

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

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 2 Reduction of <i>Escherichia coli</i> O157:H7 excretion in sheep by oral lactofer 3 administration 	rin
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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 (TTSS), than the colonization control group. The results suggest that oral lactoferrin 36 37 administration could be used to prevent persistent colonization of sheep with E. coli 38 O157:H7. 39

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- 42

43 Key words

44 Lactoferrin, E. coli O157:H7, sheep

46 1. Introduction

47 Enterohemorrhagic Escherichia coli (EHEC) are zoonotic pathogens associated with **48** haemorrhagic colitis (HC) and haemolytic uremic syndrome (HUS) in humans (Mead and 49 Griffin, 1998). The main EHEC serotype responsible for these clinical signs is E. coli 50 O157:H7 (Nataro and Kaper, 1998). Ruminants are the main reservoir of this 51 microorganism which normally resides in their gut without causing apparent illness 52 (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 53 54 key step towards protecting humans from *E. coli* O157:H7 infection could be the control 55 and/or prevention of E. coli O157:H7 colonization of the ruminant's intestine. Several 56 approaches have been suggested with variable success including vaccination, probiotic and antibiotic treatment and even diet management (Molbak et al., 2002; Potter et al., 57 58 2004; Callaway et al., 2004, 2009). Although some of these approaches seem promising, 59 none of them stop bacterial excretion for a 100 %.

60 Milk feeding protects young mammals from intestinal infections. This protection is 61 attributed to the multiple anti-microbial, anti-inflammatory and immunomodulatory 62 factors present in milk of which lactoferrin (LF) is one of the most important (Gallois et 63 al., 2007; Walker 2010). This molecule which is also present in mucosal secretions like 64 tears, saliva and vaginal and airway surface fluid (Travis et al., 1999; Gonzalez-Chavez 65 et al., 2009), is involved in host defense against pathogenic bacteria, fungi and protozoa, 66 both directly and through regulation of the inflammatory response (Vorland, 1999; Tian **67** 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 **68**

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.

79

80 2. Materials and methods

81 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 resuspended in sterile phosphate-buffered saline (PBS) to a concentration of 10¹⁰ CFU.

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89 2.2. Lactoferrin

90 Non-iron-saturated bovine lactoferrin (Ingredia nutritional, France) with 90% purity91 was used.

92 2.3. Animals

93 Seventeen 3-month-old male sheep (Belgian cross-breed, Zootechnical Centre, 94 Leuven, Belgium) were used in this study. The faeces of these sheep were free of E. coli 95 O157:H7 as demonstrated by immunomagnetic separation (IMS) and culturing 96 (procedure described further). Animals were seronegative for antibodies against EspA, 97 EspB and intimin, as determined by ELISA (described further). Selected animals were 98 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 99 100 ad libitum.

101

102 *2.4. Experimental procedures*

103 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 30 104 105 days. Animals were housed in the same pen as group 1 (n = 8). Lactoferrin was given 106 orally to group 1 in a volume of 10 ml sodium bicarbonate buffer (10%) via a syringe 107 allowing the sheep to drink the solution. The *colonization control* group 2 (n = 7) and the 108 *LF control group* 3 (n = 2) received the sodium bicarbonate buffer or the high lactoferrin 109 dose, respectively (Table 1). The LF control group allowed monitoring for visual side 110 effects of the lactoferrin administration. After 1 day of lactoferrin administration, groups 1 and 2 received 10¹⁰ E. coli O157:H7 in 10 ml PBS orally for 2 consecutive days, 111 112 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

- **115** EspB. All animal experiments were approved by the ethical committee of the Faculty of
- **116** Veterinary Medicine (approval 2009/074).
- 117
- **118** 2.5. Monitoring of E. coli O157:H7 excretion

119 Faecal excretion of E. coli O157:H7 was monitored as described by Vande Walle et 120 al., (2010 a), who formerly demonstrated the reproducibility of the E. coli O157:H7 121 excretion pattern in this sheep model. Briefly, 10 g faeces were diluted in modified 122 tryptone soy broth (Oxoid Ltd, Hanst, UK) supplemented with 20 mg/ml novobiocin and 123 subsequently homogenized using a stomacher. Ten-fold serial dilutions were spread-124 plated onto MacConkey agar plates supplemented with sorbitol, cefixime and tellurite and Nal (NalCT-SMAC) (MERCK, Darmstadt, Germany). Remaining broth was 125 126 enriched for 6h at 42°C and subjected to immuno-magnetic separation (IMS) with O157 Dynabeads[®] (Invitrogen, Merelbeke, Belgium) according to the manufacturer's 127 128 instructions. Finally, 100 µl was plated onto NalCT-SMAC. Colonies were confirmed to 129 be E. coli O157 by a latex agglutination test (Oxoid Ltd, Basingstoke, UK). Colony 130 counts were log10 transformed for data analysis. If E. coli O157 was not detected by 131 direct plating, but only detected by enrichment, a concentration of 10 CFU/g was 132 assigned (Vande Walle et al., 2010 a). Excretion results were considered negative after 2 133 successive negative IMS results.

134

135 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

138 EspB. Briefly, sera were heat-inactivated (30 min. at 56°C) and kaolin-treated. Polysorb 139 96-well plates (NUNC, Polysorb Immuno Plates, Roskilde, Denmark) were coated with 140 200 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 141 142 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 143 144 donkey antibodies (AbD Serotec, UK). Sera of sheep intramuscularly immunized with 145 intimin, EspA and EspB during a former study (Vande Walle *et al.*, 2010 a), served as 146 positive control. The cut-off values were calculated as the mean OD_{405} -values of all sera 147 (dilution 1/10) obtained at day 0 increased with three times the standard deviation (cut-148 off values for intimin, EspA and EspB were 0.387, 0.412 and 0.312, respectively).

149

150 2.7. *Statistical analysis*

151 Statistical analysis was performed using SAS software version 1999. The Proc 152 MIXED test was used to analyze bacterial excretion. The statistical model included the 153 fixed effect of LF dose (low, high), the random effect of sheep nested within treatment 154 and the residual errors. The duration of the excretion was compared in three groups of 155 infected animals by using the t- test. Serum antibody titres were statistically analyzed by 156 use of a General linear Model (repeated measures analysis of variance). Differences were 157 considered statistically significant at p < 0.05.

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162 3. Results

163 3.1. Effect of lactoferrin on faecal E. coli O157:H7 excretion in sheep

164 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 165 166 the buffer, were inoculated on the second and third day of the lactoferrin administration 167 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 168 of $7x10^7$ CFU/g (Fig 1). Subsequently, the number of excreted bacteria declined 169 170 gradually and five of seven animals stopped shedding between 18 and 28 days post 171 inoculation (PI). The two remaining animals excreted E. coli O157:H7 till the end of the 172 experiment (day 28 PI).

173 In the *high LF* group, reduced faecal shedding was observed from day four onwards. 174 On day seven, two animals already ceased excreting *E. coli* O157:H7. One week later, the 175 faeces of two additional animals became negative (day 14 PI), while bacterial excretion 176 in the single remaining animal ceased at day 21 PI. The shedding period for the *high LF* 177 group (12.6 \pm 1.17 days (mean \pm SEM)) was significantly shorter than for the 178 *colonization control* group (24.71 \pm 0.68 days (mean \pm SEM)) (p < 0.05).

179 Reduced faecal shedding was observed in the *LF low* group from day four PI
180 onwards, however this reduction was only significant on day 4 and 7 PI. Day 14, one of
181 the animals stopped excreting and day 17 the two remaining animals became negative.
182 Nevertheless, during the second week of the experiment the number of bacteria excreted
183 by the *low LF* group was higher than for the *high LF* group. Differences between the *high*184 *LF* and *low LF* groups were not significant (Fig 1). However, again the duration of

185 excretion (16.66 \pm 0.76 days (mean \pm SEM)) was significantly shortened in comparison 186 with the *colonization control* group (p < 0.05), but not in comparison with the high LF 187 group. The *LF control* group remained negative throughout the experiment. 188 189 3.2. Effect of lactoferrin on the IgG response against *E. coli* O157:H7 antigens 190 Since lactoferrin has been described to modulate the adaptive immunity (Legrand and 191 Mazurier, 2010), the effect of oral lactoferrin treatment on the serum antibody response 192 against virulence factors of E. coli O157:H7 including EspA, EspB and intimin was 193 determined (Fig. 2). Two weeks after the experimental inoculation, the *colonization* 194 control group, receiving E. coli O157:H7 only, showed a very low serum IgG response 195 against intimin, EspA and EspB with maximal log₂ titres of 4.54, 4.05 and 4.62, 196 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) 197 198 increased with a peak at 2 and 3 weeks post infection for EspB and EspA, respectively.

199 The IgG response against intimin did not significantly raised, as compared to the *LF*200 *control* and the *colonization control* group.

201

202 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.

207 Several strategies have been described to reduce excretion such as isolating animals
208 which excrete more than 10⁴ CFU/g *E. coli* O157:H7, the so called "super shedders"
209 (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),
211 bacteriophages (Callaway *et al.*, 2008) and dietary changes (Callaway *et al.*, 2009).
212 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.

235 Both lactoferrin dosages were capable to significantly reduce the concentration of E. 236 *coli* O157:H7 in the faeces and to shorten the duration of faecal excretion, indicating that 237 sufficient functional active lactoferrin reaches the intestine to interfere with bacterial 238 colonization. Interestingly, sheep, which received the high dose of lactoferrin, showed a 239 higher antibody response against EspA and EspB in comparison to the control group. The 240 peak of the antibody response was 2 to 3 weeks post inoculation, when the excretion of E. 241 coli O157:H7 had completely ceased. A similar increase in antibody response was 242 observed in chickens orally fed lactoferrin from birth on and orally vaccinated with an 243 infectious bursal disease (Gumboro) vaccine at 1 and 3 weeks of age (Hung et al., 2010). 244 As in our study, this effect was observed with the highest dose. Furthermore, a significant 245 increase in total serum IgA and IgG and an increased mitogen-induced proliferation of 246 peripheral blood lymphocytes was demonstrated. Studies in mice demonstrated increases 247 in total antibody, B-, T- and NK-cells amounts (Teraguchi et al., 2004; Varadhachary et 248 al., 2004; Wolf et al., 2007). In humans, oral supplementation resulted in an enhanced T 249 cell activation, but no effects on B-cells or antibody production have been described 250 (Mulder et al., 2008).

251 Lactoferrin has important immunomodulatory activities such as increased maturation of252 B- and T-lymphocytes and increased recruitment and maturation of antigen-presenting

253 cells which occur by influencing pattern recognition receptor-mediated cell signaling 254 (Curran et al., 2006; De la Rosa et al., 2008; Legrand and Mazurier, 2010). Whether such 255 effects are responsible for the increased antibody response in our study still has to be 256 determined. In this study, we used an Stx-negative strain and there is evidence that 257 infections with STEC can suppress the development of specific cellular immune 258 responses in cattle (Hoffman et al., 2007). Stx could counteract the immuno-modulatory 259 effects of lactoferrin. This will be examined in a future experiment in biosafety level 3 260 large animal facilities.

261

262 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.

269

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- 280

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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 ×. 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.

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404Figure 2. Mean serum antibody responses in the groups infected with *E. coli* O157:H7.405Results are presented as the mean log_2 values of antigen-specific IgG titers. DPI: days406post inoculation. Significant differences (p < 0.05) between the *colonization control*407group and the *high LF* group are marked with ×. For EspA, the observed difference408between 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)	



