manipulation	<i>E. coli</i> O157:H7	Lactoferrin (LF) for 30 days
1	5	High LF	+	1.5 g/per 12 hours (high dose)
1	3	Low LF	+	0.15 g/per 12 hours (low dose)
2	7	Colonization control	+	-
3	2	LF control	-	1.5 g/per 12 hours (high dose)