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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

45

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
53 indirectly via contaminated food or water, from exposure to ruminant's feces. Therefore a
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
57 and antibiotic treatment and even diet management (Molbak *et al.*, 2002; Potter *et al.*,
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
68 its attachment to epithelial cells *in vitro* (Atef Yekta *et al.*, 2010). This reduction is at

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

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

79

80 **2. Materials and methods**

81 *2.1. Bacterial inoculum*

82 NCTC12900, a well characterized Shiga toxin (Stx) negative, nalidixic acid (Nal)
83 resistant *E. coli* O157:H7 strain was kindly provided by Prof. M. Woodward (Woodward
84 *et al.*, 2003). A Stx negative strain was used since this allowed us to perform experiments
85 in A2 isolation units. Bacteria were grown overnight in Luria Bertani broth (LB) at 37°C
86 while shaking (200 rpm), centrifuged (550 g, 10 min, 4°C) and subsequently re-
87 suspended in sterile phosphate-buffered saline (PBS) to a concentration of 10¹⁰ CFU.

88

89 *2.2. Lactoferrin*

90 Non-iron-saturated bovine lactoferrin (Ingredia nutritional, France) with 90% purity
91 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
99 housed in groups of 8, 7 and 2 animals per pen and received grain-based pellets and water
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*)
104 and three animals at a 10 times lower dose (0.15 g per 12 hours) (*low LF group*) for 30
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
111 1 and 2 received 10^{10} *E. coli* O157:H7 in 10 ml PBS orally for 2 consecutive days,
112 whereas the *LF control group* 3 received PBS only.

113 Excretion of *E. coli* O157:H7 was monitored twice a week. Blood was collected
114 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
125 and Nal (NalCT-SMAC) (MERCK, Darmstadt, Germany). Remaining broth was
126 enriched for 6h at 42°C and subjected to immuno-magnetic separation (IMS) with O157
127 Dynabeads[®] (Invitrogen, Merelbeke, Belgium) according to the manufacturer's
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

136 As described by Vande Walle *et al.*, (2010 a), sera were tested for the presence of
137 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
141 subsequently blocked overnight at 4°C using PBS + 0.2% Tween[®]80. After washing
142 with PBS + 0.2% Tween[®]20, plates were incubated with two-fold serial dilutions of
143 serum in PBS + 0.05% Tween[®]20 and with HRP-conjugated anti-sheep IgG-specific
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 OD₄₀₅-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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161

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
165 low dose) in sodium bicarbonate buffer during 30 days and seven sheep receiving only
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*
168 *control* group shed between 10^5 and 10^8 CFU *E. coli* O157:H7/g faeces with an average
169 of 7×10^7 CFU/g (Fig 1). Subsequently, the number of excreted bacteria declined
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 ± 1.17 days (mean \pm SEM)) was significantly shorter than for the
178 *colonization control* group (24.71 ± 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 ± 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 \log_2 titres of 4.54, 4.05 and 4.62,
196 respectively. A similar response was observed in the *low LF* group. However, for the
197 *high LF* group, the IgG response against EspA and EspB significantly ($p < 0.05$)
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

203 *Escherichia coli* O157:H7 colonizes the intestinal tract of ruminants, making them the
204 main reservoir for this human pathogen (Dean-Nystrom *et al.*, 1999; La Ragione *et al.*,
205 2009). Therefore, reducing or eliminating *E. coli* O157:H7 excretion by ruminants is
206 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 10^4 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*
210 *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.

213 Lactoferrin has a direct antimicrobial effect on *E. coli* O157:H7 (Atef Yekta *et al.*,
214 2010) and is responsible for the proteolytic degradation of EspA and EspB (Atef Yekta *et*
215 *al.*, 2010). In addition, lactoferrin is an immunomodulatory protein (Legrand and
216 Mazurier, 2010). These findings resulted in the following hypothesis: lactoferrin might be
217 used to reduce *E. coli* O157:H7 colonization of the intestinal tract and consequently,
218 faecal shedding by sheep.

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

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

230 has been demonstrated to exert a stronger bactericidal, immunomodulatory and
231 inflammatory effect than lactoferrin (Gifford *et al.*, 2005). Once lactoferrin reaches the
232 small intestine it probably is only slowly degraded, since it is strongly resistant to trypsin
233 and trypsin-like enzymes (Brines and Brock, 1983). Whether lactoferrin shows a
234 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 of
252 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

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

269

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396

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.

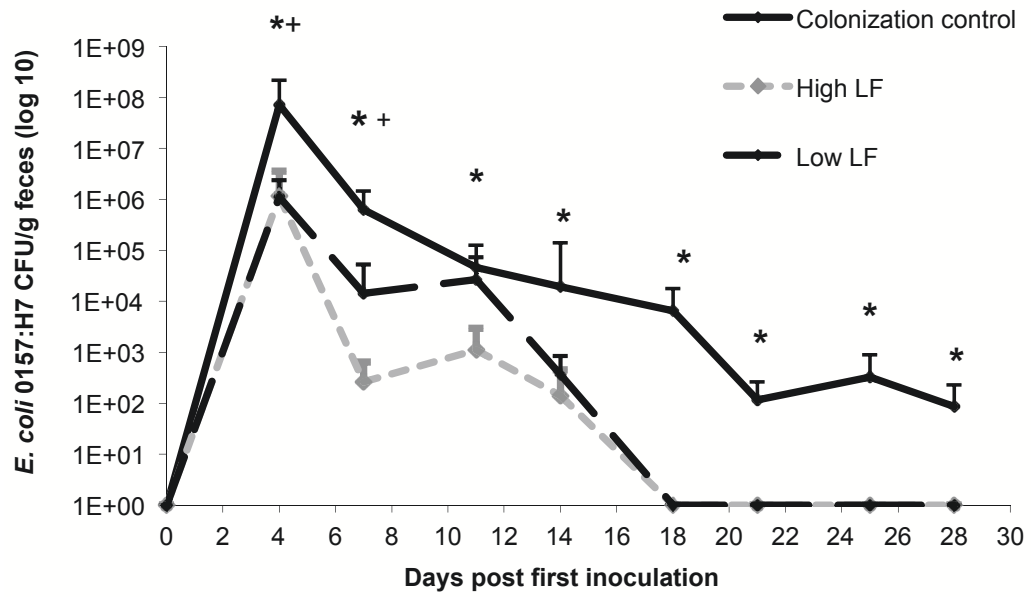
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Table 1: Experimental set-up

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)



Accepted

