New strategies for prevention of *E. coli* O157:H7 infection in sheep

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List of abbreviations

A/E	Attaching and effacing
Ab	Antibody
Ag	Antigen
APC	Antigen presenting cell
BCG	Bacille calmette-guerin
bLF	Bovine lactoferrin
CFU	Colony forming unit
CE	Competitive exclusion products
DAEC	Diffuse adherent *Escherichia coli*
DC	Dendritic cell
DFM	Direct-fed microbials
DPI	Days post infection
E. coli	*Escherichia coli*
EAEC	Enteroaggregative *Escherichia coli*
EHEC	Enterohaemorrhagic *Escherichia coli*
EIEC	Enteroinvasive *Escherichia coli*
ELISA	Enzyme linked immunosorbent assay
EPEC	Enteropathogenic *Escherichia coli*
EspA	*Escherichia coli* secreted protein A
EspB	*Escherichia coli* secreted protein A
ETEC	Enterotoxigenic *Escherichia coli*
FAE	Follicle associated epithelium
F-actin	Filamentous actin
Gb3	Globotriaosylceramide-3
GIT	Gastrointestinal tract
GRO	Growth regulated protein
HC	Hemorrhagic colitis
hLF	Human lactoferrin
HUS	Hemolytic uremic syndrome
IFN	Interferon
IL	Interleukin
IM	Intra muscular
IPP	Ileal Peyer's patches
L. acidophilus	*Lactobacillus acidophilus*
LEE	Locus of Enterocyte Effacement
LF	Lactoferrin
LFcin	Lactoferricin
LPF	long polar fimbriae
LPS	Lipopolysaccharide
SMAC	Sorbitol MacConkey
MUG	Methyl umbelliferyl-D-glucuronide
OD	Optical density
PBS	Phosphate buffered saline
RAJ	Rectoanal junction

SC	Subcutaneous (ly)
SEM	Standard error of the mean
Stx	Shiga toxin
STEC	Shiga toxin producing *E. coli*
Th	T helper
TLR	Toll like receptor
TNF	Tumor necrosis factor
Tir	Translocated intimin receptor
TTSS	Type three secretion system

Preface

Enterohaemorhagic *E. coli* (EHEC) are zoonotic pathogens that can cause hemorraghic diarrhoea and haemolytic uremic syndrome in humans. *E. coli* O157:H7 is the most well known serotype of this group. Ruminants are the main reservoir of these bacteria and contaminated food is the main source of the infection. Therefore, a key step towards protecting humans from *E. coli* O157:H7 infection is controlling *E. coli* O157:H7 infection in ruminants. Several approaches have been suggested to control the infection, including vaccination (Peterson *et al.,* 2007a; Potter *et al.,* 2004), antibiotic treatment (Molbak *et al.*, 2002), probiotics (Callaway *et al.*, 2004), bacteriophages (Callaway *et al.*, 2008) and dietary changes (Callaway *et al.*, 2009). These strategies are either too expensive, too labor intensive, only show a limited effect or hold the risk of an increase in antibiotic resistance. Therefore, there is need for new intervention strategies.

This thesis focuses on inhibition of *E. coli* O157:H7 infection in sheep by using lactoferrin as a natural antimicrobial protein or by vaccination using type III secretion system (TTSS) proteins. In the literature review of this thesis we will first highlight the most known strategies to inhibit *E. coli* O157:H7 infection particularly in ruminants. Subsequently we will review the role of lactoferrin as natural antimicrobial protein of milk in inhibiting bacterial infection.

Part I: Review of the Literature

CHAPTER 1

E. coli O157:H7; pathogenesis, infection and control

1.1 Introduction

Escherichia coli (named after Dr. Theodor Escherich and abbreviated *E. coli*), is a facultative anaerobic Gram-negative rod-shaped bacterium belonging to the family of *Enterobacteriaceae*. The optimal growth temperature of this family is 37°C. However, various *E. coli* are capable of surviving in water and food and the environment. Only 90% of *E. coli* serotypes are able to ferment lactose while 99% are indole positive. Other characteristics are: oxidase negative, catalase positive, methyl red positive, citrate negative and negative for H_2S (Orskov *et al.*, 1984).

Most *E. coli* strains are commensals which are important for the maintenance of intestinal physiology. Beneficial effects for the host include production of vitamin K and prevention of the establishment of pathogenic bacteria within the intestine. Commensal *E. coli* comprise almost 1% of the bacterial population in the gut, with approximately 10^8 -10^9 bacteria/g human faecal material (Callaway *et al.*, 2009). However, certain strains are pathogenic and have acquired virulence factors to cause intestinal disease (Kaper *et al.*, 2004). A conserved core genomic structure is common to both commensal and pathogenic strains, providing the microorganisms with mechanisms required for survival under the competitive conditions in the gut, as well as the ability to spread among hosts and survive in the environment (Dougan *et al.,* 2001). In pathogenic bacteria, virulence factors are often found in genetic islands (Wain *et al.,* 2001). These genes provide the bacteria with a higher level of adaptation, leading to specific tissue targeting and facilitating efficient dissemination to new hosts (Garmendia *et al.,* 2005). Diarrheagenic *E. coli* are divided in at least six different categories including enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), diffuse adherent *E. coli* (DAEC), Shiga toxin producing *E. coli* (STEC) and its subgroup enterohaemorrhagic *E. coli* (EHEC).

The term enterohaemorhagic *E. coli* (EHEC) denotes strains that are associated with haemorrhagic colitis (HC) and the haemorrhagic uremic syndrome (HUS) in humans, express Shiga toxins (Stx), colonise the intestine by causing a typical lesion known as attaching-effacing (A/E) lesion and possess a specific 60-MDa EHEC plasmid (Nataro and Kaper, 1998). From this group, *E. coli* O157:H7 is the most well known strain.

1.2. *E. coli* O157:H7

This organism was first recognized in 1982 following an outbreak of haemorrhagic colitis (HC) in Oregon and Michigan linked to the consumption of ground beef (Riley *et al.,* 1983). Since then, many outbreaks have been reported in developed countries and *E. coli* O157:H7 became one of the most important life-threatening foodborne pathogens (Nyachuba, 2010).

E. coli O157:H7 has unique biochemical characteristics: delayed D-sorbitol fermentation (>24 h) and inability of producing β-glucuronidase, which hydrolyzes a synthetic molecule, 4-methyl umbelliferyl-D-glucuronide (MUG). Thus, sorbitol MacConkey (SMAC) agar supplemented with MUG is used for detection of *E. coli* O157:H7. Additionally cefixime, potassium tellurite, and vancomycin can be added to SMAC agar as inhibitors of other Gram negative bacteria. This supplementation increases the chance of *E. coli* O157:H7 selection (March, 1986). A suspected *E. coli* O157:H7 colony can be further confirmed by a commercially available latex agglutination assay (Oxoid Ltd) to determine whether the isolate belongs to the O157 serogroup.

The annual incidence of reported clinical *E. coli* O157:H7 infection in humans in the United States is around 73,000 resulting in more than 2,000 hospitalizations and 60 deaths. The annual cost is estimated to be 405 million $ and includes productivity losses, medical care costs and premature deaths (Frenzen, 2003). The number of infections in developed countries is increasing and in 2005 alone 26 countries (principally European but including Japan) reported a total of 2937 *E. coli* O157:H7 infections (La Ragione *et al.*, 2009). The reported incidence of *E. coli* O157:H7 infection is inceased from 1.05 to 1.12 cases per 100,000 inhabitants during the period 2005 till 2008 (Nyachuba, 2010). According to the most recent available data from the European Food safety Authority (EFSA) 3159 cases of STEC infection occurred in 2008 in European Union (EU) member states. Fifty-three percent of these cases were caused by serotype O157:H7 and the reported incidence of *E. coli* O157:H7 infection in Belgium was 1 case per 100,000 inhabitants (European Food safety Authority, 2010).

Most cases of *E. coli* O157:H7 infection are connected to food contamination (Armstrong *et al.*, 1996). An epidemiological study showed that from 1982 till 2002, among 183 outbreaks in the USA, 41% were the result of consumption of ground beef (Rangel *et al.*, 2005). Person

to person transmission is also reported as a source of infection, as 50 outbreaks (out of 183) reported in the USA were spread by the faecal-oral route (Rangel *et al.,* 2005). Although consumption of contaminated food and/or direct contact with contaminated faecal material are still the most common routes for transmission of *E. coli* O157:H7, new transmission routes are becoming an important concern. Visiting dairy farms with contaminated dairy products, drinking fruit juice and even eating contaminated vegetables have been reported to be sources of infection (Grant *et al.,* 2008). Interestingly, Varma *et al.*, (2003) reported a potential airborne transmission of *E. coli* O157:H7 to people in a building where animals had been kept before.

1.2.1. Pathogenesis

The most important virulence characteristics of *E. coli* O157:H7 are the formation of A/E lesions and their ability to produce one or more Stx, since the role of the latter in causing HC and HUS in humans is very well established (Karmali, 2009). The contribution of Shiga toxins in colonization is of less concern, although they can bind to enterocytes and subsequently influence the colonization receptors. The role of some of the other virulence factors, such as long polar fimbriae and enterohemolysin is not fully established and may have less effect in the pathogenesis of *E. coli* O157:H7 (Torres *et al.,* 2007; Khare, *et al.,* 2010). Here we explain the most well studied virulence factors of *E. coli* O157:H7 and their contribution in pathogenesis.

1.2.2. Shiga toxin

Shiga toxin (Stx) was first discovered in *Shigella dysenteriae* by Kiyoshi Shiga in 1898 and its production by *E. coli* O157:H7 was confirmed by O'Brien (O'Brien, 1982; O'Brien *et al.,* 1983). Shiga toxin is thought to be responsible for the development of HC and HUS. There are two main types of Stx produced by EHEC, namely Stx1 and Stx2. Stx1 is almost identical to the Shiga toxin produced by *Shigella dysenteriae* (Beutin, 2006), whereas Stx2 is a more diverse molecule, with only 56% amino acid homology to Stx1 (Paton and Paton, 1998). Most *E. coli* O157:H7 strains produce Stx2 and epidemiologic data suggest that isolates producing only Stx2 are more likely to cause severe disease than those producing only Stx1 or a combination of Stx1 and Stx2. Both Stx1 and Stx2 are made up of five identical 7.7 kDa B-subunits and a

single 32 kD A- subunit (Fraser *et al.*, 1994; Stein *et al.*, 1992). The B-subunit binds specifically to the Gb3 receptor, which is a glycolipid found in varying degrees in membranes of eukaryotic cells including human gastrointestinal tract (GIT) and renal epithelium. Shiga toxins have further been reported to bind to globotetraosylceramide (Gb4) that is connected to the trisaccharide of Gb3 (Waddell *et al.*, 1996). After endocytosis, the A subunit is cleaved into an A1 (27.5 kDa) and A2 (4.5 kDa) subunit, but they remain covalently bound through a disulfide bond between two cysteine residues. When the cysteines are reduced, the catalytically active A1 enzyme cleaves a specific adenine from the 28S rRNA of the 60S ribosomal subunit (Endo *et al.*, 1988) resulting in inactivation of the 60S ribosomal subunit and subsequently blockage of protein synthesis and cell death (O'Brien and Holmes, 1987). Villous cells are more sensitive than crypt cells to the Stx-induced inhibition of protein synthesis as expression of the Stx receptor Gb3 is greater in the villi cells (Kandel *et al.,* 1989). Translocation of the toxin into the blood stream is necessary to reach the kidney, which leads to HUS. The first evidence for the possibility of Stx translocation through the intestinal barrier was obtained *in vitro* by Acheson *et al.* (1996), who showed Stx translocation across the intestinal barrier in intact polarized intestinal epithelial cells (Caco-2 and T84 cells) without apparent cellular disruption in an energy-requiring system. Later, it has been shown that the transmission of Stx through the intestinal barrier is different for Stx1 and Stx2. While Stx1 translocation through intestinal epithelial cells occurs via a transcellular route, Stx2 uses a paracellular pathway (Hurley *et al.*, 1999; Philpott *et al.,* 1997).

Once in the bloodstream, Stx binds to monocytes, platelets and polymorphonuclear leukocytes and is transferred to the kidney, thereby inducing a prothrombotic state that contributes to the pathogenesis of HUS (Stahl *et al.*, 2009). The renal tissue expresses higher concentrations of Gb3 than other tissues, and the cytotoxic effect of Stx in human renal endothelial cells is more pronounced. Expression of Gb3 is higher in the renal cortex than in the medulla. Glomerular capillaries of infants express higher Gb3 receptor levels than adults, explaining the higher risk of developing HUS following *E. coli* O157:H7 infection in children compared to adults (Lingwood, 1994).

Unlike humans, cattle lack the vascular Gb3 receptor which explains why ruminants are symptomless carriers of *E. coli* O157:H7 (Pruimboom-Brees *et al.*, 2000). Although *in vitro* experiments showed that Stx1 blocks differentiation and proliferation of bovine

lymphoblasts (Menge et al., 2003), *in vivo* studies clearly show that Stx negative mutants of *E. coli* O157:H7 are also able to colonize ruminants leading to persistent infection in cattle and sheep (Woodward *et al.,* 2003).

1.2.3. A/E lesion

Attachment of EHEC to epithelial cells is accompanied by striking changes in the local morphology of the host cell named "attaching and effacing (A/E) lesion" which is encoded by the genes on the *LEE* pathogenicity island (Nataro and Kaper, 1998). The term A/E lesion was first described by Moon *et al.* (1983) for the lesion characterized by intimate adherence between the bacterium and the host epithelial cell membrane with an intervening gap of about 10 nm, plus effacement of enterocyte microvilli. Characteristic cytoskeletal rearrangement, including the accumulation of filamentous actin (F-actin) underneath adherent bacteria is also a feature of the A/E lesion. The bacteria often sit upon a pedestal-like structure, which can extend up to 10 µm away from the epithelial cell surface (Kaper, 1998). The lesion is typically described as being formed in three stages: (i) early adhesion followed by (ii) signal transduction (leading to cytoskeletal reorganization and microvillus effacement) and finally (iii) intimate attachment.

Initial adhesion of *E. coli* O157:H7 to intestinal epithelial cells is poorly understood. Like most other bacteria, the motility of *E. coli* is due to expression of flagella, and H7 flagellin is important for motility of *E. coli* O157:H7. Besides motility, recent evidence indicates a role for H7 in primary adherence of *E. coli* O157:H7 to epithelial cells. Time-dependent expression of H7 flagella was recently reported. While Type III secretion system (TTSS) proteins becomes up-regulated after initial bacterial contact with the host cell (described further), H7 expression becomes downregulated. This can show that H7 contributes to the initial non-intimate attachment of *E. coli* O157:H7 to epithelial cells (Mahajan *et al.,* 2009). Some studies have indicated a role for the long polar fimbriae (LPF), which are homologous to *Salmonella* Typhimurium fimbriae. They suggest a primary interaction between LPF and the mucus, that probably increases the survival of *E. coli* O157:H7 in many different physiological environments (Torres *et al.,* 2007).

The second step in EHEC attachment is mainly characterized by signal transduction and cytoskeletal reorganization of epithelial cells, which ends up in formation of the A/E lesion

(Figure 1). *LEE* encodes a type-III secretion apparatus, which resembles a molecular needle extending to the surface of the host cell and through which effector molecules are injected into the host cell (Elliott *et al.*, 1998). EspA filaments form a transport channel between the bacteria and the host cell, while EspB and EspD form the translocation pore in the plasma membrane of the host cell through which bacterial effector proteins are delivered into the host cell (Figure 2) (Lai *et al.*, 1997; Kresse *et al.*, 1999; Ide *et al.*, 2001; Buttner and Bonas, 2002). Following the translocation of effector proteins, the EspA filaments are eliminated from the bacterial cell surface; this is necessary to allow intimate bacterial attachment through intimin interaction with translocated intimin receptor (Tir) (Frankel *et al.*, 1998). Tir is injected by the TTSS into the host cell membrane and adopts a hairpin loop conformation, allowing its extracellular domain to interact with intimin in the bacterial outer membrane. This interaction initiates a signalling cascade, leading to actin polymerization and pedestal formation finally resulting in strong attachment of *E. coli* O157:H7 to the host cell. The C-terminal part of intimin is responsible for the binding to Tir, and is highly variable. Intimin α is generally found in EPEC strains, whereas intimin β is produced by both EPEC and EHEC strains and intimin γ is produced by *E. coli* O157. Evidence suggests that intimin also binds a host-cell-encoded receptor(s) (Hir), and interaction of different intimin types with Hir contributes to tissue tropism of the bacteria (Mundy *et al*., 2007). For instance *in vitro* studies show that intimin *γ* is associated with colonization of the large intestinal epithelium while bacteria expressing intimin *α* colonize both the small and large intestine (Mundy *et al.*, 2007). However the interaction of bacteria with the host cells is a multifactorial process and involves also other bacterial and host factors as well as intimin.

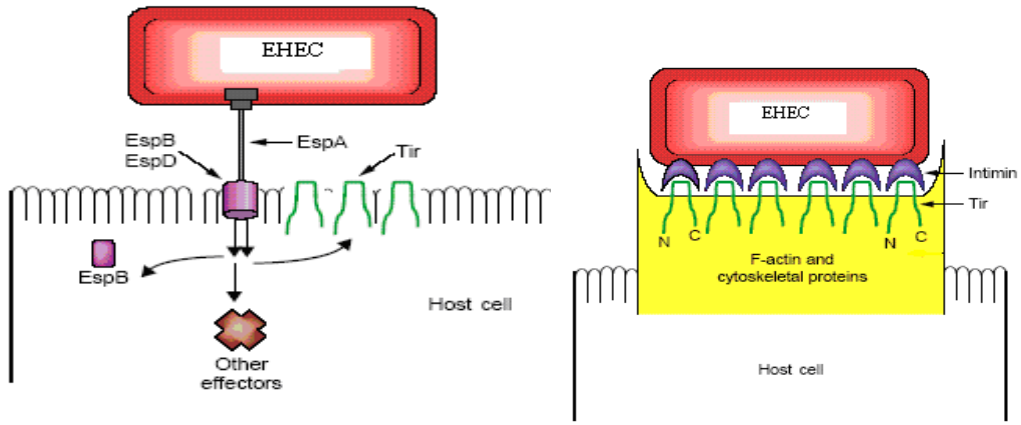

Figure 1: Initial attachment of EHEC (Left) and subsequently the strong attachment to host cells (Right), (Adapted from Campellone and Leong, 2003)

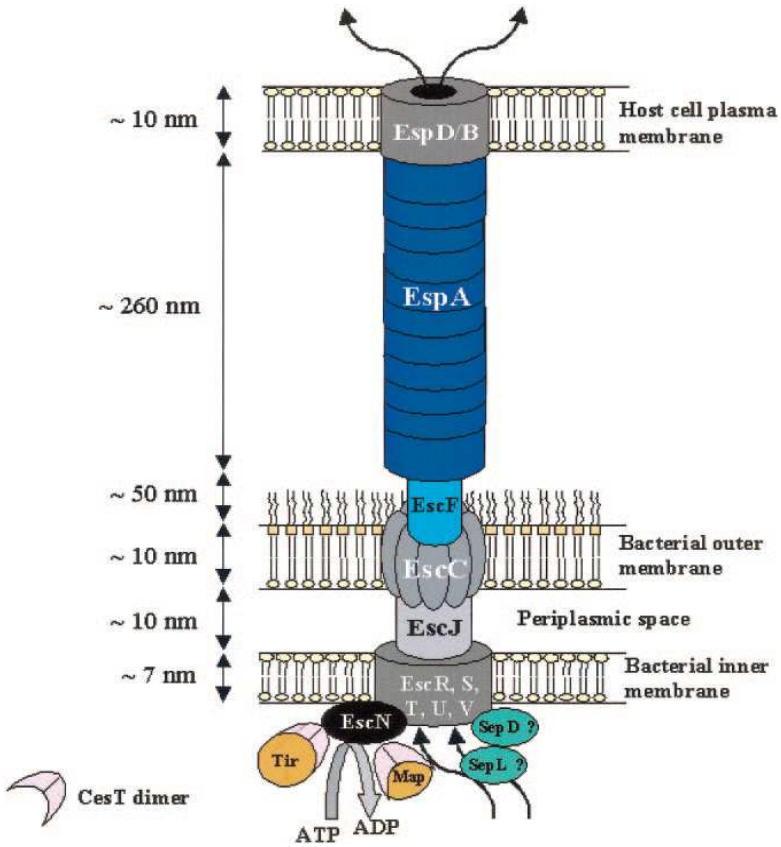

Figure 2. Schematic representation of the EPEC/EHEC type III secretion system apparatus (TTSS). The basal body of the TTSS is composed of the secretin EscC, the inner membrane proteins EscR, EscS, EscT, EscU, and EscV, and the EscJ lipoprotein, which connects the inner and outer membrane ring structures. EscF constitutes the needle structure, whereas EspA subunits polymerize to form the EspA filament. EspB and EspD form the translocation pore in the host cell plasma membrane, connecting the bacteria with the eukaryotic cell via EspA filaments. The cytoplasmic ATPase EscN provides the energy to the system by hydrolyzing ATP molecules into ADP. SepD and SepL have been represented as cytoplasmic components of the TTSS. (Adapted from Garmendia *et al*, 2005)

1.2.4. Plasmid (O157)

In addition to Stx and *LEE*, *E. coli* O157:H7 possesses a virulence plasmid named pO157. This plasmid encods several putative virulence factors including *ehxA*, *etpC* to *etpO*, *espP*, *katP*, *toxB*, *ecf*, and *stcE*. Among these genes, ehxA is the most well known which expresses Hemolysin A. Some studies show that the genes encoded on the pO157 can influence bacterial adherence to eukaryotic cells, colonization of cattle, and acid resistance. However, conflicting evidence exists and more studies have to be performed to determine the exact role of pO157 in EHEC pathogenesis (Lim *et al.*, 2007; Sheng *et al*., 2008; Lim *et al*., 2010).

1.2.5. *E. coli* O157:H7 infection

1.2.5.1. Humans

Patients infected with *E. coli* O157:H7 initially experience watery diarrhoea although some individuals may be asymptomatic. The infectious dose for *E. coli* O157:H7 is very low and it has been reported that less than 100 organisms can cause an infection in humans (Williams *et al.,* 2000). The incubation period for an *E. coli* O157:H7 infection ranges from one to eight days, but is usually three to four days. Most cases progresses to HC with severe abdominal cramps. The majority of the cases are self-resolving within a week (Figure 3).

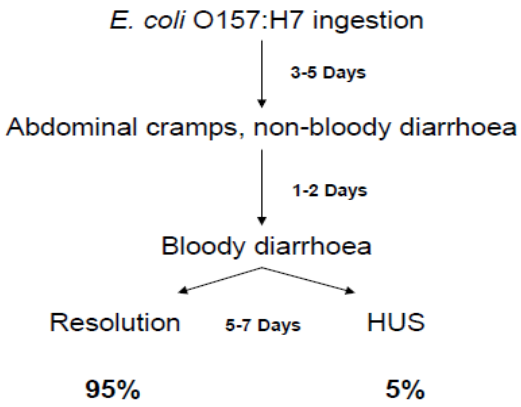

Figure 3: Progress of an *E. coli* O157:H7 infection in humans (adapted from Mead and Griffin, 1998 and Tauxe, 1991)

The infection can be complicated by the development of HUS. The mechanisms by which *E. coli* O157:H7 causes HC and HUS in humans are not fully understood. The organism is believed to adhere closely to intestinal mucosal cells, disrupting the brush border and leading to non-bloody diarrhoea (Figure 4). Shiga toxins (Stx) probably play a critical role in this process. Histopathological changes associated with the infection include haemorrhage and oedema in the lamina propria with areas of superficial focal necrosis. Post diarrhoeal HUS is primarily a disease of the microvasculature, thought to develop when Stx produced in the intestine enters the blood and binds to Gb3-rich endothelial cells in the kidneys. Damage of the endothelial cells, mediated by Stx, may trigger platelet and fibrin deposition, leading to injury of passing erythrocytes (haemolysis) and occlusion of renal microvasculature (renal failure). Inflammatory cytokines and circulating bacterial lipopolysaccharide may play an important part in augmenting this process (Van Setten *et al.*, 1998). It was shown that inflammatory cytokines such as TNFα can increase Gb3 expression and Stx toxicity. On the other hand Stx increases the expression of TNFα by monocytes/macrophages. *In vivo* and *in vitro* studies showed that the pro-inflammatory cytokines TNFα, IL-1β, IL-6 and IL-8 are highly expressed during the acute phase of Stx expression (Van Setten *et al.*, 1996; Inward *et al.*, 1997; Sakiri *et al.*, 1998) leading to inflammation in Stx-associated HUS and renal failure. Renal failure mostly occurs in children (Siegler *et al.*, 1994). The reason why children are more susceptible to develop HUS is not known. The difference in immune system, Stx transport effectors and Stx receptors expression may explain the age-associated difference in developing HUS in *E. coli* O157:H7 infection in children.

Recently Khan *et al*., (2009) reported that Stx1 and Stx2 bind less to glomeruli of adults compared to children glomeruli, and that Stx binding in adults could be “unmasked” by removal of the lipids of the plasma membranes with detergents or by cholesterol-removing drugs, thus possibly clarifying the age-restricted pathology of Stx-associated HUS. Although the kidneys are preferentially involved in the pathogenesis of HUS, other organs including the brain may be affected. Thirty-three percent of patients with HUS experience neurologic symptoms such as irritability, seizures, and altered mental status (Walker *et al*., 2004) resulting in a wide range of complications (Mead and Griffin, 1998).

At the moment there is no specific therapeutic agent available for HUS and supportive care is the only generally approved therapy for the patient. Novel strategies are still under

investigation, including vaccination, Stx receptor mimicking agents and antibodies against Stx.

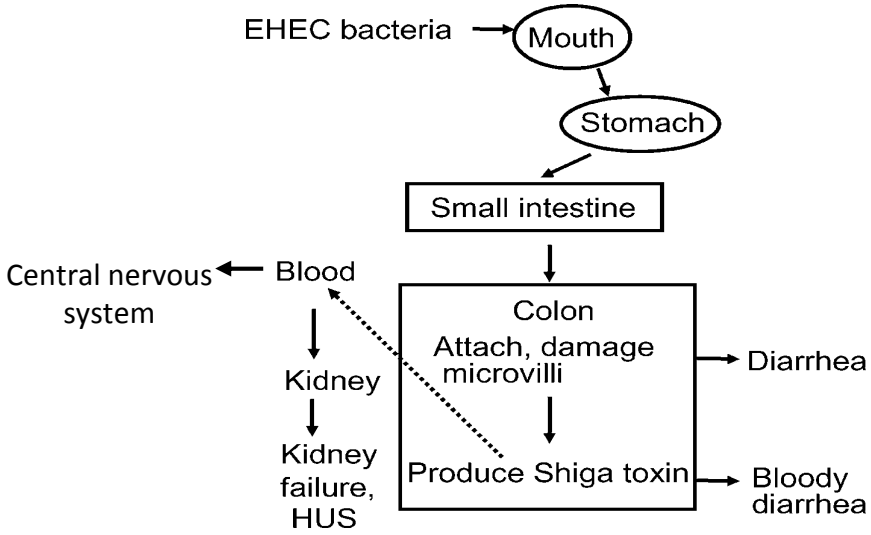

Figure 4. Overview of disease in humans due to enterohemorrhagic *Escherichia coli* (EHEC). Infection begins with entry of the bacteria through food or water taken in the mouth. Acid resistance of EHEC facilitates their survival through the low pH of the stomach. The bacteria pass through the small intestine, and virulence genes are turned on by environmental signals in the colon. The EHEC adhere to the enterocytes of the colon in a characteristic intimate adherence and cause effacement of the microvilli and diarrhoea. If sufficient Shiga toxin (Stx) is produced, local damage to blood vessels in the colon result in bloody diarrhoea. If sufficient Stx is absorbed into the circulation, vascular endothelial sites rich in the toxin receptor are damaged, leading to impaired function. The kidneys and central nervous system are sites that are frequently affected, and hemolytic uremic syndrome (HUS) may develop (Adapted from Gyles, 2007)

1.2.5.2. Cattle

Cattle are the most important source of human *E. coli* O157:H7 infections (Figure 5). Up to 30% of all cattle are asymptomatic carriers of *E. coli* O157:H7 (Stanford *et al.,* 2005; Callaway *et al.,* 2006; Reinstein *et al.,* 2007) and the prevalence of *E. coli* O157:H7 in cattle varies according to the sensitivity of the detection technique, the sampling method and the age of the animals.

A recent study in Belgium in different cattle farms indicated an overall farm prevalence of *E. coli* O157:H7 of 37.8% (68 of 180 farms). The highest prevalence was found in dairy cattle farms (61.2%, 30 of 49 farms) and the prevalence in beef, mixed dairy and beef, and veal calf farms was 22.7% (17 of 75 farms), 44.4% (20 of 45 farms), and 9.1% (1 of 11 farms),

respectively (Cobbaut *et al.,* 2009). A recent study in cattle slaughter houses in the US (Walker *et al.,* 2004) indicated that in 20.3% of cattle, the gut contents were positive for the presence of *E. coli* O157:H7 by selective enrichment, immunomagnetic separation, plating on selective medium, agglutination for O157 antigen, and presence of virulence genes. *E. coli* O157:H7 excretion in cattle is thought to be influenced by the age of the animals. Epidemiological studies in the US show that up to 5% of calves under 4 months of age excrete the bacteria while this number decreases by age. In an experimental infection model with *E. coli* O157:H7, Dean-Nystrom *et al.,* (1999) have demonstrated that *E. coli* O157:H7 could cause A/E lesions and diarrhoea in both colostrum-deprived and colostrum-fed neonatal calves. The same experimental infection could not induce diarrhoea in 3-4 months old weaned calves. They also reported that age-related pathogenesis of *E. coli* O157:H7 appears even during the neonatal period, since the virulence of *E. coli* O157:H7 was greater in 12-hour-old calves than in 30- and 36-hour-old calves.

It was in 1999 that Dean-Nystrom *et al* (1999) identified the rectum as the major site of *E. coli* O157:H7 colonization in cattle. Later Grauke *et al.* (2002) found evidence that the lower part of the GIT and specifically the cecum and the colon, are the most important colonization sites in ruminants (sheep and cattle). This work has been followed by a study of naturally-colonised cattle where significant numbers of *E. coli* O157:H7 were found on the mucosal surface of the terminal rectum (Naylor *et al.*, 2003; Low *et al.*, 2005). Although these findings indicate the terminal rectum as the principal colonisation site of *E. coli* O157:H7 infection in cattle, Naylor *et al.,* (2003) showed that the rumen, the small intestine, the proximal colon and the cecum can be other sites of *E. coli* O157:H7 colonization in cattle.

Bacterial colonization of the intestine results in bacterial faecal shedding. The number of bacteria excreted by an infected animal and the duration of shedding is highly variable and there is evidence suggesting that a kind of individual preference contributes towards the duration and the number of bacteria excreted by animals (Hancock *et al.*, 1997). Naylor *et al.,* (2005a) showed that certain positive animals, so-called “super shedders”, shed *E. coli* O157:H7 at much higher concentrations than others. A recent longitudinal study using recto–anal mucosal swabs (RAMS) defined a super-shedder on the basis of both mean concentration ($\geq 10^4$ CFU/g faeces) and persistent colonization ($\geq$4 consecutive positive RAMS) for samples taken twice a week for 14 weeks (Cobbold *et al.,* 2007). Super shedders

are thought to have a substantial impact on the on-farm epidemiology of *E. coli* O157:H7; while they constitute only a small proportion of cattle, it has been estimated that they may be responsible for over 95% of the *E. coli* O157:H7 bacteria shed by cattle (Naylor *et al.*, 2005b).

The occurrence of *E. coli* O157:H7 infection also depends on the season, with shedding being more pronounced in summer than in winter (Chapman *et al.*, 1997; Hancock *et al.,* 1997; Van Donkersgoed *et al.*, 1999).

1.2.5.3. Sheep

Sheep are the second most important reservoir of *E. coli* O157:H7 and the bacteria have been frequently isolated from both milk and meat (Espie *et al.*, 2006; Beneduce *et al.*, 2008; King *et al*., 2009; Solomakos *et al.*, 2009). Sheep can be colonized by *E. coli* O157:H7 and excretion of the bacteria up to 50 days after infection has been reported (La Ragione *et al.,* 2009). The prevalence of *E. coli* O157:H7 in small domestic ruminants is less well documented than in cattle, and reports vary between 0.2 to 35% in different surveys in developed countries (Kudva *et al.,* 1996; Battisti *et al.*, 2006). Sheep have been identified as a source of human infections and a large outbreak occurred at a boy scout camp where some of the scouts played with sheep dung (Ogden *et al.*, 2002). In addition, a high prevalence of *E. coli* O157:H7 was observed in a sheep flock in Scotland, with individuals in the flock shedding up to 10^6 CFU per gram faeces. The studies conducted by La Ragione *et al.* (2006) and Wales (2005) suggest that, unlike in calves, in sheep the rectoanal junction (RAJ) is not the site of primary colonization leading to persistent colonization and shedding of *E. coli* O157:H7 (Wales *et al.,* 2005; La Ragione *et al.*, 2006). Interestingly Woodward *et al*., (2003) showed that animals colonized beyond 14 days post-infection, and therefore considered to be persistent shedders, had *E. coli* O157:H7 organisms throughout the entire gastrointestinal tract, rather than just the large intestine which support the hypothesis that the entire gastrointestinal tract or at least other parts of GIT rather than only RAJ are the principal colonisation site in sheep.

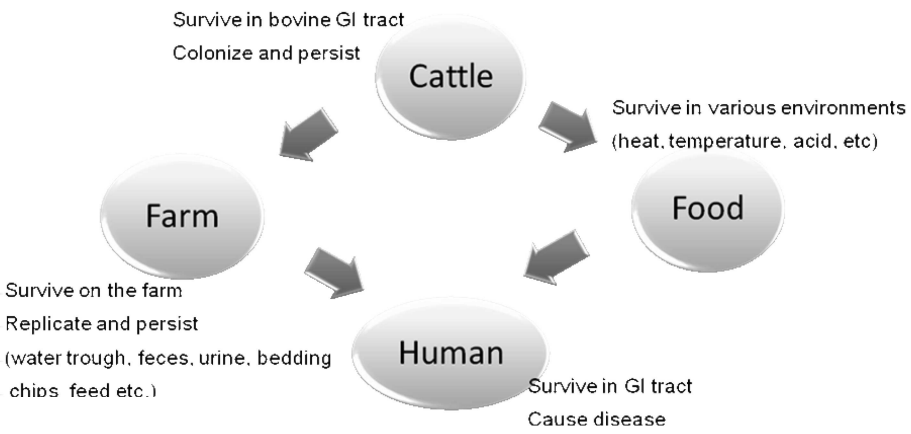

Figure 5: Schematic presentation of the spread of EHEC from cattle to humans (Adapted from Hovde, 2010)

1.2.6. Inflammatory response in EHEC infection

Although *E. coli* O157:H7 is a significant human enteropathogen and ruminants are the most important reservoir, information on the immune response in humans and ruminants against this pathogen is limited. In humans, Fitzpatrick *et al.*, (1992) reported the increase of serum IL-8 levels in patients who developed HUS. Later on, the possible involvement of other inflammatory cytokines such as TNF was reported and the role of several virulence factors was studied such as Stx. However, the effect of Stx on intestinal epithelial cells remains controversial. Some studies demonstrate the induction of chemokines such as IL-8 and growth regulated protein (GRO)-alpha, beta and gamma in a human colonic cell lines (Thorpe *et al.*, 1999). Others have shown that *E. coli* O157:H7, even in the absence of Stx, is a potent activator of NF-kB, and can markedly upregulate epithelial IL-8 production (Berin *et al*., 2002). Besides on epithelial cells, Stx also acts on macrophages, inducing the expression of TNF-alpha, IL-1 beta and IL-6 and on granulocytes, delaying the onset of apoptosis and triggering the generation of reactive oxygen intermediates (Harrison *et al.,* 2004).

Interestingly, studies in cattle, which excrete the bacteria for a long time without clinical signs, suggest not only a stimulatory effect but also a suppressive effect for Stx in *E. coli* O157:H7 infected bovines. Indeed, although cattle lack the receptor for Stx in their blood vessels, the Stx receptor is expressed on intestinal epithelial cells, B cells, CD4+ and CD8+T cells, in contrast to humans where it is expressed on endothelial cells, intestinal epithelial

cells, monocyte/macrophages and B cells, but no T cells (Lindberg *et al.,* 1987; Menge *et al.*, 1999; Stamm *et al.*, 2002). Binding of the toxin to the receptor on intraepithelial T lymphocytes can subsequently suppress the expression of IL-8 and accelerate the synthesis of IL-4 (Menge *et al.*, 2004). However, this effect was observed *in vitro* on isolated cells but could not be reproduced in a ligated intestinal loop model. Here the main toxin effect was a decrease in CD8+ T cells (Menge *et al.,* 2004). Bovine granulocytes do not express the Stx receptor (Gb3), which could be an additional reason for the absence of intestinal inflammation during a bovine EHEC infection (Menge *et al.,* 2006). In contrast, ovine granulocytes constitutively express Stx1 receptors, but although the toxin can delay apoptosis only little effect is seen on oxidative burst activity and phagocytosis (Menge *et al.,* 2006).

These data provide evidence that Stx subverts the inflammatory response and underline an essential role for Stx in the initial step of the colonization of the intestinal mucosa. Moreover, they suggest that in cattle Stx may suppress mucosal immunity, enabling the bacterium to escape the host innate defences, enhancing its colonization.

Another virulence factor of *E. coli* O157:H7 which has been studied is the H7 flagellin (Berin *et al.,* 2002). It was shown that Stx and intimin are not required for the activation of the NF-kB signalling pathways in epithelial cells. H7 flagellin is the key factor activating this pathway leading to a proinflammatory response with up-regulated expression and production of IL-8 by human intestinal epithelial cells. Flagellin is doing this via binding to Toll-like receptor TLR-5 (Steiner *et al.*, 2000; Gewirtz *et al.*, 2001; Berin *et al.,* 2002). Isogenic mutants of EHEC lacking flagellin do not significantly upregulate prototypic neutrophil and dendritic cell chemoattractants in human colon epithelia, irrespective of Stx production. This could indicate that H7 flagellin and not Stx is the major EHEC virulence factor involved *in vivo* in upregulation of proinflammatory cytokines by the human colon epithelium (Miyamoto *et al.*, 2006).

It seems that although *E. coli* O157:H7 infection stimulates the inflammatory responses in infected patients; this activation is somehow suppressed during the colonization steps. Since H7 is thought to play a role in initial colonization of EHEC, the inflammatory response might occur only in this step and then by secretion of Stx and suppression of inflammatory

cytokines, bacteria get more chance to colonize the intestine. However more investigation is needed to confirm this hypothesis.

1.2.7. Control of EHEC infection

Ground beef is the main source of *E. coli* O157: H7 infection for humans and the cause of severe outbreaks in the world and thus cattle were soon recognized as important reservoirs (Martin *et al.*, 1986; Borczyk *et al.*, 1987; Currie *et al.,* 2007; Hussein, 2007; King *et al.*, 2009). Indeed, it is well established that cattle and sheep can excrete *E. coli* O157:H7 for months in their faeces (La Ragione *et al.*, 2009). Moreover, traceable links have been established between human infection and ruminant faeces via water or direct contact indicating that contact with animal faeces is a strong risk factor for *E. coli* O157: H7 (Locking *et al.*, 2001). Therefore, methods that reduce the *E*. *coli* O157:H7 populations in food animals at the farm level and before entry in the food chain have great potential to reduce human illnesses (Callaway *et al.*, 2004b; Loneragan *et al.*, 2005; Sargeant *et al.*, 2007).

As for other zoonotic agents, obtaining animals that are free from EHEC is impossible in practice. However, their occurrence can be minimized. At the farm level, classical eradication strategies based on the elimination of positive animals is not feasible, due to the high prevalence of colonisation, its transient nature, and the technical difficulties in detection of low levels of the microorganism in animal faeces. Due to survival of *E. coli* O157:H7 during extended periods in faeces, soil and water, complete eradication of the bacteria from bovine populations is unlikely. Indeed, *E. coli* O157:H7 could survive in lake water at 15°C for 13 weeks (Wang and Doyle, 1998) and in animal faeces from several weeks to many months (Kudva *et al.*, 1998). In the USA, one well-identified *E. coli* O157:H7 strain persisted in a farm environment for more than two years (Shere *et al.*, 1998).

Therefore, control of this foodborne pathogen on the farm and preharvest interventions which should be economical, practical, and suitable from an animal welfare perspective, should be pursued. Probiotics, antibiotics, feed additives, vaccines and management procedures all have been actively evaluated as methods for reducing the shedding of *E. coli* O157:H7 by ruminants. However, until now these approaches have not been effective to reduce *E. coli* O157:H7. The reasons are unknown. At the moment no effective strategy against *E. coli* O157:H7 is available. Thus other strategies or combinations of strategies are

needed. Here we briefly discuss the most well known strategies to control EHEC infection in ruminants.

1.2.7.1. Antibiotics

Antibiotics have been shown to fail preventing progression of *E. coli* O157:H7 from diarrhoea to HUS in human and even increase the risk (Carter *et al.*, 1987; Pavia *et al.*, 1990; Safdar *et al.*, 2002; Dundas *et al*., 2005; Panos *et al.*, 2006). These results gave arguments against the use of antibiotics for treating *E. coli* O157:H7 infection in man. A first argument is the risk for increasing the concentration of Stx in the gut lumen due to the effect of the antibiotic on the bacteria (Zhang *et al.*, 2000; Ochoa *et al.*, 2007). A second argument against the use of antibiotics is the high risk for development of antibiotic resistance of the *E. coli* O157:H7 strains. Antibiotic resistance, including multiple drug resistance to streptomycin, sulfisoxazole and tetracycline, is common in *E. coli* O157:H7 (Kim *et al.*, 1994; Mora *et al.*, 2005). Some studies have shown a higher rate of antimicrobial resistance in *E. coli* O157:H7 bovine strains compared to human strains (Meng *et al.*, 1998; Maurer *et al.*, 2009; Mora *et al.*, 2005).

1.2.7.2. Probiotics

Probiotics are defined as commensal bacteria used to reduce pathogenic bacteria in the gut (Fuller, 1989; Schrezenmeir and de Vrese, 2001). Probiotics have been used in humans to promote intestinal health, and in animals to improve nutrition. Various probiotics (including yeast cultures, competitive exclusion (CE) products, and direct-fed microbials (DFM) have been widely used in the cattle industry (Yoon and Stern, 1996). Some probiotics have been developed to specifically reduce *E. coli* O157:H7 in cattle. A *Lactobacillus acidophilus* (*L. acidophilus*) culture, isolated from cattle rumen, reduced *E. coli* O157:H7 shedding by more than 50% (Brashears *et al.*, 2003; Gragg and Brashears, 2010). This product recently became available on the market and is being used in the cattle industry.

The use of probiotics in the control of EHEC infection of sheep is not well studied. In one study a mixture of the probiotics *S. faecium, L. acidophilus, L. casei, L fermentatum and L plantarum* reduced shedding of *E. coli* O157:H7 with 2-4 log10 CFU/g faeces (Lema *et al.,* 2001).

1.2.7.3. Vaccination

Vaccination to prevent *E. coli* O157:H7 intestinal colonization and faecal excretion should be based on priming of the animal's mucosal immune system against antigens of the bacterium involved in colonization of the gastrointestinal tract. *In vitro* experiments showed that *E. coli* O157:H7 attachment to HEp-2 cells is strongly inhibited by adding sera from cattle immunized with *E. coli* O157:H7 Type III secretion (TTSS) proteins. This finding supported the hypothesis that vaccination with TTSS proteins may reduce intestinal colonization (Asper *et al*., 2007). Several researchers have developed experimental vaccines using bacterial proteins which have a critical role in bacterial adherence to the intestinal epithelial cells of calves for instance using one or more TTSS proteins (Konadu *et al.*, 1999; Dean-Nystrom *et al.*, 2002; Potter *et al.*, 2004). Some of the strategies were promising, while others were not successful in protecting animals from *E. coli* O157:H7 infection (See Table 1). Vaccination with EspA induced high antibody responses but was unable to protect animals against oral challenge (Dziva *et al.,* 2007). Also vaccination with intimin in combination with the putative adhesion factor Efa was unsuccessful (Van Diemen *et al.*, 2007). So far, the most promising vaccinations against *E. coli* O157:H7 infection in literature is with a vaccine containing several TTSS proteins either EspA, EspB and Tir or these proteins combined with intimin (Potter *et al.*, 2004; Naylor *et al.*, 2005a; Naylor *et al.*, 2005b; Peterson *et al.*, 2007a; Peterson *et al.,* 2007b; Nart *et al.*, 2008a; Nart *et al.*, 2008b;) These TTSS proteins could be produced from culture supernatant (Potter *et al.,* 2004). Three vaccinations with maximal 200 µg of culture supernatant proteins significantly reduced the number of bacteria shed in the faeces, the number of animals that shed the bacteria as well as the duration of shedding in experimentally infected cattle (Potter *et al,* 2004). Already after the first immunization significant antibody responses against TTSS proteins appeared (Potter *et al.*, 2004). The effect of the immunization was dose dependent with three doses inducing the highest responses. If a two dose regime was used, the vaccination could not protect or reduce *E. coli* O157:H7 infection in a field trial (Van Donkersgoed *et al.*, 2005). Several reasons may explain the different outcome of different vaccination strategies including the use of different adjuvants, different intervals, different ratios of the antigens in the antigen-mixture and possible variability in pen prevalence of *E. coli* O157:H7 (infection pressure) (Van Donkersgoed *et al.*, 2005; McNeilly *et al.*, 2008). More recently, it was shown that

vaccination with the H7 flagellin could increase and prolong bacterial shedding compared to immunization with TTSS antigens alone. McNeilly *et al.* (2010) found evidence that IgG antibodies against H7 could block the TLR5 binding domain of flagellin (H7) so inhibiting TLR5 activation *in vitro*. This might explain the decreased efficacy of the TTSS proteins vaccine in combination with H7 which significantly reduces the efficacy of TTSS proteins vaccine in cattle (McNeilly *et al*., 2010).

Apart from TTSS proteins and vaccination with *E. coli* O157:H7antigens, recently a novel vaccination strategy has been developed to reduce the ability of Gram-negative bacteria to acquire iron. Results of vaccination of cattle against EHEC with this strategy seem promising. Animals are immunized against the outer membrane siderophore receptor and porin (SRP) proteins, the animals develop antibodies and iron transport into the cell is blocked. Blocking iron transport renders the bacteria into a competitive disadvantage in a mixed microbial environment. Thornton *et al.* (2009) showed that two time vaccinations with *E. coli* O157:H7 SRP vaccine significantly reduced the number of calves that were faecal culture positive for *E. coli* O157:H7 in an experimental infection. However, three vaccinations were more effective than two. A feedlot study of the same group demonstrated that the *E. coli* O157:H7 SRP vaccine reduces the prevalence of *E. coli* O157:H7, the number of days cattle tested positive and the number of days cattle were identified as high-shedders. They also showed that a lower dose of vaccine induced the same effect but the differences between vaccinated and non-vaccinated animals were not significant (Fox *et al.*, 2009).

Table 1: Cattle vaccination strategies against *E. coli* O157:H7

Antigen	Adjuvant and Route	Number of immunizations and the dose	Protection effect	Reference
Exp: Purified EspA, Tir and intimin	QuilA IM	3x (60 µg proteins)	Reduced shedding less than 10^4 CFU/g	McNeilly *et al.,* 2010
Exp: Supernatant proteins (Esps and Tir)	VSA3 SC and IM	3X (200µg proteins)	Reduced shedding, colonization and duration and the number of animals which shedd the bacteria	Potter *et al.,* 2004
Feedlot pen Supernatant (Esps and Tir)	Oil-water emulsion SC and IM	2x (50 µg)	No significant vaccine effect	Van Donkersgoed *et al.,* 2005
Feedlot *E. coli* O157 SRP vaccine	NA SC	2 X (2 or 3 ml of concentrated SRP supernatant)	3ml vaccine: reduced prevalence, duration of shedding, duration of being high shedder (results of 2 ml was the same but not significant)	Fox *et al.,* 2009
Exp Recombinant EspA	Freund incomplete adjuvant IM and Intranasal	3X IM (100 µg) and 1X Intranasal (300 µg)	Not effective	Dziva *et al.,* 2007
Feedlot TTSS proteins (Esp and Tir)	VSA3 SC	2X SC (50 mg protein)	Reduced the probability for *E. coli* O157:H7 colonization of the terminal rectum	Smith *et al.,* 2008
Exp: Recombinant Salmonella Dublin expressing intimin	NA Oral	3X	Reduced shedding and colonization, not associated with an enhanced IgA	Khare *et al.,* 2010
Feedlot *E. coli* O157 SRP vaccine	MVP Oral	2 or 3X 3X vaccination significantly reduced the shedding and colonization (2X : not significant)	Reduced shedding and colonization	Thomson *et al.,* 2009
Feedlot TTSS proteins (Esp and Tir)	VSA3 SC	2X N/A	Reduced the probability for *E. coli* O157:H7 colonization of the terminal rectum	Smith *et al.,* 2009

NA: not applicable. **Exp**: Experimental infection. ***E. coli* O157 SRP vaccine**: the vaccine targeting siderophore receptor and porin proteins (of *E. coli* O157).

CHAPTER 2

Lactoferrin

Lactoferrin (LF) is an 80-kDa iron binding glycoprotein which was first recognized as an unknown "red fraction" from cow's milk by Sorensen *et al.* in 1939. In 1960, the red protein from both human and bovine milk was defined as a transferrin-like glycoprotein (Groves, 1960; Montreuil *et al.*, 1960). Studies of the antimicrobial activities by Arnold *et al.* (1977), of the immunomodulatory activities by Broxmeyer *et al.* (1978) and of the structure by Spik *et al.* (1982) have been recognized as early basic research on human lactoferrin. Subsequently, a lot of research on bovine LF (bLF) was performed, from basic to clinical studies (Tomita *et al.*, 2009). Problems with multiple antimicrobial resistant pathogens and the ban on antibiotics as growth-promoter in Europe, has increased the interest in lactoferrin as an antibacterial protein as well as its availability. At the moment human and bovine lactoferrin are brought on the market by several companies as food additive. Bovine lactoferrin is more available and cheaper than human lactoferrin. It is affordable for big scale operations in animal industries and can be used in human clinical trials even in developing countries.

Several biological functions have now been ascribed to lactoferrin, including iron homeostasis, cellular growth and differentiation, host defence against microbial infection, anti-inflammatory activity and cancer protection. Here we will provide an overview of the current knowledge on the role of lactoferrin in host protection against microbial infections.

2.2. Lactoferrin structure

Lactoferrin belongs to the transferrin family of non-haem proteins. In 1984, the amino acid sequence of human lactoferrin was determined, showing 60% sequence identity with human transferrin (Metzboutigue *et al.,* 1984). The 3D structure of lactoferrin was identified three years later (Anderson *et al.,* 1987) and revealed a single polypeptide chain consisting of two lobes (N-lobe and C-lobe) linked by a short alpha-helix. Each of the lobes binds one ferric atom with very high affinity ($K \sim 10^{22}$ M) (Aisen and Liebman, 1972). Iron binding is partly due to a cooperative interaction between the two lobes. Therefore iron-saturated (holo) lactoferrin becomes a more "closed" protein with more resistance against proteolysis whereas the non-saturated (apo) form is more "open" and relatively flexible in its structure

(Baker and Baker, 2004). The unique iron binding structure of lactoferrin lies in the two domains of each lobe that can bind to one Fe3+ ion and one CO^{-2} ion. The latter may play a role in pH dependent release of iron in low pH conditions (pH 3) (Baker and Baker, 2005; MacGillivray *et al.,* 1998). Lactoferrin is remarkably resistant to trypsin and trypsin-like enzymes making it more resistant to the destructive environment in the digestive tract. However, pepsin is able to cut lactoferrin and some of the pepsin-derived peptides are more potent than the parental protein (see 2.8). Lactoferrin exists in various isoforms: two with RNase activity (namely lactoferrin–β and lactoferrin–γ) and one without RNase activity (named lactoferrin–α). The latter exhibits functional iron-binding ability, whereas the ones with RNase activity show no iron binding activity (Furmanski *et al.*, 1989).

2.3. Source, location and concentration of lactoferrin

Lactoferrin is found in mucosal secretions, including tears, saliva, vaginal fluids, semen, nasal and bronchial secretions, bile, gastrointestinal fluids and urine. However, the highest concentrations are found in milk and colostrum, making it the second most abundant protein after caseins. So, milk is the main source of lactoferrin with 1 mg/ml in natural human milk which is only 8% iron-saturated, while human colostrum contains up to 7 mg/ml (Masson and Heremans, 1971). In bovine, lactoferrin concentration in milk normally varies from 0.01 to 1 mg/ml, depending on to the stage of lactation (Cheng *et al.*, 2008; Newman *et al.*, 2009). There is a great variation in the concentration of lactoferrin in other body fluids. The concentration in human tears is as high as 2 μg/ml whereas blood contains normally only 1 μg/ml, although it can rise as high as 200 μg/ml in an inflammatory situation (Masson and Heremans, 1971). Lactoferrin is also found in considerable amounts in secondary neutrophil granules, where it plays a significant physiological role (Bennett and Kokocinski, 1978). Neutrophils are rapidly acquired to the site of inflammation during the acute phase of microbial infection where rich lactoferrin granules play a role in the innate immune response against bacterial infections. As a result, the concentration of lactoferrin in biological fluids may greatly increase during the inflammatory response.

2.4. Antibacterial effect: Mechanisms of action

The antibacterial activity of lactoferrin has been widely documented both *in vitro* and *in vivo* for Gram-positive and Gram-negative bacteria. It has been shown that lactoferrin decreases

bacterial growth and this bacteriostatic function is due to its ability to take up the Fe3+ ion and limit the use of this nutrient by bacteria at the infection site. The strong iron-binding properties of lactoferrin, coupled with its iron-free state in body secretions and neutrophil granules, allows the protein to sequester free iron and maintain an environment refractory to microbial growth (Levay and Viljoen, 1995; Ward *et al.*, 2005).

In 1977, Arnold *et al.* suggested that lactoferrin could exert an antibacterial action distinct from a simple iron deprivation. Besides this bacteriostatic effect, lactoferrin exerts a direct bactericidal activity against pathogens, resulting in disruption of LPS in the bacterial cell wall with an associated increase in membrane permeability (Ellison *et al.,* 1988). Hereto binding occurs between the cationic N-terminal part of lactoferrin and the phosphate group within the lipidA part of the bacterial membrane. Interestingly, it may be noted that most interactions of lactoferrin with cell receptors and inflammatory molecules also involve the N-terminal domain of lactoferrin.

Besides the bacteriostatic and bactericidal activities of lactoferrin, also some additional activities on bacterial attachment can have important role in the antimicrobial activity of this protein (summarized in Table 2).

Table2: Biological activity of lactoferrin on bacterial attachment to host cells

Strain	Mode of action	Lactoferrin origin	Effect	Reference
Shigella flexneri	Degradation of invasion plasmid antigens IpaB and IpaC, the key proteins responsible for bacteria-directed phagocytosis by mammalian cells	Human lactoferrin	Reduce bacterial attachment	Gomez *et al.,* (2002)
Haemophilous influenzae	Cleavage and removal of two putative colonization factors: IgA1 protease protein and the Hap adhesin	Human lactoferrin	Reduce bacterial attachment	Hendrixson *et al*., (2003)
Enteropathogenic *E. coli* (EPEC)	Proteolysis of EspA, EspB and EspD	Human lactoferrin Bovine lactoferrin	Reduce bacterial attachment	Ochoa *et al.,* (2003, 2004)
Enterotoxigenic *E. coli* (ETEC)	Binds to fimbrial colonization factor adhesion (CFA) I	Human lactoferrin Bovine lactoferrin	Reduce bacterial attachment	De Oliveira *et al.,* (2001) Giugliano *et al.,* (1995) Kawasaki *et al.,* (1999)
Salmonella Typhimurium	Possible direct interaction with bacterial surface	Human lactoferrin Bovine lactoferrin	Reduce bacterial growth and attachment	Ochoa *et al.,* (2007) Bessler *et al.,* (2006)
Chlamydophila psittaci	Possible interactin with TTSS	Human lactoferrin Bovine lactoferrin Avian transferring (Lactoferrin)	Reduce bacterial growth and attachment	Beeckman *et al.,* (2007)
Streptococcus mutans	Binding to the bacterial surface	Decaseinated human colostral whey	Reduce biofilm formation	Arnol *et al*., 1981 Dalmastri *et al.,* 1988 Bortber *et al.,* 1989
Pseudomonas aeruginosa	Inhibiting biofilm formation by decreasing the expression of cellulose	Bovine lactoferrin	Inhibit biofilm production	Odeh *et al.,* 2000 Xu *et al.,* 2010

2.5. Effect on bacterial adhesion: *in vitro* studies

In 1995 Alugupalli *et al.* showed that human and bovine lactoferrin can both reduce *Actinobacillus actinomycetemcomitans* adhesion to HEp-2 and Hela cells in a dose-dependent manner. It was the first report to suggest a kind of adhesion-counteracting mechanism in addition to lactoferrin's bacteriostatic and bactericidal properties (Alugupalli and Kalfas, 1995; Alugupalli *et al.*, 1995). It was hypothesized that the decreased adhesion may be due to blocking of both specific adhesin-ligands interaction sites as well as non-specific charge-dependent interactions (Alugupalli and Kalfas, 1997).

It has been shown that lactoferrin is capable of inhibiting the intracellular invasion of pathogens such as *Escherichia coli, Listeria monocytogenes* and *Shigella flexneri*. An *in vitro* adhesion-invasion study (Conte *et al.,* 1999) showed that bovine lactoferrin decreases the number of *L. monocytogenes* internalized by Caco-2 cells. They found two bacterial surface proteins, of approximately 80 and 60 kDa, which bind to lactoferrin. These findings strongly support the hypothesis that the anti-invasive mechanism of lactoferrin is due to its interaction with bacterial proteins.

A proteolytic effect of lactoferrin was first reported by demonstrating that lactoferrin attenuates the pathogenicity of *Haemophilus influenzae* by cleavage and removal of two putative colonization factors, namely the IgA1 protease protein and the Hap adhesin (Qiu *et al.,* 1998). Lactoferrin acts as a serine protease capable of cleaving arginine-rich sequences. Proteolytic activity of lactoferrin has also been demonstrated in *Shigella* and EPEC where lactoferrin induces release and degradation of key virulence proteins contributing to bacterial attachment to host cells. In *Shigella*, invasion plasmid antigens B and C (IpaB and IpaC), which are necessary for the adhesion to and subsequent invasion into the host cell, are degraded in presence of lactoferrin. In EPEC, lactoferrin degrades EspA and EspB, which contribute to initial attachment of bacteria to host cells (Ochoa and Clearly, 2004; Ochoa *et al.,* 2004; Ochoa *et al.,* 2003). In *Chlamydophila psittaci* (*Cp.*), lactoferrin irreversibly inhibits bacterial attachment to and entry into HD11 cells. The latter is accompanied by a dose-dependent reduction of actin recruitment at the bacterial entry site. However, once bacteria have entered the cells, lactoferrin apparently has no effect on intracellular replication. Interestingly, the attachment inhibition of *Chlamydia* to the chicken cell line is more

pronounced when ovoTF (Lactoferrin from poultry) is used than using human or bovine lactoferrin (Beeckman *et al.*, 2007).

2.6. Effect of oral lactoferrin on bacterial attachment and colonization

Whereas the antibacterial effect of lactoferrin has been very well studied *in vitro* by several investigators, there is little proof of this activity *in vivo*. Most of these studies have been performed in mice. In germ-free mice, oral administration of bovine lactoferrin inhibited adherence of *E. coli* to the intestinal tract (Kawasaki *et al.,* 2000). In addition, daily administration of 2.5 mg lactoferrin decreased hepatic colonization of *Listeria monocytogenes*, hepatic necrosis and expression of inflammatory cytokines including IL-1, TNF-α and IFN-γ in mice compared to non-treated mice in an oral infection model (Lee *et al.*, 2005). Daily oral administration of 2 mg bovine lactoferrin to mice from 2 hours before until 7 days after inoculation, could control an experimental *Salmonella* Typhimurium infection and reduced the severity, mortality and the degree of inflammation of this infection (Mosquito *et al.*, 2010). It was speculated that lactoferrin bound to LPS with disruption of the TTSS, blockage of actin polymerization, and stimulation of the immune system.

In rabbits recombinant lactoferrin could protect the animals against *Shigella flexneri*-induced inflammatory enteritis (Gomez *et al.*, 2002).

So the *in vivo* effects of lactoferrin have mainly been analyzed in rodent models. It should be noted that the mechanisms of action and more importantly the immune system in laboratory animals may vary from that of the true infection host.

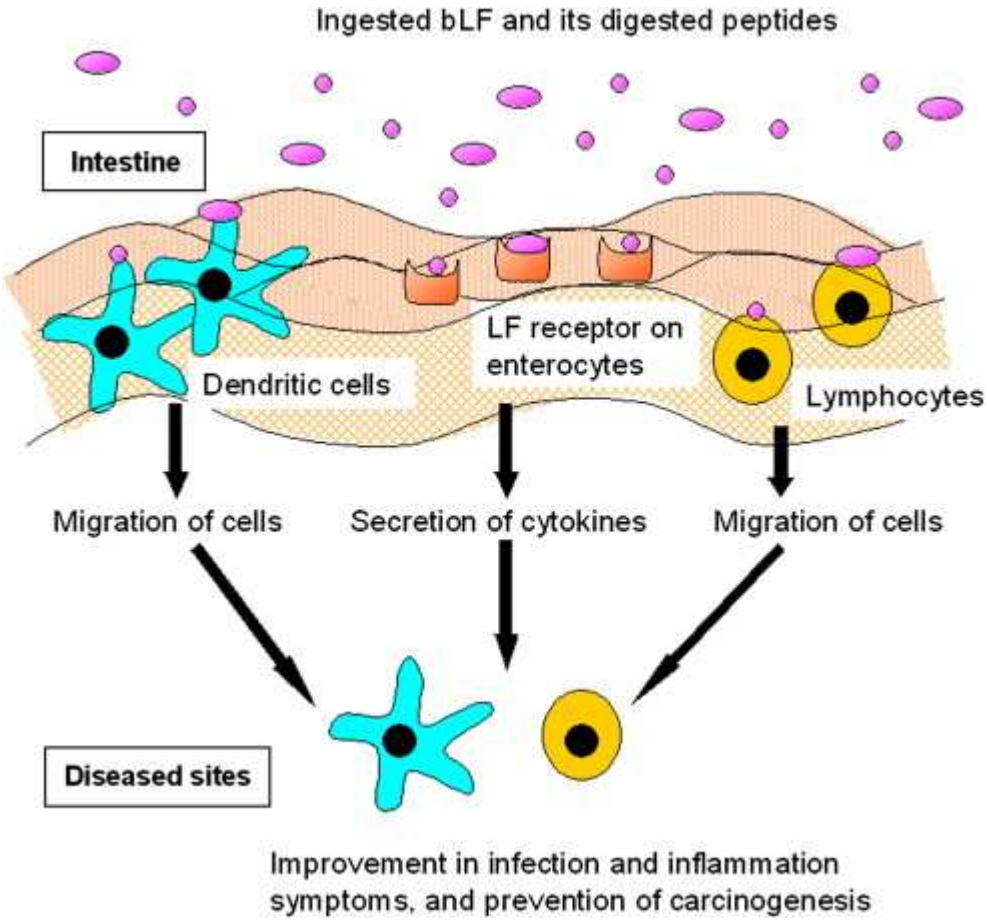

Figure 6: Deduced mechanism of action underlying the host-protective effects of orally administered bovine LF (bLF). After ingestion, bLF is partially digested to peptides by proteases in the stomach and intestine. In the small intestine, bLF and its peptides bind to receptors on enterocytes and immune cells such as dendritic cells and lymphocytes residing in the intestinal epithelium. bLF/peptides may be internalized into the cells and/or trigger intracellular signaling so activating transcription of genes. Humoral factors like cytokines become secreted and act on neighbouring cell or can reach via the circulation other target cells. The stimulated immune cells can subsequently migrate to infected and inflamed sites where they try to control the infection and inflammation and where they can also act to prevent carcinogenesis (Adapted from Tomita *et al.,* 2009).

2.7. Evidence for lactoferrin modulation of the immune system: cytokine production

Immunomodulatory effects of lactoferrin have been illustrated in several *in vitro* and *in vivo* experiments. Lactoferrin modulates the inflammatory process mainly by preventing the release of cytokines, which induce recruitment of immune cells to inflammatory sites as well as their activation. Some *in vivo* studies showed a protective effect in inflammatory processes such as septic shock, allergy or cancer (Figure 6). Injection of lactoferrin

(intraperitoneal or intravenous) could protect against lethal bacteraemia (Lee *et al.*, 1998; Zagulski *et al.*, 1989). Other studies showed that lactoferrin administration reduces gastritis induced by *Helicobacter felis* in mice and protects gut mucosal integrity during LPS-induced endotoxemia (Dial *et al.*, 2000a; Dial *et al*., 2000b; Kruzel *et al.*, 2000).

At the molecular level, lactoferrin inhibits LPS-induced IL-6, TNF-α and IL-1β expression in mice (Mattsby-Baltzer, 1996; Choe and Lee, 2000; Haversen *et al.,* 2002). Additionally, lactoferrin up-regulates the secretion of anti-inflammatory cytokines IL-10 and IL-4 in rats with colitis (Guillen *et al.*, 2002; Togawa *et al.*, 2002a; Togawa *et al.*, 2002b). Guillen *et al.* (2002) showed an enhanced Th1 response to *Staphylococcus aureus* infection by modulation of the iron supply to the spleen in lactoferrin transgenic mice. It is most likely that the immunomodulatory effect of lactoferrin is somehow related to its cell binding property. In this respect, the capacity of lactoferrin to influence cytokine production is at least partly due to its binding to both LPS and the LPS-receptor CD14 (Figure 7).

LPS is a potent activator of the immune system and stimulates host cells, mainly monocytes/macrophages and neutrophils, to produce cytokines (Elass-Rochard *et al.,* 1998). Therefore the high affinity binding of lactoferrin to LPS prevents activation of the pro-inflammatory pathways (Elass-Rochard *et al.*, 1998; Otsuki *et al.*, 2005). Lactoferrin competes with LPS-binding protein (LBP) for LPS binding and therefore prevents transfer of LPS to membrane CD14, the membrane receptor of LPS presented at the surface of macrophages (Elass-Rochard *et al.*, 1998). Furthermore, the interaction between lactoferrin and soluble CD14 (sCD14), the secreted receptor of LPS, inhibits the secretion of inflammatory cytokines by endothelial cells induced by the sCD14-LPS complex.

More importantly, a high-affinity interaction was reported between lactoferrin and membrane CD14 (mCD14) expressed on monocytes, suggesting a direct effect via receptor-mediated signalling pathways including the NF-kB pathway (Haversen *et al.*, 2002). The LPS-neutralizing effect of lactoferrin together with the great iron binding capacity which reduces the availability of iron and subsequently the toxic oxidative reactions in inflammation sites, would at least partly explain the mechanism by which lactoferrin modulates immune responses in inflammatory processes.

In contrast with the anti-inflammatory role of lactoferrin, its effect as an inflammatory stimulator has recently attracted the attention. Hwang *et al.,* (2009) showed that mice

immunized with the Bacillus Calmette-Guerin (BCG) vaccine in the presence of bovine lactoferrin demonstrated increased host protection after challenge with *Mycobacterium tuberculosis*. This protection was induced by an enhanced T-cell specific response, suggesting that bovine lactoferrin facilitates efficacy of BCG vaccination via development of Th1 cells. It has also been demonstrated that the development of Th1 and Th2 in response to lactoferrin occurs by a relative increase in the production of IL-12 while decreasing IL-10, a negative regulator of IL-12 (Fischer *et al.*, 2006; Wakabayashi *et al.*, 2006; Hwang *et al.*, 2007).

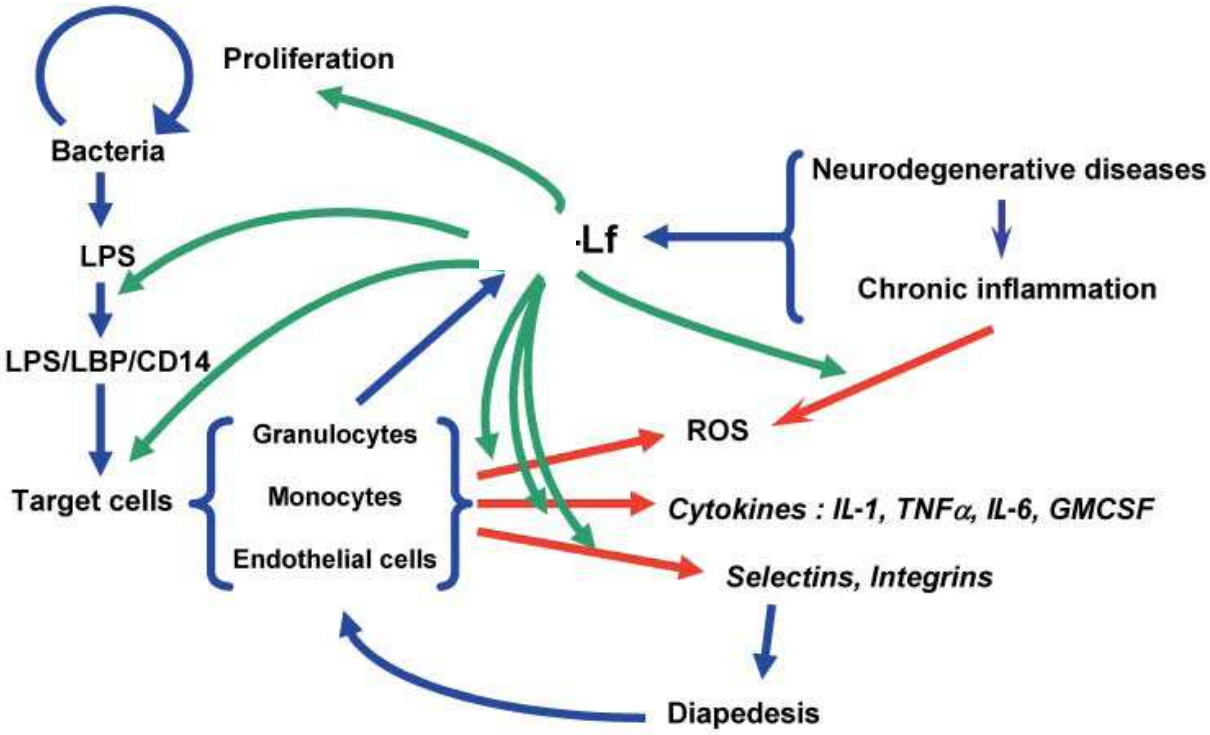

Figure 7. Regulation of the inflammatory response by Lf. Blue arrows indicate physiological processes. Red arrows indicate biological responses induced by infection, aggression or neurodegenerative diseases and green arrows indicate downregulation induced by the release of lactoferrin upon inflammatory process (Adapted from Legrand *et al.,* 2005)

2.8. Lactoferricin

Lactoferricin (LFcin) was initially identified as a cationic antimicrobial peptide derived *in vitro* via pepsin digestion from lactoferrin (Tomita *et al.*, 1991). Later on, the presence of this peptide was reported in human stomach fluid, confirming the occurrence of pepsin digestion of lactoferrin *in vivo* (Kudva *et al.,* 1998).

LFcin consists of the N-terminal region of LF which is believed to be involved in many LF functions. Various synthetic analogues of LFcin have been reported. LFcin H and LFcin B were isolated from pepsin digestion products of human LF and bovine LF respectively, and it has been shown that the antimicrobial activity of LFcin B is greater than that of LFcin H (Chen *et al.*, 2006). LFcin displays higher antimicrobial activity than its parent protein lactoferrin (Bellamy *et al.*, 1992; Bellamy *et al.*, 1993a; Bellamy *et al.*, 1993b; Kuwata *et al.*, 1998; Munoz and Marcos, 2006; Haney *et al.*, 2007). Although it lacks the iron-binding region of lactoferrin, it can rapidly bind to bacteria in a pH-dependent manner (Bellamy *et al.,* 1993a; Bellamy *et al.*, 1993b) and induce the loss of membrane integrity (Yamauchi *et al.*, 1993). Following its binding, LFcin crosses the bacterial membrane to interact with the bacterial cytoplasmic membrane. Little is known about this process, but it has been shown that this binding results in LFcin entry to the bacterial cell and acts on intracellular targets (Yamauchi *et al.*, 1993; Shin *et al.,* 1998). Moreover, it has been shown that LFcin binding to some target molecules in *E. coli* can inhibit RNA and DNA synthesis and also expression of bacterial proteins (Ulvatne *et al.,* 2004).

2.9. Effect of lactoferrin on *E. coli* O157:H7

It has been shown that lactoferrin and its peptides reduce/inhibit *E. coli* O157:H7 growth, however this effect is variable in different studies. This variation could be related to differences in strain susceptibility, lactoferrin purity, its iron saturation level, and difference in amounts of cations present in the media used (Al-Nabulsi and Holley, 2006).

Griffiths *et al.* (2003) showed that non-iron saturated bovine and human lactoferrin reduced *E. coli* O157:H7 growth, but after 24 hours treated and non-treated bacteria reached the same stationary growth phase. However, iron saturated human and bovine lactoferrin had no antibacterial effect on *E. coli* O157:H7. For 66% iron saturated bovine lactoferrin could reduce bacterial growth.

Another study using *E. coli* O157:H7 and 15% iron saturated bovine lactoferrin demonstrated that even up to 5000 µg/ml of lactoferrin was not inhibitory on *E. coli* O157:H7 growth. It could only reduce growth (Murdock *et al.*, 2007). This finding was confirmed by another group studying the effect of lactoferrin on *E. coli* O157:H7 growth, indicating that

concentrations from 6000 µg/ml are inhibitory to *E. coli* O157:H7 under similar assay conditions (Murdock and Matthews, 2002).

Unfortunately, the above reviewed antimicrobial effect of lactoferrin on *E. coli* O157:H7 in simple broth systems such as peptone or buffered phosphate has not been achieved in food or complex media because the antimicrobial activity of lactoferrin appears to be reduced in the presence of divalent cations like Ca^{2+} and Mg^{2+} (Ellison *et al.*, 1988; Shimazaki *et al.,* 2000). Shimazaki (2000) reported that divalent cations protect bacteria from lactoferrin by inducing changes in its tertiary structure yielding a tetrameric form of lactoferrin with reduced bio-functionality and at the same time generating bacterial cell membranes with increased stability (Shimazaki *et al.,* 2000). Recent studies suggest that increased NaCl concentration and lower temperatures improve the antimicrobial effect of lactoferrin towards *E. coli* O157:H7 (Al-Nabulsi and Holley, 2006).

In addition to lactoferrin, some of its peptides can also reduce *E. coli* O157:H7 growth.

Shin *et al.* (1998) studied the antimicrobial activities of bLF, its pepsin hydrolysate (bLFH) and the active peptide LFcin B against four clinical isolates of *E. coli* O157:H7. They showed membrane blisters on the cell surface of two bacterial strains after treatment with Lfcin B for 30 min. A progressive increase in cytoplasmic debris was also observed after 60 and 120 min. This finding confirmed that Lfcin B exerts its bactericidal effect on *E. coli* O157:H7 by acting on the bacterial cell surface initially, and then on the cytoplasmic contents. They also showed that 1 µg/ml Lfcin B in 1% Bactopeptone suppressed the growth of *E. coli* O157:H7 within 3 h. This peptide reduced the number of viable cells from approximately 10^6 cfu/ml, to the limit of detection (20 cfu/ml) within 3 h at concentrations above 10 µg/ml. The results indicate LfcinB as the most effective peptide which inhibits *E. coli* O157:H7 growth. Changing either the pH between 5.5 and 7.2 or the temperature from 4 till 10 °C in 1% Bactopeptone could not influence the growth inhibitory effect of bLFcin (Venkitanarayanan *et al.*, 1999).

Part II: Aims of the Study

Aims of the study

An EHEC infection, especially with the serotype O157:H7, can lead to bloody diarrhea, HUS, HC and even death in humans. Until now, there is neither a specific treatment available nor a vaccine or therapeutic agent which completely prevents *E. coli* O157:H7 infection. Antibiotic therapy does not represent a valid alternative, since it might lead to the release of the Stx from the bacteria resulting in even more severe symptoms.

The majority of the human infections are foodborne and the origin of the *E. coli* O157:H7 is mainly ruminant's feces. In this context, reduction, inhibition or clearance of an intestinal colonization of ruminants may be the strategy to protect humans. Although many intervention strategies have been studied to reduce the level of *E. coli* O157:H7 colonisation in ruminants, non of those strategies seems to be sufficient effective.

The aim of the present thesis was to evaluate two different strategies to reduce *E. coli* O157:H7 colonisation of sheep namely the use of a natural antimicrobial protein and vaccination.

Hereto the following questions were addressed:

1. What is the pattern of antibiotic resistance among *E. coli* isolates from Iranian dairy cows (Chapter 3)?
2. Can lactoferrin, a natural antimicrobial protein in milk, be used to prevent colonisation of *E. coli* O157:H7 in sheep?
    a. Does lactoferrin influence *E. coli* O157:H7 growth and if so, which mechanisms could be involved (Chapter 4)?
    b. Does lactoferrin reduce *E. coli* O157:H7 attachment to a human epithelial cell line (Chapter 4) and if so, is this also the case for adhesion to sheep epithelial cells of explants from different sites of the intestinal tract (Chapter 5).
    c. Can lactoferrin influence the cytokine response induced by *E. coli* O157:H7 in these explants (Chapter 5)?
    d. Does lactoferrin reduce *E. coli* O157:H7 excretion in sheep and does it influence the immune response in these animals (Chapter 6)?
3. Is vaccination of sheep with type III secretion system proteins and intimin γ a possible strategy to reduce fecal shedding of *E. coli* O157:H7 as in cattle (Chapter 7)?

Part III: Experimental studies

Chapter 3

Antimicrobial resistance of *Escherichia coli* isolates from dairy cows in Iran

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(Manuscript in preparation)

3.1. Abstract

In the present study, 63 *Escherichia coli* (*E. coli*) isolates from raw milk and faeces of healthy dairy cows from West Central Iran were assessed for their susceptibility to 11 antimicrobial agents used in Iran: ampicillin, amoxicillin-clavulanic acid, ceftiofur, chloramphenicol, colistin, enrofloxacin, florfenicol, spectinomycin, sulfafurazole, tetracycline and trimethoprim. Seventy-five percent of the isolates showed acquired resistance to three or more antimicrobial agents. The highest percentage of acquired resistance was detected for tetracycline (46%), followed by ampicillin (43%), the highest susceptibility was found to ceftiofur (94%) followed by colistin (86%). Even though resistance against colistin is low, it is higher than in most other studies. The overall high antimicrobial resistance and a high multiple antimicrobial resistance of the commensal *E. coli* should alert veterinarians and authorities to take measures for decreasing antimicrobial usage.

3.2. Introduction

Acquired antimicrobial resistance is an increasing threat to human and animal health. Several data suggest that antimicrobial use in live stock industry may have an impact on antimicrobial resistance of human bacterial pathogens (Bywater *et al.*, 2004; Aarestrup *et al.*, 1998). One possible route of transfer of resistance genes from animal-associated to human-associated bacteria is through the presence of resistant commensal bacteria in the food chain (Guillemot, 2001). *Escherichia coli* (*E. coli*) is an important commensal of the intestinal tract of different animal species and humans, and may serve as a reservoir for antimicrobial resistance genes. Internationally, it is often used as an indicator bacterium reflecting resistance levels present in Gram-negative bacterial populations (Osterblad *et al.,* 2000). Animal-associated *E.coli* strains may be present on the carcass of slaughtered animals and in raw milk, especially if collected in less hygienic conditions. Resistance genes from these strains may be transferred to bacteria belonging to the normal commensal human microbiota through handling and consumption of meat and milk (Smet *et al.*, in press).

Currently, very little data are available regarding the presence of antimicrobial resistance in animal-associated commensal bacteria in developing countries such as Iran, where antimicrobial agents are often overused in veterinary medicine and especially in food animals such as cattle. Therefore, in the present study, the presence of antimicrobial resistance in *E. coli* isolates from raw milk and faeces of healthy dairy cows was studied.

3.3. Materials and methods

3.3.1. Isolation and identification of *E. coli*

Thirty-nine and 24 *E. coli* isolates were obtained from raw bulk milk and faecal samples, respectively. Samples were collected between March and September 2007 in West Central Iran (Chaharmahal Bakhtiari province) on 35 dairy farms with an average of 50 cows per farm. For raw bulk milk samples, 200 ml tank milk was collected. The faecal samples were collected from healthy dairy cows using rectal swabs. Milk samples and swabs were transported on ice to the lab where samples were plated onto Mac Conkey agar (Oxoid, Basingstoke, United Kingdom) and incubated aerobically for 20 h at 37°C. The isolates were identified as *E. coli* by colony morphology and standard biochemical methods (Quinn *et al.*, 1994).

The strains were transported to Belgium in bacterial transport tubes (Venturi Transystem, Copan, Brescia, Italy) under a transport licence of the"Federal Agency for the Safety of the Food Chain" (FASFC) (n.191362). At the day of arrival, bacteria were cultured on Luria Bertani (LB) agar at 37°C, and subsequently confirmed as *E. coli* by positive glucose/lactose fermentation, gas production, absence of H_2S production (Kligler iran agar; Oxoid), indole production and absence of aesculin hydrolysis (bile aesculin agar; Oxoid). Then one colony was picked up and incubated overnight in LB broth at 37°C, whereafter 0.6 ml of the broth was mixed with 0.6 ml of sterile glycerol in a 2 ml Sarstedt tube (Corning, Mexico) and frozen at -80°C until tested for antimicrobial susceptibility.

3.3. 2. Antimicrobial susceptibility by agar dilution method

A quantitive agar dilution method using Mueller Hinton II agar (Becton, Dickinson and Company, Cockeysville, US) supplemented with 5% lysed horse blood was used for determination of the minimal inhibitory concentrations (MIC) of 11 antimicrobials (ampicillin, amoxicillin-clavulanic acid, ceftiofur, chloramphenicol, colistin, enrofloxacin, florfenicol, spectinomycin, sulfafurazole, tetracycline and trimethoprim) as described by De Leener *et al.* (2005). Hereto, two-fold dilutions of the antimicrobials were incorporated in the agar in final concentrations ranging from 0.03 till 128 µg/ml, except for the sulfonamide sulfafurazole for which additionally the concentrations of 256, 512 and 1024 µg/ml were tested. Clinical

Laboratory Standards Institute (CLSI) standards guidelines were followed for inoculum standardization, medium and incubation conditions and internal quality organisms (CLSI, 2009). *E. coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213 were used as internal control strains. The inoculum was standardized in phosphate-buffered saline to 0.5 McFarland (Densimat Biomerieux, France) and triple streaked on the agar. MIC values were recorded after incubation for 16-18 h at 35°C and were determined as the lowest concentration that inhibited visible growth. The strains were considered to have acquired resistance when their MIC was higher than the wild type cut-off value as described by EUCAST (2009).

3.4. Results and discussion

E. coli are prevalent enteric bacteria in healthy animals which rapidly acquire antimicrobial resistance and therefore are internationally used as Gram-negative indicator bacteria for monitoring the selection pressure on Gram-negative bacteria exerted by antibiotic use (Van den Bogaard and Stobberingh, 2000; Saenz *et al.,* 2001). Their high prevalence increases the risk of a transfer of these strains between animals or from animals to man via faecal contamination (Costa *et al.,* 2008). These resistant commensal *E. coli* provides a pool of transferable resistance genes (Schmieger and Schicklmaier, 1999; Winokur *et al.,* 2001; (Smet *et al.*, in press).

The distribution of the MICs for the 63 *E. coli* isolates is given in Table 1. For 93% of these isolates, resistance to at least one of the antimicrobial agents was detected. The highest resistance occurred against tetracycline (46%) followed by ampicillin (43%) and the lowest against ceftiofur (6%), followed by colistin (14%). For the other antibiotics resistance varied between 30 and 17%. Recently a survey in five European countries of antimicrobial susceptibility towards human-use antimicrobials was published demonstrating a high susceptibility of faecal commensal *E. coli* isolated from cattle at the slaughterhouse to most of the tested antimicrobials (de Jong *et al*., 2009). Nevertheless, there were difference between countries with a significantly higher resistance in Italy for e.g. tetracycline (20%), ampicillin (12%) and trimetroprim/sulfamethoxazole (11%) than in Germany, France, Ireland and the UK. However, this is still clearly lower than in our study. In the European study, resistance was overall the highest for tetracycline going from 20% in Italy to 3% in the UK. Also in the Iranian isolates, tetracycline resistance was most prevalent, but resistance was

more than two times as high as in Italy. In the European study, no resistance was observed against colistin, whereas in the Iranian isolates 14% of the isolates where resistant.

Higher resistance rates than in Europe were observed in a recent study in the western United States (Berge et al., 2010). Faecal samples were collected on farms. Again, the highest resistance was observed against tetracycline (50 %), followed by streptomycin (47%), sulfisoxazole (45%) and ampicillin (31%). Resistance against the other tested antibiotics was much lower and varied between 16 and 1%. Colistin was not tested. The study compared the prevalence of multiple antimicrobial resistance between different farm types namely calf ranches, feedlots, dairies and beef cow-calf operations. The lowest multiple antimicrobial resistance was observed in isolates from dairies and from beef cow-calf operations. Within dairies, the antimicrobial resistance was higher in calves than in adult cows, probably reflecting the higher exposure of calves to antimicrobials. In our study, samples were collected from cows. Increase in antimicrobial resistance by farm type has been described in other studies and could reflect differential antimicrobial selective pressure on the faecal E. coli by these different farm types (Sawant *et al*., 2007; Call et *al., 2008*).

Only few data have been published on the use of antibiotics in veterinary medicine in Iran (Salehi and Bonab, 2006; Moniri *et al*., 2007). These data and information obtained via personal communications suggests that sulfonamides, beta-lactams, tetracyclines and enrofloxacin are the most frequently used antimicrobials in veterinary medicine in Iran, followed by trimethoprim, spectinomycin and aminoglycosides. This could be one of the reasons for the very high resistance in our study against ampicillin and tetracycline followed by amoxicillin-clavulanic acid, trimethoprim and suflafurazole (Table 1).

Nevertheless, except for ceftiofur, there was also an important resistance of the Iranian isolates against the other tested antimicrobials. For florfenicol, only 10 years ago introduced into the veterinary practice in Iran (Zahraei *et al*., 2006), resistance was clearly higher in our study (20%) (Table 1) than observed by Sawant *et al*. (2007) for *E. coli* isolates from dairy cattle in Pennsylvania (5%).

Also for colistin, a remarkably high resistance was observed (14%). In the past, acquired resistance to colistin has only occasionally been described. However, the last years, this is becoming more common (Boyen et al.,2010) . In the present study 14% of the *E. coli* isolates were resistant to colistin which was higher than in most previous studies (Harada *et a*l., 2005, Wang *et al.,* 2008, de Jong *et al*., 2009; Stannarius *et al.*, 2009; Boyen *et al*., 2010). Harada *et*

al. (2005) observed a higher resistance in isolates from pigs (36%) as in isolates from cattle (12%) using 2 µg/l as cut-off and Magwira *et al*. (2005) found a high resistance in O157:H7 isolates from beef products (26 %). We found eight isolates (12.6%) resistant to 16 µg/ml colistin and one isolate from bulk milk was even resistant to 32 µg/ml colistin. A previous study in Iran by Ebrahimi *et al.* (2007a), using 4 µg/ml as cut-off, found even 53% of 17 *E. coli* isolates from mastitis resistant against colistin. This high percentage of resistance against colistin in Iranian pathogenic *E. coli* together with our finding in commensal *E. coli* isolates, seems to suggest that colistin is extensively used in Iran. It highlights the urgent need to perform constant monitoring of *E. coli* isolates for resistance against this antimicrobial agent. As in our study, in most other studies, susceptibility of E. coli isolates to cephalosporins such as ceftiofur is very high, varying between 89 and 100% (Hariharan *et al.*, 2004; Harada *et al*., 2005; Sawant *et al*., 2007; de Jong *et al*., 2009; Berge *et al*., 2010).

Although the overall antimicrobial resistance in the present study was high, it was still lower than in a previous Iranian study (Ebrahimi *et al.,* 2007a). In the latter study only pathogenic *E. coli* have been studied. Pathogenic *E. coli* tend to be more resistant to antimicrobials than commensal *E. coli*, due to constant exposure to antimicrobials (Chulasiri and Suthienkul, 1989; Holland *et al.*, 1999).

The multi antimicrobial resistance (MAR) rate of isolates in our study was higher than has been reported for pathogenic *E. coli* from humans in Iran (Moniri *et al.,* 2003). In the present study 75% of the isolates were resistant to al least three, 43% to at least four and 32% to at least five antimicrobials. In a study on dairy cattle in Pennsylvania, MAR (resistance to at least 3 antimicrobials) was observed in 40% of the *E. coli* isolates (Sawant et al., 2007), in a study in western United States in < 48% *E. coli* isolates (Berge et al., 2010) and in a European study 0,2 % of the isolates showed MAR against at least four antimicrobials (de Jong et al., 2009).

We are aware that our study shows serious limitations. The sample size was rather small not enabling us to perform statistical analysis and no information on usage of antibiotics within the herds was available. More continuous sampling on more animals of different age groups (calves versus cows), at different time points throughout the year and in more Iranian provinces with a questionary on antimicrobials usage, housing, management, etc., should allow us to generalize results and to analyze some of the factors responsible for the antimicrobial resistance determined.

There still is debate on the risks antimicrobial resistance in non-pathogenic animal *E. coli* exerts for public health. Nevertheless, the potential of animal-associated strains to transfer resistance genes to the normal commensal human microbiota has been demonstrated *in vitro* (Zhao *et al.*, 2001; Costa *et al.*, 2008, Smet *et al.*, in press). Irrespective of such *in vitro* prove, given the uncertainty principle, measures should be taken to reduce antimicrobial resistance in production environments. Therefore, the high prevalence of antimicrobial resistance and the multiple antimicrobial resistance in the Iranian *E. coli* isolates observed in our study should alert the veterinarians and authorities to take all necessary measures for decreasing antimicrobial usage in Iran.

3.5. Acknowledgements

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Table 1: Distribution of minimal inhibitory concentrations (MIC) for 63 *Escherichia coli* isolates from milk and faecal samples of 35 Dairy farms in the Iranian province of Chaharmahal Bakhtiari.

	MIC WT[a]	Number of isolates[b] with MICs (µg/ml)																		**% R**[c]
Antimicrobial agent	**µg/ml**	**≤0.03**	**0.03**	**0.06**	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**>128**	**256**	**512**	**1024**	**>1024**	
Amoxicillin-clavulanic acid (2/1)	**16**								3	23	19	**5**	**2**	**8**	**1**	**2**	_	_	_	_	29
Ampicillin	**16**								1	19	16	**2**	**1**	**10**	**1**	**13**	_	_	_	_	43
Ceftiofur	**2**				3	18	31	7	**2**	**1**		**1**					_	_	_	_	6
Chloramphenicol	**32**								2	5	39	6	**5**			**6**	_	_	_	_	17
Colistin	**4**					4	50					**8**	**1**				_	_	_	_	14
Enrofloxacin	**0.25**	37		10	2	**5**	**1**	**1**				**6**			**1**		_	_	_	_	22
Florfenicol	**32**								4	5	30	10	**4**		**5**	**5**	_	_	_	_	22
Spectinomycin	**128**											9	36	7	**7**	**4**	_	_	_	_	17
Sulfafurazole	**512**[d]			2					1		2	10	17	5	8	_	1			**17**	27
Tetracycline	**16**							6	26	1	1	**6**	**2**	**8**	**13**		_	_	_	_	46
Trimethoprim	**4**	1		1	7	31			4	**7**					**1**	**11**	_	_	_	_	30

[a] MIC for the wild-type (WT) organism according to EUCAST in µg/ml.

[b] The results in bold show number of isolates with MIC that exceeds the breakpoint criteria for resistance.

[c] %R, percentage of isolates showing acquired resistance to the respective antimicrobial agent.

[d] MIC is not available in EUCAST. The MIC for CLSI has been used.

_: not applicable.

Chapter 4

Lactoferrin inhibits *E. coli* 0157:H7 growth and attachment to intestinal epithelial cells

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4.1. 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 for hLF. Nevertheless, both lactoferrins had bacteriostatic effect even at high concentration (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 proteolytic effect of lactoferrins on the type III secreted proteins EspA and EspB.

4.2. Introduction

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 “attaching and effacing” (A/E) lesion characterized by localized destruction of brush border microvilli and intimate attachment of the bacteria to the 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 strong interaction between bacteria and host cells (Cookson and Woodward, 2003; Vilte *et al.,* 2008). Virulence arises also from Shiga toxins- production, coded by Shiga toxin genes (*stx1* and *stx2*), which are the primary factors responsible for the hemorrhagic aspect of the 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 an *E. coli* O157:H7 infection without sequelae. Children and the elderly however are more likely to develop 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 treatment. 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 rendering the price more feasible, especially for developing countries.

Lactoferrin exhibits anti-oxidant, antiviral, anti-inflammatory, immune modulating as well as anti-cancer activities, and interestingly can promote the growth of probiotic bacteria such as *Bifidobacterium* (Aguila *et al.,* 2001; Al-Nabulsi and Holley, 2007; Jenny *et al.,* 2010; Tsuda *et al.,* 2010; Xu *et al.,* 2010). Lactoferrin's bacteriostatic effect is due to its ability to

bind iron and limit its availability in the growth environment (Orsi, 2004). Binding of lactoferrin 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).

4.3. Material and methods

4.3.1. Organisms and cell culture

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 Stx 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 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% CO2 (approximately 72 h).

4.3.2. Recombinant intimin, EspA and EspB

Plasmids pCVD468 and pCVD469 (kind gift of Dr. D. Karpman, Lund, Sweden) were used for recombinant expression of respectively EspA and EspB as described earlier (Karpman *et al.,* 2002). Plasmid pMW103 (kind gift of Dr. A. O'Brien, Bethesda, USA) was used to express the Cterminal 380 amino acids of intimin-g (referred to as intimin) as previously described (Sinclair and O'Brien, 2002).

4.3.3. Lactoferrins

Iron saturated bovine lactoferrin (bLF) (Sigma, Bornem, Belgium), with 90% purity (SDS-PAGE) and > 85% iron saturation (as describe by manufacturer) 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.

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

E. coli O157:H7 overnight cultures were prepared by inoculating a colony into a 10-ml tube containing 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 hours 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}) by hour for 8 subsequent hours. At the same time, viable bacteria were counted by spread plating appropriate bacterial serial dilutions onto LB medium plates. After 8 hours, bacteria were washed three times with LB medium and inoculated into a 10-ml tube containing LB broth and incubating the tube at 37°C for 5h with shaking (200 rpm). In addition, we also examined the surface of lactoferrin treated bacteria, one and 8 h after adding lactoferrin using scanning electron microscopy (SEM) as described by Vandekerckhove *et al.* (2009). Briefly bacterial pellet were fixed in a HEPES-buffered 2% paraformaldehyde-2.5% glutaraldehyde solution for 24 hours 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 concentration, which did not inhibit bacterial growth. They were subsequently used in cell attachment assays.

4.3.5. 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, actually 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 ballanced 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.

4.3.6. 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. 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 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 hours at 37°C and 5% CO_2. After incubation for 4 h at 37°C, non-adherent bacteria were removed by washing preparations 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 CFU were enumerated. The attachment efficiency of *E. coli* O157:H7 was expressed as a percentage based on the CFU that was recovered as adherent *E. coli* O157:H7 cells to the control cells have not been treated with LF. The attachment efficiency of each isolate was measured in duplicate wells in at least three independent experiments (Fig 5).

4.3.7. 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 hours 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.

4.3.8. 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 CFU ± SD and mean

colony forming units (CFU) ± SD for the bacterial growth studies. The model was used to analyze the effect of hLF and bLF in different time points on the growth of bacteria, included the fixed effect of LF dose, time and two ways interaction terms of fixed effects.

For the cell adhesion study, the results were presented as mean percentage of bacterial attachment ± SD. The above software was used to analyze the effect of hLF and bLF on the reduced bacterial attachment with the repeated measurement (n# 3). The statistical model was used to analyze the data included the fixed effect of hLF and bLF doses in two separate statistical analyzes. The significant level in all the stadies was $p<0.05$.

4.4. Results

4.4.1. 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. *E. coli* O157:H7 growth was significantly inhibited during 3 to 6 hours post incubation (PI) using 0.5 to 10 mg/ml and 0.1 to 10 mg/ml of bovine or human LF, respectively (Fig 1 and 2).

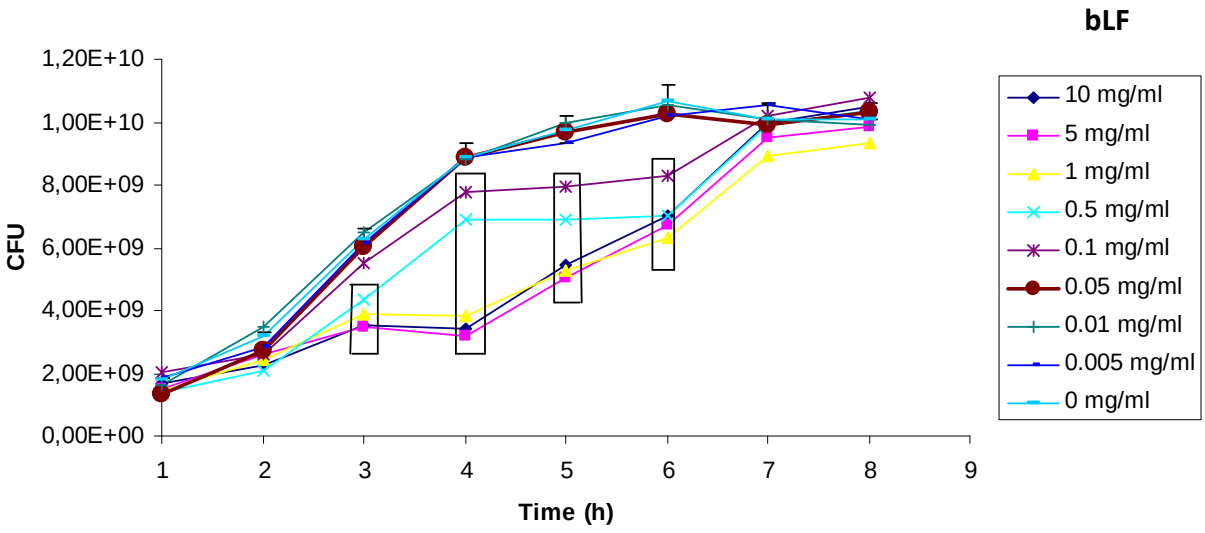

Figure1: Inhibitory effect of bovine lactoferrin on the growth of *E. coli* O157:H7. The results are represented as the mean CFU ± S.E.M. (n = 3). Error bars are only mentioned for the 0.05 mg/ml bovine lactoferrin. The data in the rectangles are significantly different from the control (0 mg/ml bovine lactoferrin).

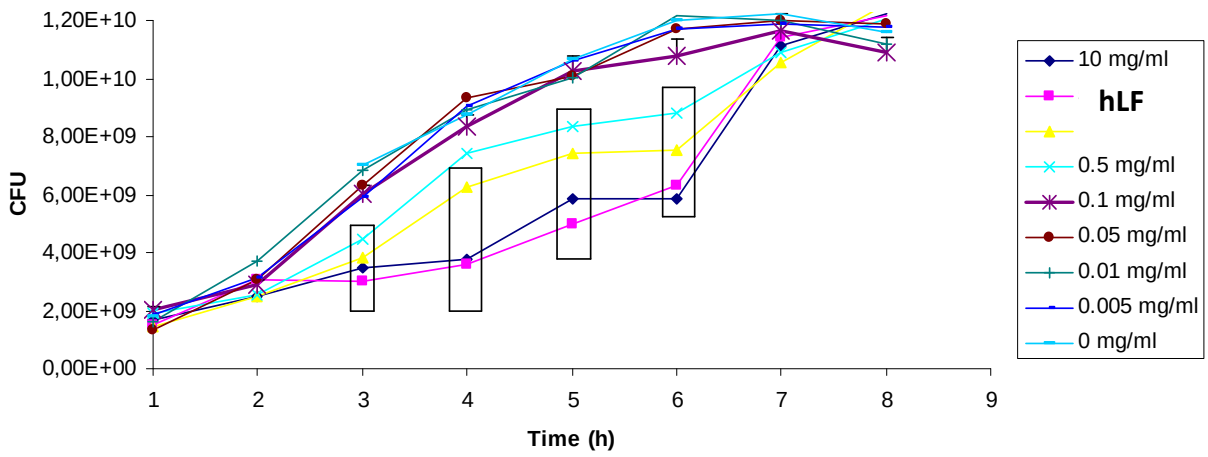

Figure2: Inhibitory effect of human lactoferrin on the growth of *E. coli* O157:H7. The results are represented as the mean CFU ± S.E.M. (*n* = 3). Error bars are only mentioned for the 0.1 mg/ml human lactoferrin. The data in the rectangles are significantly different from the control (0 mg/ml human lactoferrin).

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 highest 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 hours, 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 (Fig 3).

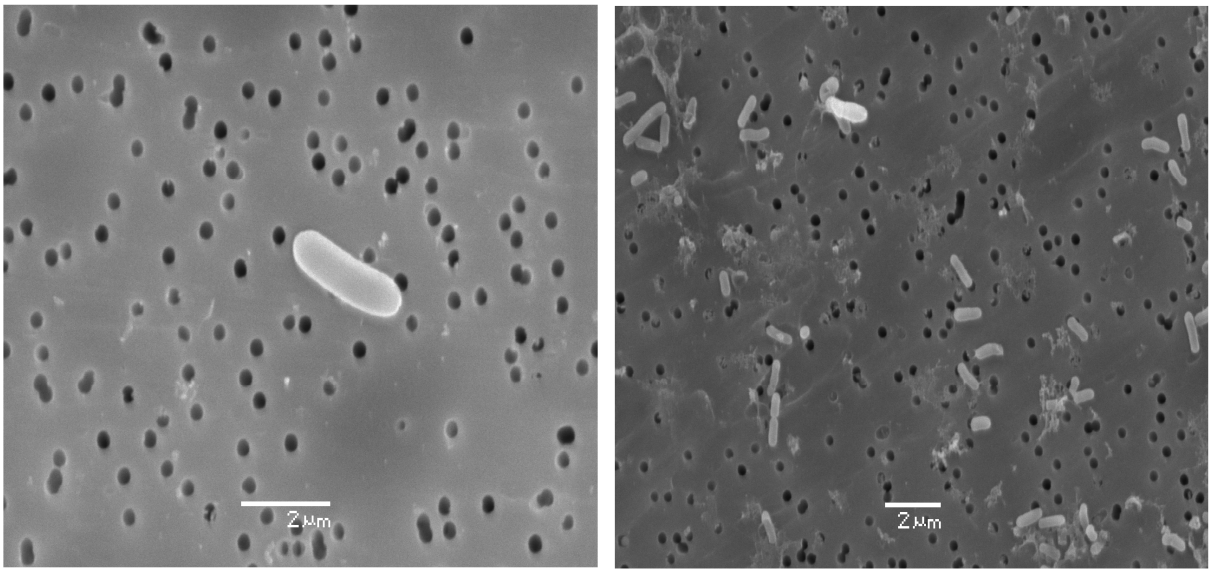

Figure 3: Scanning electron microscopy of *E. coli* O157:H7 after 8 hours incubation with 10 mg/ml bovine lactoferrin (left) and no lactoferrin (right).

4.4.2. Lactoferrin cytotoxicity assay

None of the lactoferrin concentrations tested was cytotoxic to Caco-2 cells, as compared to untreated control cells (Fig 4). Thus, maximum non-growth-inhibitory concentrations of lactoferrins could be used in a subsequent cell attachment assay.

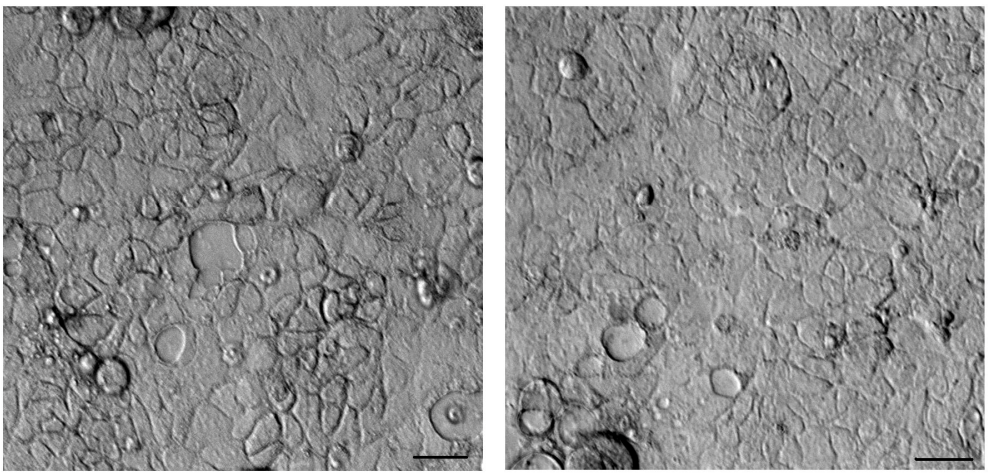

Figure 4: Light microscopic view of Caco-2 cells after 4 hours incubation with 0.1 mg/ml human lactoferrin (left) and no lactoferrin (right). Bars represent 20 µm.

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

Lactoferrins had no effect on Caco-2 cells (Fig 4). In the absence of LF, a mean of 4×10^4 CFU/well (100%) was recovered from Caco-2 cells. In the presence of lactoferrins, *E. coli* O157:H7 attachment to Caco-2 cells decreased in a concentration dependent manner (Fig 5).

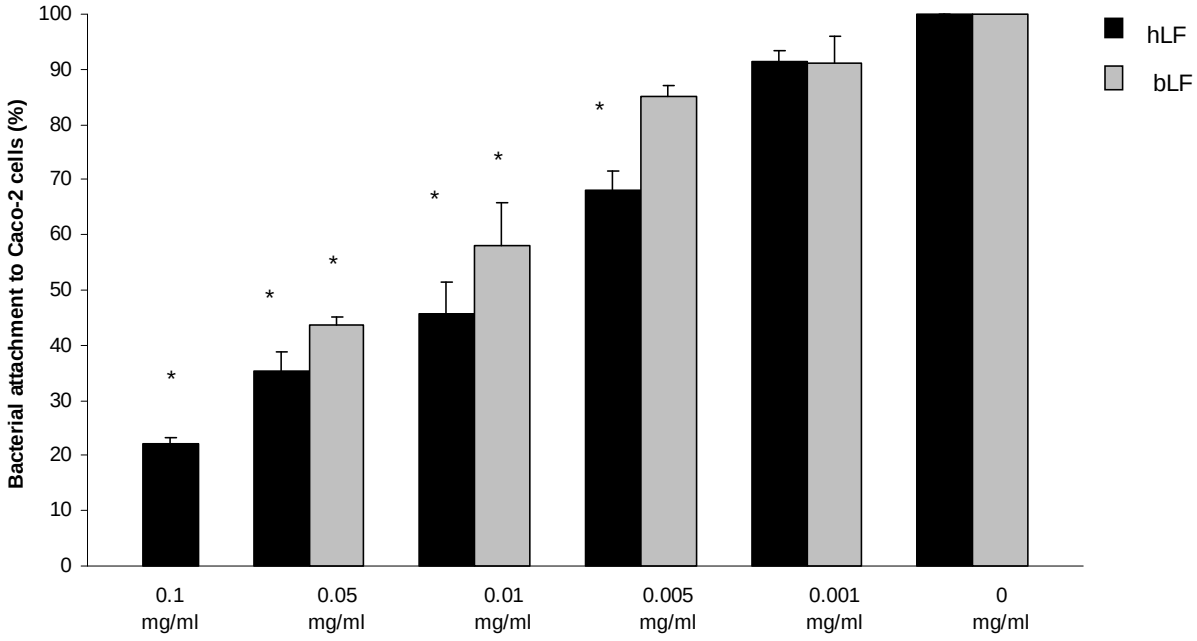

Figure 5: Lactoferrin significantly reduced *E. coli* O157:H7 attachment to Caco-2 cells. Results are represented as the mean values ± S.E.M (*n=3*). Asterisks indicate statistically significant different between lactoferrins treated groups and the control (0 mg/ml lactoferrin) ($p < 0.05$).

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 reduced with 78% and 57%, respectively as compared to untreated bacteria (100% attachment; $p < 0.05$).

4.4.4. Effect of lactoferrins on TSSS proteins

Lactoferrins both reduced *E. coli* O157:H7 attachment to Caco-2 cells significantly at non-growth-inhibitory concentrations indicating that other mechanisms 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 (Fig 6), but not intimin (Data not shown). The proteolytic effect of LF was prevented by the serine protease inhibitor.

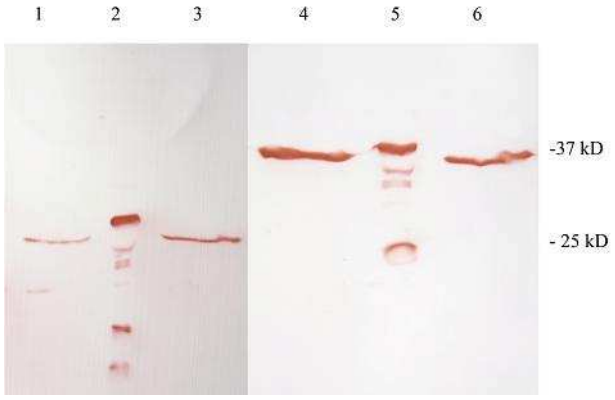

Figure 6: Illustration of the proteolytic effect of LF on EspA and EspB (Western blot). The degradation by LF was prevented by adding the serine protease inhibitor PMSF (0.25mM). Molecular mass (kilodaltons) is shown on the right. Lane 1: EspA; lane 2: EspA + LF; lane 3: EspA + LF + PMSF. Lane 4: EspB; lane 5: EspB + LF; lane 6: EspB + LF + PMSF.

4.5. 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 already demonstrated that antibiotic resistant *E. coli* O157:H7 could be isolated from humans, cattle, feed and even form 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. At present, we examined the effect of human and bovine lactoferrin on *E. coli* O157:H7 growth and on attachment to human cells. Growth inhibition was more pronounced when using bLF. Groenink *et al.,* (1999) observed the same. 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, result 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 antimicrobial activity resides mainly in the basic N1-domain of lactoferrins containing two stretches, designed lactoferricin and lactoferrampin (reviewed by Baker and Baker, 2009). Others observed the same. 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.,* 1999). Anti-microbial properties of bovine lactoferrampin are also stronger than for their human counterparts (Haney *et al.,* 2009).

Nevertheless, none of the lactoferrin concentrations used in our study gave 100% killing. To our knowledge 100% killing 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 few minutes in *E. coli* K12, concurrently with disrupting membrane integrity and killing of *E. coli* (Van der Kraan *et al.,* 2005). However, in the present study, CFU's for controls and treated bacteria were statistically the same till 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 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), bacterial growth was completely arrested for 1 hour. Thus, at sublethal concentrations, human and bovine lactoferrins acted bacteriostatic 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 also have developed a bacterial defence system leading to blockage of lactoferrins. Maybe, this explains why low and high (1, 5 and 10 mg/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 the 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 respectively 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 for 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 namely more effective than human and bovine lactoferrin in preventing attachment and entry of *Chlamydophila psittaci* in avian macrophages (Beeckman *et al.,* 2007). On the other hand, uptake of bLF in Caco-2 cells might be more effective than for hLF as demonstrated by Shin *et al.* (2008), studying the interaction between human and bovine

LF and intelectin, a lectin present on the brush border of intestinal cells. So, 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) (Ochoa *et al.,* 2003). 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 antimicrobial activity.

In conclusion, the direct antibacterial effect of bLF on *E. coli* O157:H7 was stronger than for hLF. Nevertheless, both lactoferrins acted bacteriostatic 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 towards the use of LF for supporting human treatment and/or for preventing *E. coli* O157:H7 infections in ruminants.

4.6. Acknowledgments

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Chapter 5:

Lactoferrin reduces *E. coli* O157:H7 attachment to ovine intestinal explants and subsequent IL-8 and TNF-α gene expression

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Wattegedera Sean, Cox Eric, Vanrompay Daisy

(Manuscript in preparation)

5.1. Abstract

The present study investigates the potential use of lactoferrin, a natural antimicrobial-immunomodulatory protein of milk, for preventing intestinal *E. coli* O157:H7 colonization in sheep. Sheep intestinal explants of ileal sections with and without Peyer's patches and of colon were infected with *E. coli* O157:H7 in the presence or absence of lactoferrin. At the same time, IL-8 and TNF-α gene expression was investigated by real-time RT-PCR. In the absence of lactoferrin, *E. coli* O157:H7 preferably attached to ileal explants with Peyer's patches as compared to ileal explants without Peyer's patches or to colon explants. Attachment to the latter two explants was not significantly different. Interleukine-8 gene expression was significantly upregulated following bacterial attachment to ileal explants with and without Peyer's patches. On the other hand, tumor necrosis factor-α gene expression was only significantly upregulated following bacterial attachment to ileal explants with Peyer's patches. Lactoferrin (0.05 mg/ml) significantly inhibited *E. coli* O157:H7 attachment to all explants. Attachment inhibition gave reduced IL-8 and TNF-α gene expression levels, although IL-8 and TNF-α gene expression was still significantly upregulated as compared to non-treated infected control explants or to treated non-infected control transplants. Results could contribute to the development of a preventive strategy for diminishing *E. coli* O157:H7 infections in ruminants, with the purpose of reducing food-borne EHEC infection in humans.

5.2. Introduction

Enterohemorrhagic *Escherichia coli* (EHEC) are emerging foodborne pathogens leading to HC and HUS characterized by thrombocytopenia, hemolytic anemia, and kidney lesions and to hemorrhagic colitis in humans. The majority of EHEC infections are caused by the serotype O157:H7, a member of the Shiga-toxin producing *E. coli* (Nataro and Kaper, 1998), frequently isolated from ruminant feces. In ruminants, this microorganism resides in the gut without causing apparent illness (Besser *et al.,* 1999). Many human *E. coli* O157:H7 infections originate, either directly or indirectly from exposure to ruminant's feces. Knowledge on the pathogenesis of EHEC infections in ruminants is crucial to control and/or prevent faecal shedding. However, less is known regarding the pathogenesis and of EHEC and in particular *E. coli* O157:H7 infection.

In humans, *E. coli* O157:H7 colonization generally results in a striking histopathological future known as the attaching and effacing (A/E) lesion characterized by an actin-rich pedestal formed by the host cell around the bacteria, destruction of brush border microvilli, and intimate adhesion of EHEC to the enterocyte surface. Bacterial genes, expressed from a chromosomal pathogenicity island named locus for enterocyte effacement (LEE), are essential for adherence. The attachment factor intimine, encoded by the *eae* gene, binds to Tir and nucleolin on the host cell membrane. Tir, a bacterial effector protein secreted though the bacterial type III secretion system of the LEE locus, is inserted into the host cell membrane to serve as a receptor for intimin (Hartland *et al.,* 2000; Wolff *et al.,* 1998).

Milk feeding protects young mammals from intestinal infections. Protection is attributed to multiple anti-microbial, anti-inflammatory and immunoregulatory milk components (Morrow *et al*., 2004). Lactoferrin (LF) is the main multifunctional protein in milk. It is also present in mucosal secretions like tears and saliva and in surface fluid of vaginal and respiratory tissues (Beisswenger and Bals, 2005). Anti-microbial functions ascribed to lactoferrin or its peptides include iron sequestration, destabilization of the outer membrane of Gram-negative bacteria through binding of bacterial lipopolysaccharides (LPS), selective permeation of ions, modulation of bacterial entry into host cells through host gene regulation and disrupting the bacterial type III secretion system (Arnold *et al.*, 1977, Ashida *et al.*, 2004, Ellison, 1994, Rossi *et al.,* 2002, Orsi, 2004).

Previously, we demonstrated the direct bactericidal activity of bovine and human lactoferrin on *E. coli* O157:H7. Moreover, bovine and human lactoferrin inhibited *E. coli* O157:H7 attachment to HEp-2 (human laryngeal carcinoma cells) and Caco-2 cells (human epithelial colorectal adenocarcinoma cells). Attachment inhibition was more pronounced when using bovine lactoferrin and was attributed to proteolysis of EspA and EspB, two structural proteins of the EHEC type III secretion system (TTSS) (Atef Yekta *et al.,* 2010).

In the present study, we investigated whether bovine lactoferrin can prevent *E. coli* O157:H7 colonization of sheep intestinal explants. Concurrently, we examined the expression of sheep IL-8 (CXCL8), one of the major mediators of the local inflammatory response, and of sheep TNF-α, a cytokine involved in systemic inflammation and member of a group of cytokines stimulating the acute phase reaction. Lactoferrin can bind and sequester bacterial LPS, one of the most powerful bacterial virulence factors in terms of pro-

inflammatory properties. Thus, lactoferrin could prevent pro-inflammatory pathway activation, sepsis and tissue damage. However, the interplay between lactoferrin and LPS is complex, and may result in different outcomes, including both suppression of the inflammatory response and immune activation (Puddu *et al.,* 2010). The outcome of this interplay is critically relevant in the development of a LF-based prophylactic strategy for ruminants. Reduction in carriage in ruminants is likely to lower the incidence of human infections.

5.3. Material and methods

5.3.1. Bacterial strain

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 a BSL2 laboratory. Bacteria were grown overnight in Luria Bertani broth at 37°C while shaking (200 rpm), centrifuged (550 g, 10 min, 4°C) and subsequently re-suspended in sterile phosphate-buffered saline (PBS) at a concentration of 10^6 CFU.

5.3.2. Lactoferrin

Bovine lactoferrin (bLF; Sigma, Bornem, Belgium), originated from bovine milk. The purity (SDS-PAGE) and iron saturation (manufacturer information) were 90% and > 85%, respectively. Lactoferrin was iron-saturated to avoid the occurrence of an anti-EHEC effect by iron-sequestration. This allowed the examination of direct anti-EHEC effects of bLF.

5.3.3. Generation of intestinal tissue explants

Sheep intestinal tissue explants were generated as previously described (Baehler *et al.,* 2000). Briefly, 4 months-old sheep (n = 3) were euthanized to collect ileal segments with and without Peyer's patches (ileum PP and ileum, respectively) as well as segments of the distal colon. Intestinal segments (1 cm^2) were rinsed using cold (4°C) sterile PBS and subsequently

immersed in cold (4°C) RPMI 1640 medium (Invitrogen, Merelbeke, Belgium) for 30 min. Tissue explants were deposed, mucosal side up, on biopsy foam pads (Fisher Scientific Company, USA). All biopsy foam paths were placed individually in 6-wells tissue culture plates and submersed in 5 ml RPMI 1640 medium/well. Plates were incubated at 37°C and 5% CO_2. All animal procedures were in accordance with the animal welfare regulations of the Veterinary Ethical Committee of Ghent University.

5.3.4. Inoculation and examination of tissue explants

To determine the anti-adhesive effect of bLF, the highest possible concentration with no effect on *E. coli* O157:H7 growth, as determined in previous experiments (0.05 mg/ml) (Atef Yekta *et al.,* 2010) was added to each submersed biopsy foam pad. Subsequently, *E. coli* O157:H7 (10^6 CFU/well) were added. For each sheep, an equal number of uninfected explants and infected explants without the addition of bLF served as controls. After 6 h, wells were washed three times to remove non-adherent bacteria. Explants were cut in half, using one part for bacterial culture and the other for scanning electron microscopy (SEM).

Parts for bacterial culture were immediately examined by direct plating according to the method described by Laegreid *et al.,* (1999). Briefly, explant parts were individually homogenized (Lab Blender - Stomacher, Labequip, Canada) in modified tryptone soy broth (Bio Rad, Eke, Belgium) containing novobiocin (20 mg/ml) (Sigma, Bornem, Belgium). Subsequently, ten-fold dilution series of all individually homogenized tissue explant parts were plated onto cefixime-tellurite sorbitol MacConkey agar supplemented with nalidixic acid (15 µg/ml) (Nal CT-SMAC plates, MERCK, Darmstadt, Germany). Plates were incubated at 37°C for overnight. EHEC identification was confirmed by the latex agglutination test (Oxoid, Ltd, Basingstoke, UK). Counted colony numbers were $\log_{10}$ transformed.

Explant parts for SEM were submersed (24 h) in HEPES-buffered 2% paraformaldehyde and 2.5% glutaraldehyde (VWR, Brussels, Belgium). Scanning electron microscopy was performed as described by Vandekerckhove *et al*. (2009). Briefly, post-fixation was performed in osmium tetroxide (1%) (EMS, Hatfield, USA) for 2 h and dehydrated in ascending grades of alcohol. Hereafter, the specimens were critical point dried with CO_2 (CDP 030, Balzers, Sercolab, Germany), mounted on metal stubs, platinum-coated (JFC-1300

Auto Fine Coater, Jeol, Germany) and finally examined using a JSM 5600 LV scanning electron microscope (Jeol).

5.3.5. Cytokine expression analysis

Total RNA was extracted from all explants at 6 h post infection using the RNeasy® Mini kit (Qiagen, West Sussex, UK) according to the manufacturer's instructions, including the column DNaseI digestion step to remove contaminating genomic DNA. RNA integrity was assessed by visualization (UV transillumination) of amplified 18S and 28S rRNA bands on agarose gels. Fifteen µl of RNA from each sample was reverse-transcribed into cDNA using the High-Capacity cDNA Archive Kit (Applied Biosystems, Warrington, Cheshire, UK) according to the manufacturer's instructions. cDNA was stored at -20°C until use (Wattegedera *et al*., 2010).The quality of the cDNA was tested (Nano Drop ND-1000, Thermo Fisher, Belgium). cDNA was subsequently used for real time qPCR Quantitative PCR's for IL-8 and TNF-α were performed as described by Leuteneger *et al*. (2000) and Budhia *et al.* (2006), respectively. Primers and probes for IL-8, TNF-α and for the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GADPH) are mentioned in Table 1 (Montagne *et al*., 2001; Tudor *et al.,* 2009). Amplification was achieved by use of the TaqMan® Universal PCR Master Mix (Applied Biosystems). Each sample (5 µl) was PCR amplified (in triplicate) using thermal cycling conditions of 50°C for 2 min, 95°C for 10 min and 40 cycles of 95°C for 15 s and 60°C for 1 min. Cycle threshold (Ct) values were converted into template quantities using the ABI Prism 7000 SDS software 1.2.3. Relative quantification was performed as described by Wattegedera *et al.* (2010), using the comparative cycle threshold (C_T) method. The method measures the relative difference in IL-8 and TNF-α gene expression for EHEC infected and non-infected explants, and for EHEC infected explants in the presence or absence of bLF. Explants submersed in medium alone served as calibrator. The cytokine gene expression C_T values were normalized against the GADPH C_T values for each sample, giving ΔC_T values. The $\Delta\Delta C_T$ was calculated by subtracting the calibrator ΔC_T from each sample ΔC_T, and the fold-change determined by the equation $2^{-\Delta\Delta C}{}_T$ (Cikos *et al.,* 2007).

Table 1: Sequence of primers and probes for ovine IL-8, TNF-α and GADPH

Primer or Probe	Sequence (5′-3′)	Length (bp)	Accession No.	Ref.
Ov TNF-α F4	GGTGCCTCAGCCTCTTCTC	136	X56756	Budhia *et al.* (2006)
Ov TNF-α R3	GAACCAGAGGCCTGTTGAAG			
Ov TNF-α probe	TGGTTCAGGAGCCACCACG			
Ov IL-8.177f	CACTGTGAAAAATTCAGAAATCATTGTTA	113	S74436	Leutenegyer *et al*. (2000)
Ov IL-8.282r	CTTCACCAAATACCTGCACAACCTTC			
Ov Il-8 probe	AATGGAAACGAGGTCTGCTTAAACCCCAG			
Ov GAPDH F1	GGCGTGAACCACGAGAAGTATAA	120	AF030943	Montagne *et al*., (2001)
Ov GAPDH R1	CCCTCCACGATGCCACCGT			
Ov GAPDH probe	CACTGTCCACGCCATCACTGCCA			

5.3.6. Statistical analysis

To analuze the data with repeated measures, the MIXED procedure of the SAS software program (version 8.2, SAS Institute Inc, USA) was used. The statistical model used to analyze the data included the fixed effect of animal (sheep), tissue, treatment, three ways interaction terms of fixed and the residual errors. The significant difference level was set as $p < 0.05$.

5.4. Results

5.4.1. Attachment of *E. coli* O157:H7 to ovine intestinal explants

First, we determined the amount of bacteria recovered from 3 ileum PP, 3 ileum and 3 colon explants per sheep. For each sheep (A, B and C), the amount of bacteria isolated within the group of ileum PP, ileum and colon explants was statistically the same. The highest number of *E. coli* O157:H7 (1.9×10^8) was recovered from an ileum PP explant of sheep A, while the lowest number of *E. coli* O157:H7 (1.17×10^7) was obtained of an ileum explant of the same sheep. For all sheep, the number of *E. coli* O157:H7 recovered from ileum PP explants was significantly higher than those recovered from ileum or colon explants ($p > 0.001$). Attachment to ileum and colon explants did not significantly differ, although *E. coli*

O157:H7 was found more often on colon explants (Figure 2). Results were confirmed by SEM, as we observed differences in the presence of well-developed EHEC micro-colonies, which is typical for intimately attached *E. coli* O157:H7 (Figure 1).

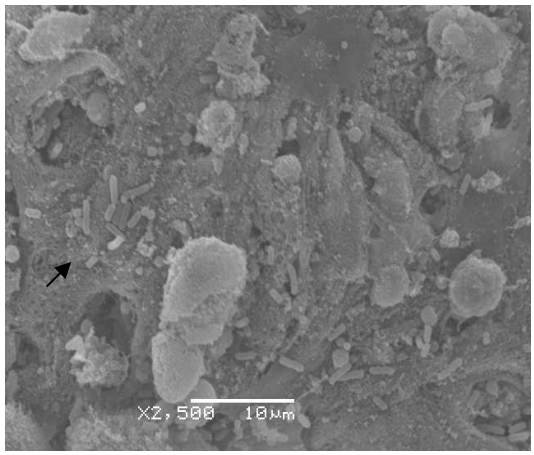

Figure 1: Scanning electron microscopy of sheep intestinal explants (ileal Peyer's patches) infected with *E. coli* O157:H7. Arrows: *E. coli* O157:H7 colonization.

5.4.2. Lactoferrin reduces *E. coli* O157:H7 attachment to ovine intestinal explants

Bovine lactoferrin significantly reduced *E. coli* O157:H7 attachment to ileum PP, ileum and colon explants, as compared to the controls for each tissue ($p < 0.05$) (Figure 2).

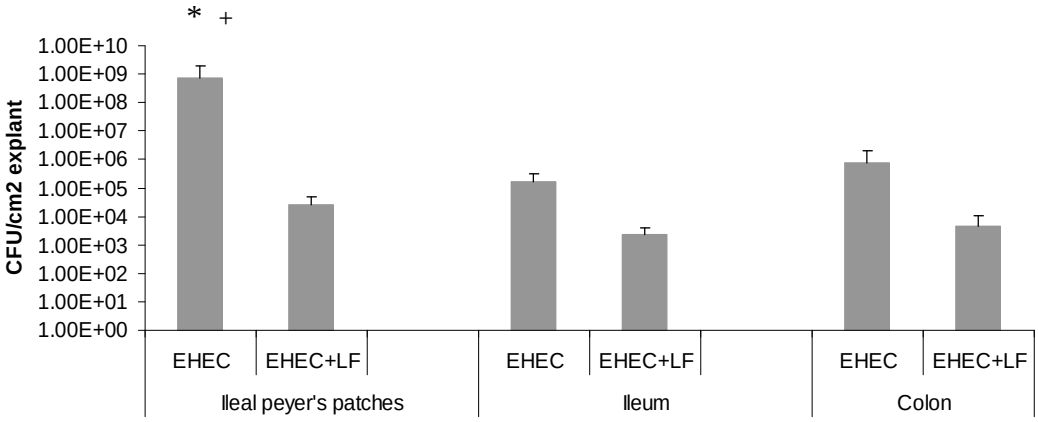

Figure 2: Mean number of *E. coli* O157:H7 attached to intestinal explants. Results present mean values for ileal Peyer's patches, ileum and colon explants of 3 sheep. Bovine lactoferrin significantly inhibited *E. coli* O157:H7 attachment to all intestinal explants. Error bars: SEM. * represents significant difference in bacterial attachment to Ileal peyer's patches (inoculated with bacteria) compare with colon and Ileum. $P < 0.05$. + represents significant difference in bacterial attachment to Ileal peyer's patches inoculated with EHEC compare with Ileal peyer's patches inoculated with the bacteria and incubated with LF.

5.4.3. Cytokine gene expression in sheep intestinal explants

E. coli O157:H7 attachment resulted in an upregulation of IL-8 gene expression in ileum PP and ileum explants of all sheep ($p < 0.05$) (Fig 3) and in an upregulation of TNF-α gene expression in ileum PP explants of all sheep.

E. coli O157:H7 attachment did not significantly change mean cytokine expression levels in the colon, although the level of IL-8 and TNF-α expression in the colon explant of one sheep significantly increased (data not shown).

We also examined the effect of bLF on IL-8 and TNF-α gene expression induced by *E. coli* O157:H7 attachment to intestinal explants. Lactoferrin itself seemed to have no effect on IL-8 or TNF- α gene expression at 6 h post infection, as $\Delta\Delta C_T$ values of all non-infected bLF-treated controls and all non-infected, non-bLF treated controls were statistically the same. However, bLF significantly inhibited *E. coli* O157:H7 induced expression of TNF-α and IL-8 at 6 h post infection, as shown by the $\Delta\Delta C_T$ values for *E. coli* O157:H7 infected explants, non-bLF treated explants and for infected, bLF treated explants (Figure 3).

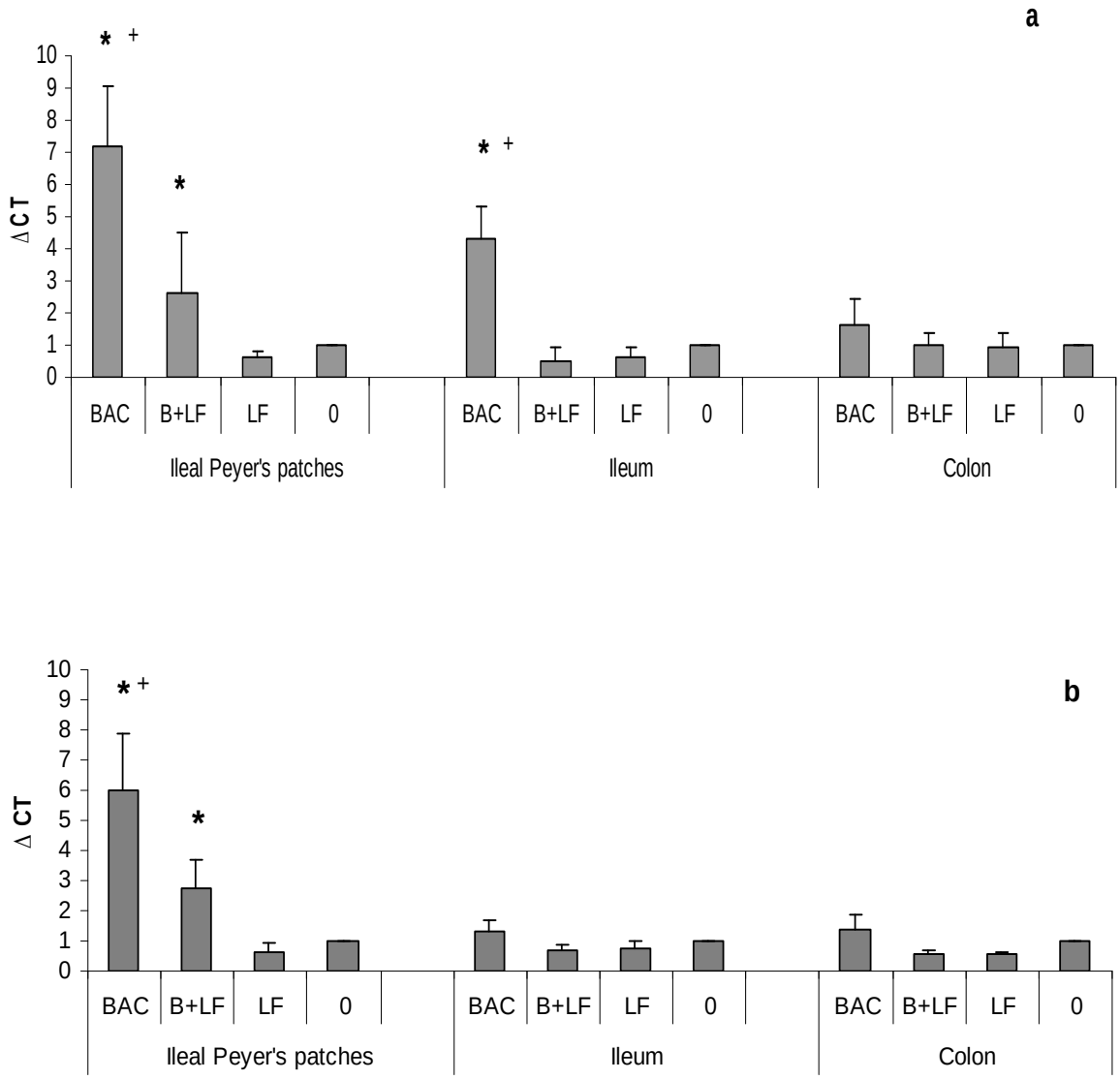

Figure 3: Expression of IL-8 (a) and TNF-α (b) genes in sheep intestinal explants. BAC: *E. coli* O157:H7. B+LF: *E. coli* O157:H7 + Lactoferrin. LF: Lactoferrin, no *E. coli* O157:H7. 0: explants + culture medium. *E. coli* O157:H7 infection of intestinal explants significantly up-regulated IL-8 and TNF-α gene expression. Bovine lactoferrin (LF) significantly suppressed the expression of *E. coli* O157:H7 induced IL-8 and TNF- α genes. *: Significant difference in the expression of cytokine compare with non-infected, non-bLF treated control. +: Significant difference in the expression of cytokine in the presence or absence of bLF in *E. coli* O157:H7 infected explants. Error bars: SEM. $P< 0.05$

5.5. Discussion

Previously we have shown that bLF inhibited *E. coli* O157:H7 adhesion to Caco-2 cells (Atef Yekta *et al.*, 2010). In the present study we examined the effect of bLF on *E. coli*

O157:H7 attachment to sheep intestinal tissues using an *ex vivo* model. Indeed, bLF significantly reduced *E. coli* O157:H7 attachment to epithelial surfaces of sheep intestinal explants. Moreover, bLF significantly reduced *E. coli* O157:H7-induced upregulation of IL-8 and TNF-α genes in sheep intestinal explants.

E. coli O157:H7 attached preferably to ileum PP explants. This appears to agree with a previous report wherein Girard *et al.* (2007) showed variation in tissue tropism for *E. coli* O157:H7 attachment using calf intestinal explants. In their study, *E. coli* O157:H7 attached significantly better to terminal ileum explants as compared to colon explants. Similar was observed in a study using human intestinal explants, in which *E. coli* O157:H7 strongly attached to ileal Peyer's patches (Philips *et al.*, 2000). However, we have shown that *E. coli* O157:H7 adhesion to ovine intestinal tissue is not limited to the distal part of the small intestine, but includes regions with normal absorptive epithelium.

Cantey *et al.* (1989), infecting rabbits with EPEC, showed that EPEC initially colonized Peyer's patches and afterwards spread to other intestinal parts. Since we also found more attachment to ileal Peyer's patches, we suggest that colonization of the other intestinal regions by *E. coli* O157:H7 might be a subsequent event following colonization of ileal Peyer's patches. However, to proof this hypothesis, we have to exam bacterial attachment at earlier stages, before 6 h post infection. The specificity of this initial adherence is uncertain as Peyer's patches are considered to be sites of antigen sampling. However, M cells in the epithelial surface above the ileal Peyer's patches express β-1 integrins, a receptor for intimin γ. This could explain the selective attachment of *E. coli* O157:H7 to ileal Peyer's patches. The infective dose for *E. coli* O157:H7 is very low (10^2 CFU/ml) (Cornick *et al.,* 2004) which is possibly due to selective attachment and colonization of Peyer's patches allowing subsequent spread to lower intestinal parts. Unfortunately, we were unable to examine a possible spread, as we could not monitor *E. coli* O157:H7 attachment as the cells died after more than 6 h post infection.

In our previous study we showed that a non-cytotoxic, non bactericidal concentration of bLF inhibited *E. coli* O157:H7 attachment to Caco-2 cells and this inhibition was at least partly due to the proteolytic effect of bLF on EspA and EspB, both secreted by the *E. coli* TTSS (Atef Yekta *et al.,* 2010). Results generated by use of our *ex vivo* explant model suggest that this mechanism might also be active in sheep.

In the second part of our study we investigated the expression of IL-8 and TNF-α genes in sheep intestinal explants at 6 h post *E. coli* O157:H7 infection in the presence or absence of bLF. We were the first to provide evidence that an *E. coli* O157:H7 infection increased both sheep IL-8 and TNF-α gene expression in ileal Peyer's patches and ileal epithelium.

Bacterial LPS is a strong mediator of the inflammatory response. Although Cario *et al.* (2000) reported that LPS activates NF-κB probably through Toll-like receptors (TLR-4), activation depends on the presence of serum soluble factors including CD14 and BLP (Cario *et al.,* 2000). Since our experiments were conducted in serum-free medium, we speculated that LPS is not the major player in the observed pro-inflammatory response following *E. coli* O157:H7 infection of sheep intestinal explants, even if LPS is a key activator of subepithelial macrophages. Recently it has been shown that other bacterial factors including flagellin and TTSS proteins mediate the pro-inflammatory responses of *E. coli* infected cells by activating the NF-κB signaling pathway (Steiner *et al.,* 2000; De Grado *et al.,* 2001). In agreement with our results, Zhou *et al.,* (2003) showed that *E. coli* O157:H7 lacking Stx is able to activate IL-8 expression and they suggested that additional factors like flagellin and TTSS were responsible for IL-8 up-regulation following *E. coli* O157:H7 infection.

Previously, we have shown that bLF exerts a proteolytic effect on the *E. coli* O157:H7 TTSS proteins EspA and EspB (Atef Yekta *et al.,* 2010). Although the lower *E. coli* infection rate in the presence of bLF could simply explain the reduction of cytokine expression in bLF treated samples, the above findings suggests additional mechanisms, such as the inhibition of the NF-κB pathway, for down-regulating IL8 and TNF-α gene expresssion by bLF (Haversen *et al.,* 2002). Lactoferrin itself did not stimulate the intestinal tissues and in most of the bLF treated explants even a slight suppression of cytokine gene expression, compare with non-treated controls, was observed.

In conclusion, the present study shows that: 1) *E. coli* O157:H7 preferentially binds to ileal Peyer's patches of sheep, 2) binding is inhibited by bLF, 3) *E. coli* O157:H7 attachment induces IL-8 and TNF-α gene expression and the gene expression levels correlate with the amount of bacteria attached to the sheep intestinal explants, 4) bLF inhibits *E. coli* O157:H7 induced IL-8 and TNF-α gene expression. Our data provide insights regarding the intestinal immune response triggered by an *E. coli* O157:H7 infection in ruminants. Moreover, the

present study implies that bLF could reduce *E. coli* O157:H7 colonization and subsequent excretion in ruminants diminishing the risk for *E. coli* O157:H7 transmission to humans.

5.6. Acknowledgements

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Chapter 6:

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

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(Submitted to Veterinary Microbiology)

6.1. Abstract

The majority of human *Escherichia coli* O157:H7 infections are the result of exposure to ruminant's feaces, therefore reducing *E. coli* O157:H7 excretion by ruminants could play a key role in reducing human infections.

The present study investigates the potential of bovine lactoferrin, a natural antimicrobial-immunomodulatory protein of milk, to prevent colonization and excretion of *E. coli* O157:H7 in sheep. The effect of two different doses of lactoferrin (1.5 g or 0.15 g per 12 hours) was evaluated on colonization of sheep intestine and faecal excretion of the NCTC12900 strain. Hereto, lactoferrin was orally administered to sheep during 30 consecutive days and sheep were experimentally infected with *E. coli* O157:H7 on the second day of the lactoferrin administration. Interestingly, both lactoferrin dosages significantly reduced the number of *E. coli* O157:H7 in faeces as well as the duration of faecal excretion. The high dose group showed a significantly higher antibody response against EspA and EspB, two structural proteins of the bacterial type III secretion system (TTSS), than the infection control. The results suggest that oral lactoferrin administration could be used to reduce persistent colonization of sheep with *E. coli* O157:H7.

6.2. 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, non 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 more 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 (EPEC) (Ochoa *et al.*, 2003; Ashida *et al.*, 2004). 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 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.

6.3. Materials and methods

6.3.1. 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.

6.3.2. Lactoferrin

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

6.3. 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.

6.3.4. Experimental procedures

Five animals received lactoferrin at a 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. Lactoferrin was given orally in a volume of 10 ml sodium bicarbonate buffer (10%) via a syringe allowing the sheep to drink the solution. As control, seven animals received sodium bicarbonate buffer *(Infection control* group) and two animals received the high lactoferrin dose (*LF control* group) (Table 1). The *LF control group* allowed us to see if: a) no spontaneous or cross infection occurred, b) no antibodies appeared against the *E. coli* O157:H7 or c) visual side effects of the lactoferrin administration occurred.

After 1 day of lactoferrin administration the first 3 groups received 10^{10} *E. coli* O157:H7 in 10 ml PBS orally for 2 consecutive days, whereas the *LF control* was not infected. 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).

Table 1: Experimental set-up

Group	Number of sheep	*E. coli* O157:H7	Lactoferrin
high LF	5	+	3 g/day day: (high dose)
low LF	3	+	0.3 g/day: (low dose)
Infection control	7	+	-
LF control	2	-	3 g/day day: (high dose)

6.3.5. 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.

6.3.6. 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 OD_{405}-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).

6.3.7. Statistical analysis

Statistical analyses were performed using SAS software. The Proc MIXED test was used to analyse the excretion of the bacteria. The statistical model used to alanyse data 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 t-test. Statistical analysis of serum antibody titers was done by using General linear Model (repeated mesures analysis of variance). Differences were considered statistically significant at $p < 0.05$.

6.4. Results

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

To analyze the effect of lactoferrin on *E. coli* O157:H7 colonization, 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 *infection control* group shed between 10^5 and 10^8 CFU *E. coli* O157:H7/g faeces with an average of $7x10^7$ CFU/g (Fig. 1).

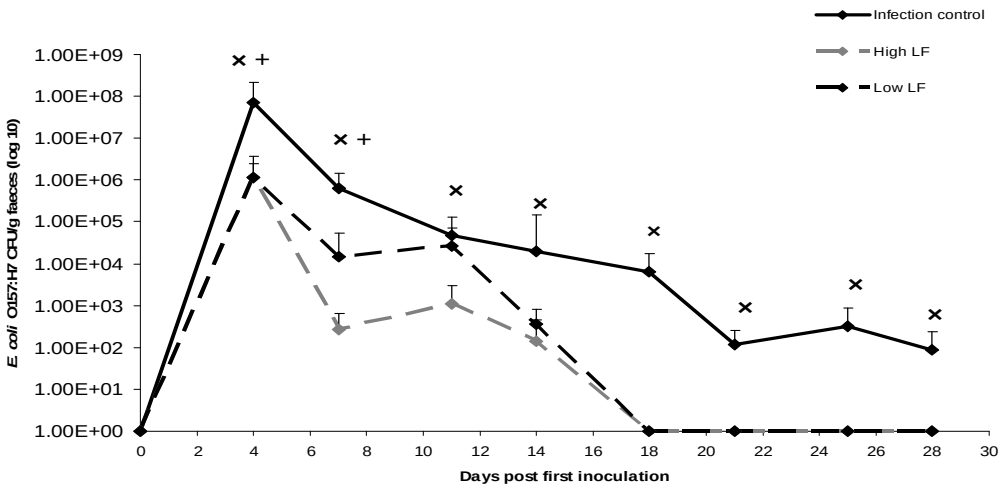

Figure 1: Effect of lactoferrin administration on faeacal excretion of *E. coli* O157:H7 by sheep. Results are presented as the mean log10 values of colony forming units (CFU)/ g faeces ± SEM. Significant differences at $p < 0.05$. × between Infection Control and *LF high* group; + between Infection Control and *LF low* group.

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 *LF high* 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 *LF high* group (12.6 ± 1.17 days (mean ± SEM)) was significantly shorter than for the *colonization control* group (24.71 ± 0.68 days (mean ± 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. On day 14, one of the animals stopped excreting and on day 17 the two remaining animals became negative. Nevertheless, during the second week of the experiment the number of bacteria excreted by the *LF low* group was higher than for the *LF high* group (Fig 1). However, again the duration of excretion (16.66 ± 0.76 days (mean ± SEM)) was significantly shortened in comparison with the *colonization control* group ($p < 0.05$), but not in comparison with the *LF high* group. The *LF control* group remained negative throughout the experiment.

6.4.2. 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 log2 titres of 4.54, 4.05 and 4.62, respectively. A similar response was observed in the *low lactoferrin* group. However, for the *high lactoferrin* dose 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 raise, as compared to the *LF control* and the *infection control* group.

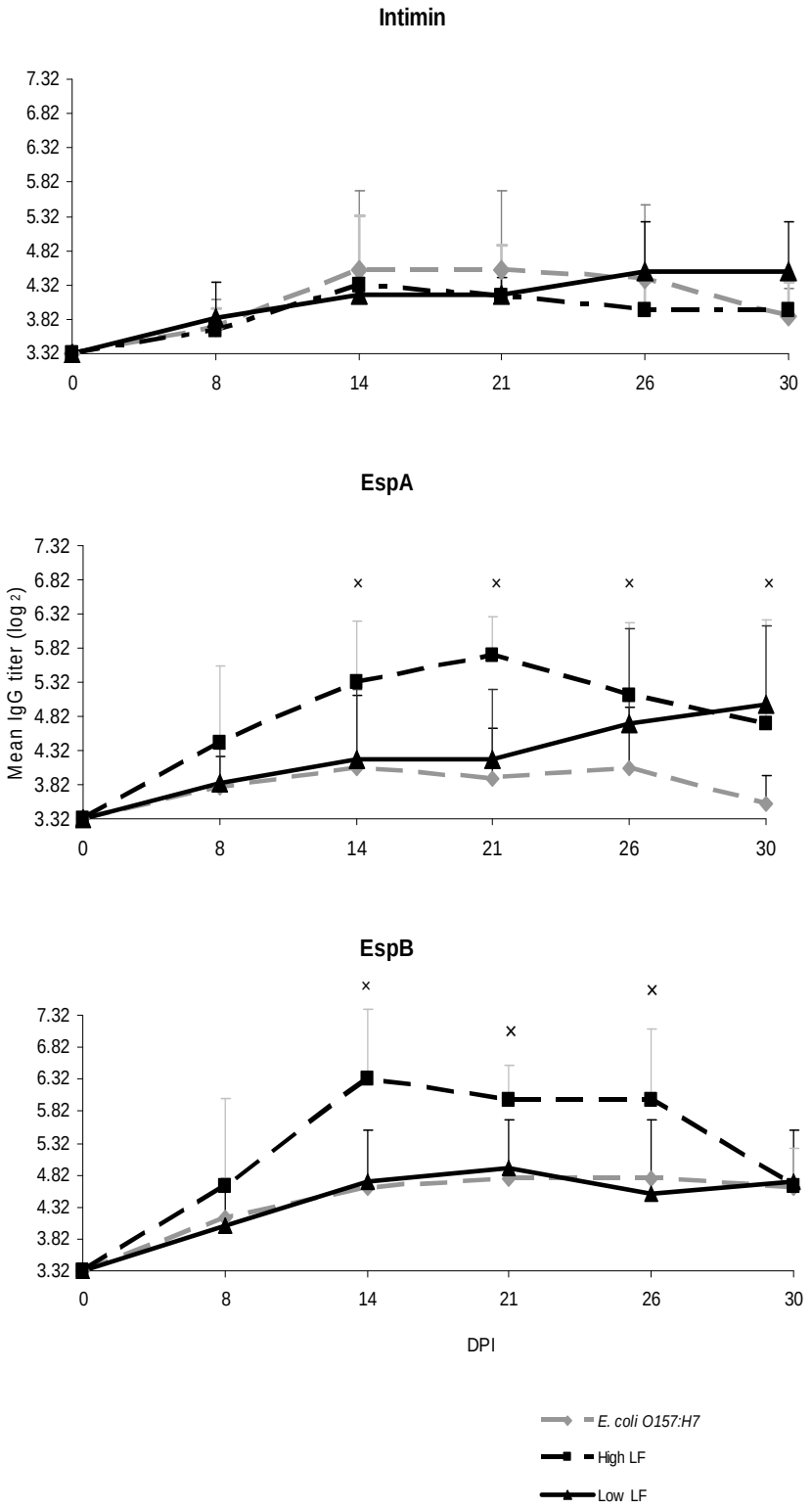

Figure 2: Mean serum antibody responses in the 3 groups infected with *E. coli* O157:H7. Results are presented as the mean $\log_2$ values of antigen-specific IgG titers. DPI: days post inoculation. × marks a significant difference between the high dose LF group and the *E. coli* O157:H7 group. Error bars indicate SEM. $p < 0.05$.

6.5. Discussion

Escherichia coli O157:H7 colonizes the intestinal tract of ruminants, often leading to a persistent bacterial excretion making ruminants 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). These strategies are either too expensive, too labor intensive, only show a limited effect or hold the risk of an increase in antibiotic resistance. Therefore there is need for new intervention strategies.

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 b). This 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.

6.6. 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.

6.7. Acknowledgements

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Chapter 7:

Immunization of sheep with a combination of intiminγ, EspA and EspB decreases *Escherichia coli* O157:H7 shedding

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(Accepted, Veterinary Immunology and Immunopathology)

7.1. Abstract

Enterohaemorrhagic *Escherichia coli* O157:H7 are zoonotic pathogens associated with haemorrhagic colitis (HC) and the haemolytic uremic syndrome (HUS). Ruminants are the main reservoir of this organism and most outbreaks of *E. coli* O157:H7 infections are food borne. Food contamination by ruminant manure has been reported as the primary source of human infection, therefore inhibition of *E. coli* O157:H7 colonization and shedding in ruminants could control the risk of human exposure to this pathogen. In the present study a vaccine based on the translocon proteins EspA and EspB and the outer membrane adhesion factor intiminγ significantly reduced faecal shedding of *E. coli* O157:H7 by orally infected sheep. Protection correlates with serum antibody responses to the defined antigens and validates the targeting of these colonization factors. Whereas vaccination has been described in cattle, this is the first study describing a significant decrease in faecal shedding following systemic immunization of sheep.

7.2. Introduction

Enterohaemorrhagic *Escherichia coli* (EHEC) O157:H7 is a serious human health risk in many countries with ruminants recognized as asymptomatic carriers (Borczyk *et al.,* 1987; Besser *et al.,* 1999). Following initial adherence of *E. coli* O157:H7 to the intestinal epithelium, a LEE-encoded type III secreted protein translocation tube is formed, which connects the pathogen with its target cell (Frankel *et al.,* 1998). EspA is a major component of this tube (Ebel *et al.,* 1998; Knutton *et al.,* 1998), through which EspB, EspD and Tir are delivered to the host cell. EspB and EspD form pores in the host cell membrane. EspB is also translocated into the host cell cytosol, where it triggers signal transduction events that mediate effacement of the microvilli and replacement with a pedestal-like structure. Tir becomes translocated to the host cell membrane, where it forms the receptor for intimin γ, expressed on the surface of the bacteria, resulting in intimate attachment to the host cell (Wolff *et al.,* 1998; Hartland *et al.,* 2000). A consequence of this interaction is a striking histopathological change known as attaching and effacing (A/E) lesion. Meanwhile, the bacteria produce toxins such as the Shiga toxins Stx1 and Stx2 (variants). However, unlike humans, ruminants lack vascular receptors for Stxs. They do have Gb3 on their intestinal crypt epithelial cells. Nevertheless, binding does not result in cytotoxicity due to exclusion of

the toxin from the endoplasmatic reticulum (reviewed by Moxley, 2004). In neonatal colostrum-deprived calves experimental *E. coli* O157:H7 infection could induce oedema of the colon and diarrhoea (Dean-Nystrom *et al.,* 1997) and even in 3 to 4 month old weaned calves A/E lesions could be found in cecum and rectum following experimental infection (Dean-Nystrom *et al.,* 1999). Therefore, *E. coli* O157:H7 appear to readily induce disease in young calves. However, this serotype is generally considered non-virulent in older cattle (Naylor *et al.,* 2003). One of the reasons for the absence of clinical signs in older cattle could be a quite localized colonization. Indeed, *E. coli* O157:H7 can colonize the large intestine, but preferentially colonizes the terminal rectum where it binds to the lymphoid follicle dense mucosa (Naylor *et al.,* 2003). Furthermore, large numbers of bacteria need to be present before sufficient A/E lesions occur. Indeed 10^6 CFU/g is the threshold for recognition of adherent layers of bacteria in histological sections, suggesting that A/E lesions will not be detected unless bacterial counts in the gut lumen are quite large, which only seems to occur at the preferential sites (Cray and Moon, 1995). In sheep no clinical signs have been attributed to *E. coli* O157:H7 infections, even though A/E lesions can be observed (Naylor *et al.,* 2005b).

Reduction of *E. coli* O157:H7 infection and faecal excretion of these asymptomatic shedders will seriously reduce the risk of human exposure to this pathogen. Therefore, several studies focused on interventions which could limit colonization and shedding from ruminants including vaccination, probiotic and antibiotic treatment (Brashears *et al.,* 2003; Callaway *et al.,* 2004; Moxley, 2004; Potter *et al.,* 2004; Dziva *et al.,* 2007). Vaccination of cattle showed variable results depending on antigens (type III secretion proteins; siderophore receptor and porin-protein), doses (2 or 3) and adjuvant (Moxley *et al.,* 2009; Smith *et al.,* 2009). In bovine, intramuscular immunization with type III secretion proteins of *E. coli* O157:H7 resulted in a significant reduction in shedding. Such vaccinations have not yet been performed in sheep. The present study was the first to examine if vaccination of sheep with intimin, EspA and Esp B is capable of reducing fecal shedding of *E. coli* O157:H7 in an oral infection model.

7.3. Material and methods

7.3.1. Bacterial strains and culture conditions

NCTC12900 is a well characterised Shiga toxin-negative *E. coli* O157:H7 strain with spontaneous nalidixic acid (Nal) resistance (Dibb-Fuller *et al.,* 2001; Wales *et al.,* 2002; Woodward *et al.,* 2003). For preparation of bacterial inocula, NCTC12900 was grown overnight in Luria Bertani broth at 37°C while shaking (200 rpm). Following centrifugation (2000 rpm, 10 min, 4°C), the pellet was resuspended in sterile phosphate-buffered saline (PBS) to a concentration of 10^9 CFU per ml for inoculating sheep (10^{10} CFU/ animal).

7.3.2. Preparation of proteins and vaccine formulation

Plasmids pCVD468 and pCVD469 were used for recombinant expression of EspA and EspB, respectively and plasmid pMW103 to express the C-terminal 380 amino acids of intimin-γ (referred to as intimin. Briefly, transformed bacteria were induced with 1 mM isopropyl-β-d-thiogalactopyranoside and recombinant His-tagged proteins were purified by nickel-affinity chromatography. For intramuscular (i.m.) vaccination, intimin, EspA and EspB were formulated at 100 µg/ protein in total volume of 1 ml PBS. Subsequently, this solution was suspended 1/1 (vol/vol) in incomplete Freund's adjuvant (Sigma, Bornem, Belgium).

7.3.3. Vaccination and experimental infection of sheep

Eight 3-month-old male sheep (Belgian crossbreed, Zootechnical Centre, Leuven, Belgium) were used in this study. Sheep were housed at the Faculty of veterinary medicine where they were divided into two groups of three and five animals per pen, respectively. They received daily a pelleted grain-based diet and water ad libitum. The experiments were approved by the ethical committee of Ghent University, Faculty of Veterinary Medicine and Bioscience Engineering (n°: 2009/074). All animals were screened prior to immunization to be negative for serum antibodies against intimin, EspA and EspB as well as for faecal shedding of *E. coli* O157:H7.

Five animals were intramuscularly immunized three times with two weeks interval in the neck (m. *rectus capitis*) with 2 ml of vaccine (Vaccine group). At the same time, three sheep were injected with the suspension without antigen (Placebo group). Animals were challenged 10 days after the last immunization with 10^{10} CFU of *E. coli* O157:H7 on two consecutive days and the faecal excretion was monitored twice a week for three weeks as described by Tutenel *et al.* (2003). Briefly, 10 g feces was diluted in modified tryptone soy

broth supplemented with 20 mg/ml novobiocin and homogenized in a stomacher. Ten-fold dilution series were spread-plated onto cefixime-tellurite sorbitol MacConkey agar (CT-SMAC) containing nalidixic acid. Remaining broth was enriched for 6h at 42°C and subjected to IMS with O157 Dynabeads® (Invitrogen, Merelbeke, Belgium) according to the manufacturer's instructions. Finally, 100 µl was plated onto the CT-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 by enrichment the assigned concentration was 10 CFU/g (Vande Walle *et al,* 2010a).

7.3.4. Antibody response

The antibody response following immunization was measured in serum samples taken at the time of each vaccination and weekly after the challenge using enzyme-linked immunosorbent assays (ELISA) (Vande Walle *et al*., 2010a). Briefly serum samples were heat-inactivated (30 min. at 56°C) and kaolin-treated. Polysorp 96-well plates (NUNC, Polysorb Immuno Plates, Roskilde, Denmark) were coated with 200 ng/well of intimin, EspA or EspB in PBS for 2h at 37°C and blocked overnight at 4°C with PBS supplemented with Tween®80. After washing with PBS supplemented with Tween®20 (washing solution and diluent), plates were subsequently incubated with two-fold serial dilutions of serum and with HRP-conjugated anti-sheep IgG-specific donkey antibodies (AbD Serotec, UK). Sera from animals intramuscularly immunized with intimin, EspA and EspB were used as positive controls. The cut-off values were calculated as the mean OD405-values of all sera (dilution 1/10) at day 0 increased with 3 times the standard deviation. Cut-off values for intimin, EspA and EspB were 0.365, 0.329, and 396, respectively.

7.3.5. Localisation of *E. coli* O157:H7

One non-vaccinated (placebo) and three vaccinated animals were euthanized 21 days after the *E. coli* O157:H7 challenge, to determine the intestinal localisation of *E. coli* O157:H7. The intestinal tissues were examined by direct culturing and IMS as described by Vande Walle *et al.,* (in press) except intestinal tissue was used instead of faeces. Tissue samples were collected from the rectoanal junction (RAJ), rectum, colon, spiral colon, cecum, ileum, jejunum, duodenum, rumen and abomasum of all four animals.

7.3.6. Statistical analysis

Statistical analyses were performed using SAS software. The Proc MIXED test was used to analyse the excretion of the bacteria. The statistical model used to alanyse data included the fixed effect of vaccination, the random effect of sheep nested within treatment and the residual errors. The duration of the excretion was compared in two groups of infected animals by using t-test. Statistical analysis of serum antibody titers was done by General linear Model (repeated mesures analysis of variance). Differences were considered statistically significant at $p < 0.05$.

7.4. Results

The first intramuscular immunization with intimin, EspA and EspB at day 0, resulted in significant ($p<0.05$) increases in serum IgG responses against all 3 antigens 15 days later, at the moment of the second immunization (Fig. 1).

The second immunization only increased antibodies against EspA and EspB but not against intimin. No further significant rise was seen after the third immunization, suggesting that the antibody response had reached its peak with mean log2 titers ranging between 14.34 and 15.68. Indeed also after the infection, no increase in IgG response was observed in the immunized animals. In the placebo group, there were no antibodies against intimin, EspA and EspB before the infection while after the infection the mean log2 titers increased till 4.32, 4.54 and 5.05, respectively.

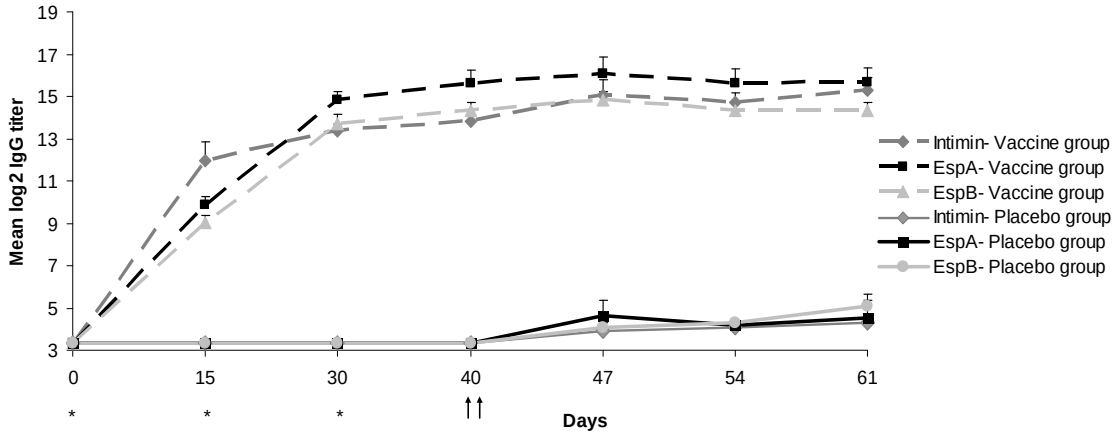

Figure 1: Serum IgG responses following intramuscular immunisation of sheep with intimin, EspA and EspB and challenge infection with *E. coli* O157:H7. Broken lines represent vaccinated sheep and full lines placebo-injected sheep. Results are presented as log 2 values of mean total IgG titre per group and per antigen. * = Intramuscular immunization. Arrows = *E. coli* O157:H7 oral inoculation.

After the challenge infection, vaccinated animals shed significantly less *E. coli* O157:H7 than sheep of the placebo group ($p< 0.05$) (Fig. 2). Four days post challenge, animals in the placebo group shed between 10^5 and 10^6 CFU O157:H7/g feces while vaccinated animals excreted 10^4 CFU/g feces ($p<0.05$).

These numbers gradually declined in both groups during the experiment. Nevertheless, the duration of bacterial excretion by the vaccinated sheep was significantly shorter than by the placebo-vaccinated animals ($p< 0.05$). Interestingly, *E. coli* O157:H7 could not be detected in the feces of two vaccinated animals from day five on, even using IMS. A third animal became negative on day 17 and the last two animals, were only positive by IMS and not with direct culture at the end of the experiment, whereas all three animals in the placebo group remained positive in direct culture till the end of the experiment (Day 21).

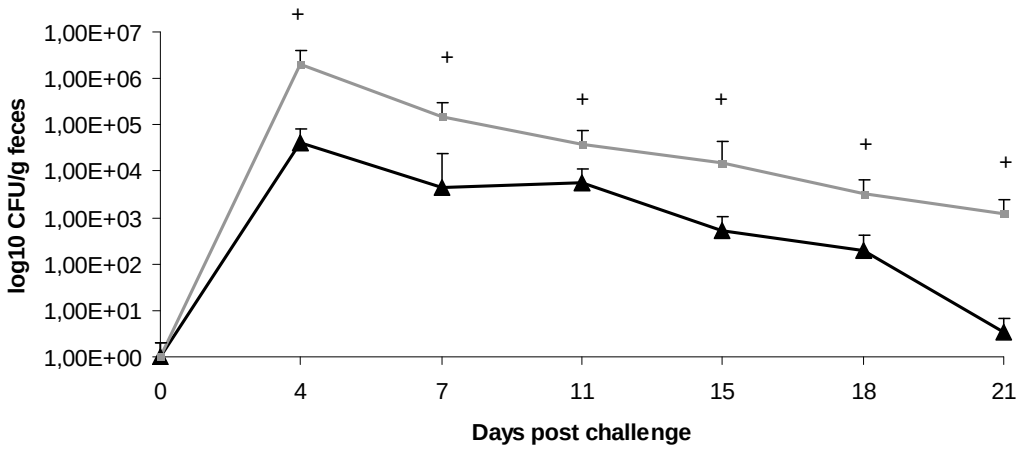

Figure 2: Excretion of *E. coli* O157:H7 in sheep. Results are represented as log values of colony forming units (CFU)/g faeces. Black line: vaccinated group, Gray line: non-vaccinated group. +: significant differences of log values of colony forming units (CFU)/g faeces between vaccinated and non-vaccinated group ($P<0.05$).

After euthanasia, direct culture was only positive for RAJ of the placebo-vaccinated animal (6x10 ^{2} CFU *E. coli* O157:H7 /g. After the enrichment and IMS, all tissues throughout the intestinal tract of the placebo-vaccinated animal were positive, whereas *E. coli* O157:H7 was only found in RAJ of two and the abomasums of one vaccinated animal indicating that only a low amount of bacteria remained in the intestinal tract after the vaccination (Table 1).

Table 1: Intestinal localization of *E. coli* O157:H7 in vaccinated and non-vaccinated (placebo) animals three weeks after bacterial inoculation.

Sheep	RAJ	Rectum	Colon	Spiral colon	Cecum	Ileum	Jejenum	Deudenum	Rumen	Abomasum
Con	* +	+	+	+	+	+	+	+	+	+
V 1	+	-	-	-	-	-	-	-	-	+
V 2	+	-	-	-	-	-	-	-	-	-
V 3	-	-	-	-	-	-	-	-	-	-

+ = Positive after the enrichment and IMS

***** = Direct culture was only positive for RAJ of the placebo-vaccinated animal (6x10 ^{2} CFU *E. coli* O157:H7 /g)

7.5. Discussion

This is the first study demonstrating that systemic vaccination of sheep against *E. coli* O157:H7, using antigens which play a role in intestinal colonization, can significantly reduce bacterial shedding following a challenge with *E. coli* O157:H7. In cattle, vaccination with TTSS proteins of *E. coli* O157:H7 has been analysed before (Potter *et al.,* 2004; Smith *et al.,* 2008; Smith *et al.,* 2009). A variable efficacy has been reported depending on the selected antigen(s). For instance, a subunit vaccine using EspA as well as intimin in combination with the putative adhesion factor Efa elicited non-protective humoral immune responses (Dziva *et al.,* 2007; Van Diemen *et al.,* 2007). In contrast, vaccination of cattle with the supernatant of *E. coli* O157:H7 culture containing TTSS factors offered partial protection in challenged animals, characterized by decreased shedding (Potter *et al.,* 2004; Van Donkersgoed, *et al.,*

2005). Beside TTSS colonisation factors, bacterial supernatant can contain other adherent factors that may contribute to vaccine efficacy including flagellin and LPS whereas it does not contain or contains only low amounts of non-secreted proteins such as intimin, the critical intimate colonization factor of *E. coli* O157:H7 (McNeilly *et al.,* 2010).

In the present study we wanted to evaluate whether a combination of purified recombinant TTSS proteins and intimin would protect against *E. coli* O157:H7 colonization and excretion. Intimin, EspA and EspB were chosen because they could induce an immune response which would affect different stages of the attachment process. Antibodies that bind to EspA and EspB could inhibit the assembly and the insertion of the delivery channel via which different colonization factors are translocated into the host cell. Targeting intimin may block its binding to host cells and the interaction with Tir.

Furthermore we have chosen incomplete Freund's adjuvant since this is effective for inducing high antibody response and increased T-helper1/ T-helper2 responses (Cox *et al.,* 2006). Another study in cattle used IFA adjuvant in combination with EspA, whereas clear antibody responses were induced, no protection was observed (Dziva *et al.,* 2007). In our study, EspA was combined with EspB and intimin. The immunization induced high serum antibody titres against all three antigens and a reduced bacterial colonisation. The mechanism for this reduction needs further investigation, but preliminary data show the presence of antigen-specific IgG ASC in the mucosa, suggesting that antibodies might play a role (Vande Walle *et al.,* unpublished data).

Previous studies showed that EHEC is not maintained in a group of infected calves if the shedding counts decreased to less than 10^4 CFU/g faeces (Matthews *et al.,* 2006). Interestingly in the present study, our vaccine formulation restricted shedding of all five vaccinated animals below 10^4 CFU/g faeces at day 7 day after the infection while the non-vaccinated animals where still excreting between 10^5 and 10^6 CFU/g faeces suggesting that this vaccination might have a significant impact on the spread of the bacteria among a group of animals. This is supported by the observation that bacterial shedding in non-vaccinated sheep was still 10^3 till 10^4 CFU/g feces three weeks after the infection whereas most vaccinated animals stopped excreting or where only positive after enrichment and IMS. In line with the decreased excretion was the low prevalence of *E. coli* O157:H7 in the intestinal tract of vaccinated animals and not of the placebo injected animal.

7.6. Conclusion

In conclusion, the present study shows that vaccination with TTSS proteins EspA, EspB and intimin can reduce *E. coli* O157:H7 excretion in a sheep infection model. Our data suggest that systemic vaccination of sheep can control the spread of *E. coli* O157:H7 which would be an important step in preventing *E. coli* O157:H7 infections in humans.

7.7. Acknowledgements

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Part IV: General Discussion and Future perspectives

Chapter 8

General discussion

Ruminants are the main reservoir of *E. coli* O157:H7, which can be associated with diarrhoea, HC and HUS in humans (Borczyk *et al.,* 1987; Besser *et al.,* 1999)

Recent investigation has shown that 80% of the *E. coli* O157:H7 strains isolated from beef carcasses at processing did not originate from the feedlot where the cattle came from (Arthur *et al.,* 2007). The origin of these isolates was determined to be the environment or even the trailers used to transport the animals (Arthur *et al.,* 2007). In areas where there is high cattle density and a rapid turnover, a few animals shedding bacteria can contaminate the hides of many animals. Indeed, nine percent of cattle presented for slaughter at an abattoir were responsible for 96% of all *E. coli* O157 shed by all animals entering the abattoir at that time (Omisakin *et al.,* 2003). Therefore reducing the number of bacteria shed by the animals and at the same time decreasing the number of animals shedding the bacteria, could dramatically reduce the contamination of carcasses and as a consequence of meat so decreasing the numbers of humans infected by the pathogen.

The specific mechanisms responsible for the persistence of *E. coli* O157:H7 in ruminants are unknown, making it difficult to find an appropriated approach to combat this pathogen. Many strategies have been tested to reduce *E. coli* O157:H7 prevalence in cattle including vaccination, use of antibiotics and probiotics and even diet management. Although some of these approaches seem promising, a 100% fail-proof method is currently not available.

Some interventions may have a direct anti-bacterial effect, while others stimulate the immune system, allowing it to better resist the pathogen. By reducing the fecal pathogen load, the pathogen prevalence and the amount of contamination on hides will be reduced and consequently, carcass contamination rates decrease. Therefore there is a definite need to find new strategies, which could effectively reduce *E. coli* O157:H7 infection at the farm level. Most importantly, the chosen approach should be practically implementable on the farms and feedlots, as well as affordable even for the local farmers.

The use of natural antimicrobial agents which form part of the immune system can be an alternative for antibiotics since ***i***) no resistance seems to occur against these molecules and ***ii***) they could have a broad activity against different stages of the interaction of the bacterium with the host.

One of the aims of the present thesis was to learn more about the effect of lactoferrin, a natural antimicrobial protein of milk, on *E. coli* O157:H7 infection in sheep.

The choice of lactoferrin as a therapeutic or prophylactic agent for *E. coli* O157:H7 was based on several factors. Lactoferrin exhibits anti-oxidant, antiviral, anti-inflammatory, immunomodulating as well as anti-cancer activities, and interestingly, lactoferrin can also 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).

Besides the need of finding new strategies to control *E. coli* O157:H7 infection on farms, the lack of an optimal treatment for *E. coli* O157:H7 infections in humans, rather than simply supportive care, motivated us to examine an approach which possibly could be used in both reservoir (farm animals) and actual host (humans). The high concentration of lactoferrin in milk and the reported therapeutic effect on gastrointestinal infections of several species makes lactoferrin a potential candidate for the treatment of *E. coli* O157:H7 (Yamauchi, *et al.,* 2006; Teraguchi *et al.,* 2004).

However, whereas lactoferrin could be therapeutically used or even prophylactic in well-defined conditions, it is unlikely to continuously administer this kind of proteins via the food. Therefore a vaccine, which will completely protect animals against colonization, is another important approach. Vaccination studies in cattle showed promising results (Potter *et al.,* 2004; Smith *et al.,* 2008; Smith *et al.,* 2009). Vaccination of cattle could decrease the number of animals colonized as well as the degree and duration of excretion of individual animals. However, complete protection was not obtained. Until now, no such vaccination studies had been performed in sheep. So, an additional aim of this thesis was to evaluate if vaccination of sheep could result in a similar protection as in cattle.

8.1. Antimicrobial susceptibilities of commensal *Escherichia coli* isolates from cattle in Iran

Concerns for the spread of resistant bacteria from the large reservoirs in food animals led the countries in the European Union to abandon the use of antimicrobial agents for growth promotion in food animals by 1 January 2006 (Anette and Heuer, 2009). The USA, Canada and most of the developing countries, such as Iran, did not show the same concern and still use many antimicrobial agents in animals and animal feed for therapeutic or prophylactic purposes. Almost no data are available regarding the epidemiology and prevalence of antimicrobial resistance in domestic animals in Iran.

Indicator bacteria, next to pathogenic and zoonotic bacteria, are used for resistance monitoring in animals and humans. Indicator bacteria are of special interest because of their possible role as a resistance reservoir, harbouring resistance genes that may be transmitted to other bacterial populations within the same host or other hosts. *E. coli* isolates from food animals are a potential reservoir of resistance genes which could transfer this resistance to organisms that might cause disease in animals and/or humans, making them important indicator bacteria (Aarestrup, 2004, Smet *et al*., 2008, Checkley *et al.,* 2010).

In Chapter 3, the antimicrobial susceptibility profile of *E. coli* isolates obtained from raw milk and feces of healthy dairy cows for 11 antimicrobial agents was determined. Seventy-five percent of the isolates showed acquired resistance to three or more antimicrobial agents. The highest resistance was detected for tetracycline (46%) followed by ampicillin (43%). Around 30 % of the isolates showed resistance against trimethoprim, amoxicillin-clavulanic acid and sulfafurazole. The highest susceptibility was seen for the cephalosporin, ceftiofur (94%), followed by colistin (86%). This order in resistance with the highest resistance against tetracyclins is comparable with results of several other studies (Sawant *et al.*, 2007; de Jong *et al*., 2009; Berge *et al*., 2010). However, the percentages of isolates with resistance against one of the tested antimicrobials as well as the percentage with multiple antimicrobial resistance, resistance, is higher than in most other studies. Also in a previous Iranian study, very high resistance rates were found (Ebrahimi *et al*., 2007a). Even though we only tested a limited number of isolates (63) from a limited number of farms (35) in only one Iranian Province, so that

results can not be generalized, the high antimicrobial resistance in our study and in the previous Iranian study are serious arguments for a restriction of the use of antimicrobials. These data are arguments for the development of alternative strategies for treatment of diseases or for reducing colonization with potential zoonotic microorganisms.

8.2. Lactoferrin reduces *E. coli* O157:H7 growth and attachment to epithelial cells

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; Magwira *et al.*, 2005; Fincher *et al.,* 2009). In addition, treatment with antibiotics cannot always shorten the duration of diarrhoea and can even increase the risk of developing HUS (Besser *et al.,* 1999; Wong *et al.,* 2000). Thus, there are several reasons for developing new strategies to control an O157:H7 infection.

In the present PhD thesis one of the strategies examined was to use a natural antimicrobial agent. As already mentioned, one of the advantages of using natural antimicrobial proteins is that no antimicrobial resistance seems to occur against these molecules. One of the important challenges was to implement such a therapy in ruminants in order to prevent *E. coli* O157:H7 infections or at least reduce fecal shedding significantly.

As already mentioned, lactoferrin, a natural protein of milk, has not only antimicrobial effects, but also immunomodulatory properties (Tomita *et al.,* 2009). Moreover, recent studies propose lactoferrin as a bactericidal agent for antibiotic resistant strains of *E. coli*. This effect was more pronounced against a multidrug resistant *E. coli* O157:H7 strain than against EPEC and *Staphylococcus aureus* with a growth inhibition of 81, 76.8 and 36.7%, respectively (Flores-Villasenor, *et al.,* 2010). These properties of lactoferrin and the fact that bulk amounts can be produced from milk are important reasons why we have selected this protein as an interesting candidate.

In **Chapter 4** we could demonstrate that lactoferrin is capable to reduce *in vitro* the growth of *E. coli* O157:H7. This effect was more pronounced for lactoferrin purified from bovine milk than for human lactoferrin. Although the reason for this difference is not known, a greater

affinity of bovine lactoferricin, a peptide derived from bovine lactoferrin, for LPS has been shown than of human lactoferricin (Bellamy *et al.,* 1992).

In our study non-iron saturated lactoferrin had a stronger effect than the saturated form. The non-iron saturated form can still capture iron, which is important for the growth of the bacteria. Important to notice is that none of the lactoferrin concentrations in any form used in our study resulted in 100% growth inhibition. The growth inhibition of bovine and human lactoferrin was significant between 3 and 6 hours after incubation. The lag period of 3 hours could be due to the relatively slow interaction of lactoferrin with bacterial LPS. This interaction is known to result in bacterial killing (Ellison *et al.,* 1991). The fact that the effect lasted for only 6 hours was most likely due to consumption of lactoferrin. Indeed, preliminary data of experiments in which lactoferrin was added repeatedly each two hours until four hours after the start of the experiment, showed a complete growth inhibition during at least 8 hours for the samples treated with 5 or 10 mg lactoferrin/ml.

In **Chapter 4** we also demonstrated that both human and bovine lactoferrin can inhibit *E. coli* O157:H7 adherence to Caco-2 cells in a dose-dependent manner. The anti-adhesive effect of human lactoferrin was higher than for bovine lactoferrin. This could be due to a higher effectiveness of lactoferrin in the homologues system (human lactoferrin and human cells). Beeckman *et al.* (2007) described a similar finding for ovotransferrin. A second explanation could be a more efficient binding of bovine lactoferrin to and uptake within Caco-2 cells so that its extracellular concentration wanes more rapidly than this of human lactoferrin (Shin *et al.*, 2008).

In this chapter, evidence was provided that the anti-adhesive effect of lactoferrin, in doses which cannot kill the bacteria, might be due to its proteolytic effect on the *E. coli* O157:H7 colonization factors EspA and EspB. Such an effect has also been shown for the related pathogen EPEC (Ochoa *et al.,* 2002, 2003).

8.3. Lactoferrin downregulates the inflammatory cytokine response of *E. coli* O157:H7 in a sheep intestinal explant model

So, lactoferrin can inhibit *E. coli* O157:H7 growth and its adhesion to Caco-2 cells. To investigate the effect of lactoferrin in a system closer to the *in vivo* situation, and to get more insights in the effect of lactoferrin on *E. coli* O157:H7 infection in sheep, an e*x vivo* model was developed in **Chapter 5** in which intestinal explants were incubated with *E. coli* O157:H7 and cell tropism and cytokine responses were studied. Results showed for the first time that in sheep, in contrast to humans, *E. coli* O157:H7 does not only have a tropism for the FAE of ileal Peyer's patches but also for the normal ileal absorptive epithelium. However, the adhesion to the FAE was almost nine times higher than to the absorptive ileal epithelium and more than 3 times higher than to the colonic epithelium. Due to this higher tropism, one could hypothesize that the ileal FAE of sheep might be the initial attachment site for *E. coli* O157:H7, whereafter the microorganism spreads to the ileal absorptive epithelium and the colon. Although it is not known why *E. coli* O157:H7 targets ileal FAE of sheep, evidence from mice studies showed that β-1 integrin, a receptor for intimin γ, is highly expressed on M cells of the ileal FAE. Such an expression still has to be demonstrated for cattle and sheep. Interestingly, initial attachment to the FAE followed by spread to other parts of the intestine has previously been shown for EPEC infections in rabbits (Cantey *et al.,* 1981).

One of the aims of this chapter was to determine if lactoferrin could diminish the bacterial adhesion to explants, similar as it did to the human epithelial cell line. The effect of lactoferrin was quite consistent over the different tissues and a reduction in adhesion with a factor 2.5 to 3.1 was observed. Effects could have been more pronounced if lactoferrin was submitted with intervals or if we could have simulated intestinal peristalsis. The latter could flush away bacteria, which initially are prevented to adhere, but in a static system can try to adhere again. Nevertheless we clearly demonstrated that lactoferrin could inhibit *E. coli* O157:H7 attachment to the sheep intestinal mucosa.

It was unclear until now, whether *E. coli* O157:H7 infection in sheep could induce inflammatory signals. Especially, since we could not demonstrate an antibody response and only a low cellular immune response in rectally inoculated sheep (Vande Walle *et al*., 2010a). This seemed to suggest that the colonic colonization could dampen the immune system. However, oral inoculation resulted in a clear antibody and cellular immune response, suggesting that small

intestinal mucosa might respond differently to colonisation than colonic mucosa (Vande Walle *et al*., 2010b). Therefore, another aim of this chapter was to analyze the cytokine response of the intestinal explants following incubation with the *E. coli* O157:H7 strain. The data of this study seem to confirm our hypothesis that the infection has a different effect in ileum and colon since the strain significantly induced IL-8 mRNA in ileal explants with and without Peyer's patches and TNF-α mRNA in the explants with Peyer's patches, whereas no such response was seen for the colon explants. We did not identify the mechanism by which *E. coli* O157:H7 stimulated IL-8 and TNF-α mRNA expression, however it has been shown that bacterial proteins including TTSS proteins may contribute to pro-inflammatory cytokine up-regulation (Steiner *et al.,* 2000; Dahan *et al*., 2002; Hauf and Chakraborty, 2003). However, this cannot explain the different response between ileal and colonic tissue.

Interestingly, lactoferrin diminished the cytokine expression. Although the lower rate of infection due to lactoferrin (**Chapter 2**) could be an explanation, we cannot rule out that TTSS protein degradation after lactoferrin treatment (**Chapter 2 and 3**) additionally helps to decrease cytokine expression.

8.4. Lactoferrin reduces shedding of *E. coli* O157 in sheep

In an effort to combat pathogenic or zoonotic bacteria from cattle, it is logical to envision strategies that specifically target and kill the bacteria. In this regards, the growth inhibiting (**Chapter 4**), the adhesion-inhibiting (**Chapter 4 and 5**), the proteolytic activities (**Chapter 4**) and the possible immunomodulatory role of lactoferrin on *E. coli* O157:H7 infection (**Chapter 2**), made us hypothesize that oral lactoferrin might be used to reduce in sheep the colonization of the intestinal tract and as a consequence, faecal shedding of *E. coli* O157:H7. That the adhesion-inhibiting effect of lactoferrin on *E. coli* occurs fast, was an additional argument (Naidu *et al.*, 1991; **Chapter 4 and 5**), as well as, that oral lactoferrin administration to rat has been validated before without side effects being reported (Appel *et al.*, 2006).

Since it is well known that the time required for *E. coli* to pass the small intestine is not more than a few hours, a more or less continuous presence of lactoferrin in the intestinal tract had to be obtained. This could be achieved by supplementing sheep feed with lactoferrin. However, in

the current experiments it was important to know the exact dose the sheep received. Therefore, we chose to orally administer lactoferrin twice a day.

Another important factor we had to consider was the iron binding property of lactoferrin. Iron is essential for bacterial growth and therefore bacteria have efficient iron acquisition mechanisms (Suits *et al.,* 2009; Torres *et al.,* 1997). However, in body secretions, blood plasma, neutrophils and macrophages lactoferrin is present in a relatively iron-free state, e.g. In milk the saturation is 10 to 30 %, allowing the protein to sequester free iron resulting in a limited availability of iron for bacteria at the infection site (Rainard, 1986; Levay and Viljoen, 1995). Since this enhances the antibacterial effect of lactoferrin, iron-free lactoferrin was used in this study. Based on our finding that lactoferrin seemed to be more effective in a homologues system (**Chapter 4**), bovine and not human lactoferrin was chosen for the *in vivo* trial on sheep, since cattle and sheep are more closely related.

In our study, the lactoferrin was given in bicarbonate buffer because this buffer is capable to close the oesophageal groove, so that lactoferrin could pass rumen, reticulum and omasum and directly reach the abomasum (Rosenberger G., 1979). This delivery in the abomasum prevents its consumption and/or bacterial degradation in the rumen. Furthermore, pepsin, which is highly present in abomasum, degrades lactoferrin in different peptides (Gifford *et al.,* 2005). Some of these peptides, such as lactoferricin, show stronger antimicrobial and immunomodulatory properties than the intact protein, lactoferrin (reviewed by Baker and Baker, 2009). So this mode of administration could increase the potential of lactoferrin to reduce *E. coli* O157:H7 infection in ruminants.

Two different dosages of lactoferrin (1.5 or 0.15 g/animal/ 12 hours) were given for 30 days and *E. coli* O157:H7 challenge infection was performed at the second day of the lactoferrin administration. For both dosages a reduction in the concentration of *E. coli* O157:H7 in feces and in the duration of fecal excretion was observed with the highest dose being more effective. Interestingly, this high dose increased the antibody response against EspA and EspB. A similar increase in antibody response was observed in chickens orally fed lactoferrin from birth on following oral vaccination with an infectious bursal disease vaccine at 1 and 3 weeks of age (Hung *et al.,* 2010)**.** It has been demonstrated that lactoferrin can stimulate maturation of B-

and T-lymphocytes and can increase recruitment and maturation of antigen-presenting cells. This occurs by influencing pattern recognition receptor-mediated cell signalling (Curran *et al.*, 2006; De la Rosa *et al.,* 2008; Legrand and Mazurier, 2010). However, the immunomodulatory effect of lactoferrin has not been studied here and whether lactoferrin exerted a direct effect on the immune response or whether its degradation of EspA and EspB, as demonstrated in **Chapter 4**, was responsible for the increased antibody response needs further clarification.

8.5. Vaccination of sheep with TTSS proteins reduces EHEC shedding after experimental challenge

Whereas oral administration of lactoferrin could be an interesting approach to use in specific situations such as the treatment of animals which shed high numbers of EHEC for a long period (super shedders) or animals which are going to be slaughtered, prevention of infection via vaccination could become the method of choice on a long term. Indeed, several studies in cattle demonstrated the possibility to decrease *E. coli* O157:H7 shedding via systemic vaccination (**Chapter 1**). However, variable efficacy has been reported, depending on the selected antigen(s), with TTSS proteins of *E. coli* O157:H7 seeming to be the most effective, on the dose, on the number of vaccinations and on the adjuvant (Potter *et al.*, 2004; Naylor *et al.*, 2005a; Naylor *et al.*, 2005b; Peterson *et al.*, 2007a; Peterson *et al.,* 2007b; Nart *et al.*, 2008a; Nart *et al.*, 2008b). In none of these studies complete protection was obtained. Therefore, this strategy needs further optimization. Furthermore, it had not yet been examined if vaccination could also reduce colonisation and shedding in sheep. The aim of this chapter was to evaluate if reduced shedding could be obtained via vaccination using the oral *E. coli* O157:H7 sheep model developed in our lab (Vande Walle *et al.,* 2010b).

EspA and EspB were chosen as vaccine antigens because antibody responses against both antigens could interfere with the assembly and the insertion of the delivery channel via which different colonization factors are translocated into the host cell. Intimin was chosen as a third antigen since intimin-specific antibodies might block the interaction of intimin expressed by the bacteria with receptors on the host cells such as Tir.

In **Chapter 7**, we showed that the immunization induced high serum antibody titres against all three antigens and that this was accompanied with a reduced bacterial colonisation. Indeed, in line with the decreased excretion was a low presence of *E. coli* O157:H7 in the intestinal tract of vaccinated animals and not of the placebo injected animal. The mechanism for this reduction has not been investigated in the present study, but preliminary data of our laboratory show the presence of antigen-specific IgG ASC in the mucosa, suggesting that antibodies might play a role (Vande Walle, 2010).

Whereas the results of the vaccination are promising some comments should be made. Firstly, unpublished data of our lab showed that the response induced by the vaccination followed by challenge infection did not remain very long and some months later sheep could be easily recolonized with the same strain of *E. coli* O157:H7 (Vande Walle *et al.,* 2010). Secondly, it should be taken into the account that the strain we have been using is Stx negative. Recent data indicate a possible immunosuppressive role for Stx in ruminant's colonisation (Bretschneider *et al*., 2007). Thirdly, results in cattle indicate that a vaccination using antigens from *E. coli* O157:H7 could reduce colonisation with the homologous strain, but were less protective against heterologous EHEC strains. This indicates that there is need for additional studies to optimize this vaccination.

Chapter 9

Future perspectives

The results obtained in this thesis indicate that oral lactoferrin as well as parenteral vaccination against TTSS proteins and intimin, could be tools to reduce *E. coli* O157:H7 infection in sheep. However, conclusions should be drawn with caution and further validation of both strategies has to be performed. The high variation in colonization and excretion in natural settings, but also following experimental infection is a complicating factor. While the size of our animal experiments in both lactoferrin and vaccination studies could be criticized, carrying out these studies in a larger number of animals was practically impossible during this thesis. This issue should become one of the future steps to be set.

Apart from using a well-established *in vivo* model with a sufficient number of animals, there is an absolute need to further understand the mechanism of *E. coli* O157:H7 colonisation in ruminants and the role of the immune system. Such studies are ongoing in our laboratory and might help us to understand how we could manipulate the immune system of ruminants to improve clearance of the bacteria (Vande Walle, 2010; Vande Walle *et al.*, 2010 a, b).

For lactoferrin, some important issues have to be clarified. One issue is the administration form; giving lactoferrin twice a day orally in a buffer is impractical. Therefore a formulation has to be developed that can be given together with the feed. Development of novel oral delivery systems which allow the selective release of the drug close to its effector site, in a highly dispersed way, prevent lactoferrin of being consumed by ruminal bacteria. Different strategies could be used to achieve this objective such as the use of slow release and/or pH sensitive release systems to deliver lactoferrin into the small intestinal tract.

Another question, which should be resolved is, if lactoferrin will reach in a sufficiently active form the rectal mucosa, a predilection site for *E. coli* O157:H7 in cattle. Here, mucosal adhesive dosage forms, which can be rectally applied, could be an option. Mucosal adhesive dosage forms have been extensively investigated as an external preparation that can be administrated effectively and safely (Machida 1979). Recent investigations in mice showed that tablets, which are adhesive for the mucosa and gradually release bovine lactoferrin, improved the healing of

artificially made oral mucosal ulcers (Takahashi *et al,* 2007). Applying lactoferrin to the oral and/or rectal mucosa of ruminants using such dosage forms might provide more effective anti-bacterial and immunomodulatory effects.

On the other hand, recently genetically modified rice was produced which expresses lactoferrin (Brooke *et al.,* 2002). The use of such a product in animal feed could have the advantage of eliminating the need to purify the protein from the producing organism or milk and of easier administration of lactoferrin (Conesa *et al.,* 2010). In support of this strategy, it has been shown that feeding genetically modified rice to mice, results in antibiotic-like and immunomodulatory effects (Brooke *et al.*, 2002).

Another issue is the optimal dose. Economically the lowest effective dose would be the choice. However, it is important to know if the enhanced antibody response in the animals which have received the highest lactoferrin dose, prevents or diminishes future infections.

In Chapter 7, we investigated a vaccination strategy against *E. coli* O157:H7 infection in sheep. This was previously shown to be effective in cattle. The idea of ruminant vaccination against *E. coli* O157:H7 has always been interesting for many researchers, since it could have a huge impact on public health. Different formulations, different adjuvants and even different delivery methods have been developed to improve the potency of *E. coli* O157:H7 vaccines in cattle (Potter *et al.*, 2004; Naylor *et al.*, 2005a; Naylor *et al.*, 2005b; Peterson *et al.*, 2007a; Peterson *et al.,* 2007b; Nart *et al.*, 2008a; Nart *et al.*, 2008b). At present, systemic vaccination of cattle against *E. coli* O157:H7 TTSS proteins seems very promising and a vaccine, Econiche, is already for a few years on the market in Canada and the USA. Recently, a second vaccine has been provisionally approved for cattle, namely Epitopix, based upon Siderophore Receptor and Porin proteins (Fox *et al.,* 2009).

In this thesis, we demonstrated that in sheep, *E. coli* O157:H7 excretion could be greatly reduced by vaccination against TTSS antigens, as in cattle. Although preliminary data obtained in our laboratory suggest a role for mucosal antibodies in protection, the exact mechanism of how vaccination reduces bacterial shedding needs more investigation (Vande Walle *et al.,* 2010). McNeilly and co-workers (2010) showed that systemic immunization of cattle against TTSS, not only induced serum but also rectal IgG antibodies. Our preliminary results also showed a

mucosal IgG response in sheep (Vande Walle, 2010). Whether these antibodies are playing a role in the mechanism affecting the *E. coli* O157:H7 colonization in sheep or cattle still has to be demonstrated.

Vaccination with different antigens and different adjuvants in cattle resulted in a variable protection efficacy (Potter *et al.,* 2004; Babiuk *et al.,* 2008; McNeilly *et al.,* 2008). Future work on sheep vaccination has to target additional antigens or different combinations of antigens. Furthermore, our vaccination was performed using Freund's adjuvant. This adjuvant induces a mixed T-helper 1 like/T-helper 2 like immune response. Other adjuvants such as ISCOMs or CpG motifs modulate the response towards T-helper 1 cells whereas adjuvants such as aluminium salts or the thermolabile enterotoxin modulates the response toward T-helper 2 cells (reviewed by Cox *et al*, 2006). A T-helper 1 like response directs the response towards cellular immunity and a T-helper 2 like response towards antibodies. Since it is currently not known which responses are involved in protection, using different adjuvants could help clarify the immune mechanisms as well as optimize the vaccine formulation.

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Summary

Enterohaemorhagic *Escherichia coli* (EHEC) are a group of bacteria responsible for numerous foodborne outbreaks in humans which can lead to bloody diarrhea, haemorrhagic uremic syndrome and in some cases in children even in death. *E. coli* O157:H7 is the most well known serotype of this group. Ruminants are the most important reservoir of these microorganisms and many outbreaks have been traced to ruminant's origin. Ruminants typically continue to shed bacteria in their feces for weeks to months. Carcasses of non-colonized animals have regularly been found to be contaminated with *E. coli* O157:H7 in the abattoir, suggesting that cross-contamination during meat processing can be a major source of the contamination of beef products and subsequently of human's infection. Therefore reducing *E. coli* O157:H7 shedding by ruminants would be a way to control the infection of humans. Up till now, no efficient treatment is available against *E. coli* O157:H7 infection and some traditional treatments, including antibiotic therapy may even increase the chance of developing severe disease in humans. Therefore, there is need for alternative ways to reduce the risk of *E. coli* O157:H7 infection of humans for instance by diminishing or preventing *E. coli* O157:H7 excretion at the farm level.

The first part of this thesis reviews current literature. **Chapter 1** of this thesis gives an overview of the literatures on the pathogenesis of *E. coli* O157:H7 in human and ruminants. Also the reported intervention strategies in ruminants were discussed. **Chapter 2** reviews present knowledge on lactoferrin as the main antibacterial immunomodulatory protein of milk. The focus of this chapter is on the antimicrobial mechanism of lactoferrin and its peptides, in particular the antimicrobial mechanism against gram-negative pathogens. In addition the role of lactoferrin as immunomodulatory protein is briefly reviewed.

A second part of the thesis describes the aims. Following questions were addressed in this thesis:

√ What is the pattern of antimicrobial susceptibility among *E. coli* isolates from dairy cows in Iran?

√ Can lactoferrin, a natural antimicrobial protein of milk, be used to prevent or reduce colonisation of the ovine intestinal tract with *E. coli* O157:H7 and so reduce or prevent fecal shedding?

√ Is systemic vaccination of sheep with type III secretion system proteins and intimin γ a possible strategy to reduce fecal shedding of *E. coli* O157:H7 as in cattle?

A third part, comprising **Chapters 3 to 7,** presents the experimental work of this thesis.

In **Chapter 3** the antimicrobial susceptibility of *E. coli* isolates from milk and healthy cattle feces in Iran has been investigated. The results identified ceftiofur (6%) and colistin as the most effective antibiotics. However the incidence of colistin resistance (14%) among the isolates was remarkably higher than in previous reports on antibiotic susceptibility of commensal bacteria. The highest percentage of acquired resistance was detected for tetracycline (46%), followed by ampicillin (43%). %). For the other tested antimicrobials (amoxicillin-clavulanic acid, chloramphenicol, enrofloxacin, florfenicol, spectinomycin, sulfafurazole and trimethoprim) resistance varied between 30 and 17%. Seventy-five percent of the isolates showed multiple antimicrobial resistance. This means resistance to three or more antimicrobial agents. This overall high antimicrobial resistance should alert veterinarians and authorities to take measures for decreasing antimicrobial usage and should stimulate research towards alternative strategies for treating cattle against bacterial infections.

Since traditional treatment including antibiotic therapy is not a suitable option in the control and/or treatment of *E. coli* O157:H7, the use of lactoferrin, a natural antimicrobial protein of milk has been investigated in the present thesis for reducing *E. coli* O157:H7 shedding in ruminants feces. First the effect of lactoferrin on this bacterium was tested. In **Chapter 4** lactoferrin from bovine and human milk were compared for their direct effect on *E. coli* O157:H7 growth. Bovine lactoferrin (bLF) had a significantly stronger inhibitory effect than human lactoferrin (hLF). The non iron-saturated form of bovine lactoferrin (apo-bLF) reduced *E. coli* O157:H7 growth significantly stronger than the iron-saturated form. In addition, all lactoferrins, apart of their origin or iron saturation level, reduced *E. coli* O157:H7 attachment to Caco-2 cells at a dose that had no effect on bacterial growth. Therefore other factors rather

than simply scavenging iron from the environment and/or reducing bacterial growth plays a role in the anti-adhesive effect of lactoferrin. In **Chapter 4** we showed that this effect is at least in part due to a catalytic effect of lactoferrin on TTSS proteins of *E. coli* O157:H7; EspA and EspB which are essential for *E. coli* O157:H7 colonisation were cleaved. This proteolytic activity of lactoferrin has already been shown on the TTSS apparatus of EPEC, a similar pathogen. In conclusion, the results reported in this chapter, support the idea that lactoferrin may reduce the colonisation of *E. coli* O157:H7 *in vivo*.

To obtain more insights in the effect of lactoferrin on the interaction of *E. coli* O157:H7 with the sheep intestinal mucosa, a sheep intestinal explant model was developed. In **Chapter 5** we examined using this sheep specific *in vitro* model 1) the tropism of *E. coli* O157:H7 for ileal or large intestinal mucosa, 2) the intestinal mucosal cytokine response induced by the mucosal *E. coli* O157:H7 colonisation and 3) the effect of lactoferrin on colonisation and cytokine response. The data presented in this chapter show that *E. coli* O157:H7 preferentially binds to ileal follicle-associated epithelium, but can also bind to the normal absorptive ileal mucosa and colonic mucosa, although to a lesser extent. We also demonstrated that this bacterial attachment could enhance IL-8 and TNF-α mRNA expression with the increase in expression being correlated with the number of bacteria attaching to the sheep intestinal explant. Furthermore results presented in this chapter clearly showed that lactoferrin inhibits *E. coli* O157:H7 attachment to the explants with lower IL-8 and TNF-α mRNA expression as a consequence.

In **Chapter 6**, we examined if lactoferrin, administered via the oral route, could reduce *E. coli* O157:H7 excretion of sheep. It is not evident that the administration via this route would be effective since the protein perhaps could become degraded in the rumen. Therefore, lactoferrin was solublized in a bicarbonate buffer which can close the esophageal groove allowing lactoferrin to pass rumen, reticulum and omasum so reaching the abomasum. Oral lactoferrin was given every 12 hours for 30 days in two different doses (1.5 or 0.15 g) and the inoculation with *E. coli* O157:H7 occurred 24 hours after the first lactoferrin administration. Both dosages reduced the number of *E. coli* O157:H7 in sheep feces as well as the duration of fecal excretion. Furthermore, sheep, which received the high dose of lactoferrin (1.5 g, every 12 hours) showed a significantly higher serum antibody response against EspA and EspB in comparison with the

control group. Since the peak of this antibody response was seen when the excretion of *E. coli* O157:H7 had completely ceased, it could be that this immune response plays a role in reducing the bacterial excretion in sheep orally treated with lactoferrin. Results of this study show that lactoferrin can become a new tool in controlling *E. coli* O157:H7 but also other EHEC infections by decreasing their excretion by reservoir animals.

In the last chapter of the experimental part of the present thesis, the potential of systemic vaccination against *E. coli* O157:H7 in sheep was examined. Vaccination studies have previously been performed in cattle. Some of these studies in which cattle were systemically vaccinated with an antigen mixture containing several type III secretion system (TTSS) proteins could significantly reduce shedding. In this chapter we wanted to know if similar result could be obtained in sheep. This could allow us to study in a next step the mechanism of this protection and to further optimize vaccination in sheep. Sheep were intramuscularly vaccinated with a mixture of the TTSS proteins EspA and EspB and the surface antigen intimin γ in incomplete Freund's adjuvant or received only the adjuvant. The data obtained in **Chapter 7** showed that the vaccination significantly reduced the fecal excretion of the bacteria. These results are consistent with the previous studies in cattle. Although the vaccination strongly increased the level of serum IgG against intimin, EspA and EspB, the mechanism for the observed *E. coli* O157:H7 reduction in the feces still needs to be determined. Results suggest that besides oral lactoferrin treatment also vaccination could become an important tool to reduce *E. coli* O157:H7 excretion by sheep.

The fourth part of the thesis consists of the general discussion and future perspectives. Although important steps were set towards new strategies in the control of *E. coli* O157:H7 infections, many questions still remain to be resolved such as: 1) Is there a tropism for the rectal mucosa in sheep and can lactoferrin via the oral route reach the rectal mucosa and clear the colonizing bacteria?; 2) What is the mechanism for the enhanced antibody response following lactoferrin treatment?; 3) Is this antibody response also present in the mucosa?; 4) Can we develop an administration form that is feasible in practice?; 5) What is the mechanism of the reduction in excretion following vaccination?; 6) Can we still improve this clearance using more

or different antigens and different adjuvants?; 7) What is the duration of this immune mechanism in the mucosa?

Future research has to direct these questions. For some of these questions the first steps to resolve them have already been taken.

Samenvatting

Enterohaemorrhagische *Escherichia coli* (EHEC) vormen een groep bacteriën die verantwoordelijk zijn voor verscheidene voedselgerelateerde uitbraken. Ze veroorzaken bij de mens bloederige diarree, het hemorragisch uremisch syndroom (HUS) en kunnen leiden tot de dood en dit voornamelijk bij kinderen. *E. coli* O157:H7 is het best gekende serotype binnen deze groep. Herkauwers vormen het belangrijkste reservoir van dit micro-organisme en de bron van meerdere uitbraken werd herleid naar herkauwers. Herkauwers scheiden de bacterie uit in de faeces gedurende weken tot maanden. In het slachthuis wordt soms vastgesteld dat karkassen van niet-gekolonizeerde dieren besmet zijn met *E. coli* O157:H7, wat erop wijst dat kruiscontaminatie tijdens het vleesverwerkingsproces een belangrijke bron van contaminatie van vlees kan zijn, en kan leiden tot infecties bij de mens. Vandaar dat een reductie in *E. coli* O157:H7 uitscheiding bij herkauwers zou resulteren in een verminderde infectie bij de mens.
Tot nu toe is er geen efficiënte behandeling van een *E. coli* O157:H7 infectie beschikbaar en bepaalde traditionele behandelingen zoals antibiotica toediening kunnen de kans op het ontwikkelen van complicaties bij de mens zelfs verhogen. Daarom is er nood aan alternatieve manieren om *E. coli* O157:H7 excretie door herkauwers te reduceren.

Het eerste deel van deze thesis, die een literatuurstudie omvat, bestaat uit twee hoofdstukken. **Hoofdstuk 1** geeft een overzicht van de literatuur over de pathogenese van *E. coli* O157:H7 bij mensen en herkauwers. Daarnaast worden bekende interventiestrategieën bij herkauwers besproken. **Hoofdstuk 2** geeft de huidige kennis over lactoferrine. De focus ligt op het antimicrobieel werkingsmechanisme van lactoferrine en zijn peptiden. Bijkomend wordt de rol van lactoferrine als immunomodulatorisch eiwit kort besproken.

Het tweede deel van de thesis beschrijft de doelstellingen van dit werk. Het doel van deze thesis was een antwoord te formuleren op volgende vragen:

- ✓ Wat is het antibiogram van *E. coli* isolaten afkomstig van gezonde dieren in Iran?
- ✓ Kan lactoferrine dienen als beschermingsmiddel om *E. coli* O157:H7 infectie en excretie bij schapen te reduceren?
- ✓ Kan systemische vaccinatie schapen beschermen tegen *E. coli* O157:H7 infectie?

Het derde deel van de thesis, **hoofdstukken 3 tot 7** omvattende, geeft het experimenteel werk van deze thesis weer.

In **hoofdstuk 3** wordt de antimicrobiële gevoeligheid van *E. coli* isolaten afkomstig van gezonde melkkoeien uit Iran onderzocht. De resultaten tonen aan dat ceftiofur en colistine de meest efficiënte antibiotica zijn, hoewel de incidentie van colistine resistentie (14%) opvallend hoger was dan vermeld in eerdere rapporten over commensale bacteriën. Daarentegen vertonen een hoog percentage isolaten resistentie tegen tetracycline (46%) en tegen ampicilline (43%). Voor de andere geteste antimicrobiële producten (amoxicillin-clavulanic acid, chloramphenicol, enrofloxacin, florfenicol, spectinomycin, sulfafurazole and trimethoprim) ligt de resistentie tussen 17 en 30 %. Ongeveer 75% van de isolaten hebben resistentie verworven tegen ten minste 3 van 11 geteste antibiotica. Dit toont een hoge antimicrobiële resistentie die kan te wijten zijn aan een hoog gebruik van antimicrobiële producten bij melkkoeien . Dit wijst op de nood naar ontwikkeling van alternatieve therapieën voor bestrijding van infecties.

Aangezien traditionele behandelingen inclusief het gebruik van antibiotica geen gepaste optie zijn voor de controle en/of behandeling van *E. coli* O157:H7, werden andere strategieën onderzocht om de hoeveelheid *E. coli* O157:H7 bacteriën in de faeces van herkauwers te reduceren. Eén van de strategiën onderzocht in deze thesis is het gebruik van lactoferrine, een natuurlijk antimicrobieel eiwit in melk. Om lactoferrine te kunnen gebruiken als bescherming van schapen tegen *E. coli* O157:H7, moesten we weten welk effect lactoferrine heeft op de bacteriën. Daarom werd in **hoofdstuk 4** het effect van lactoferrine afkomstig uit runder- en menselijke melk bepaald op groei van *E. coli* O157:H7. Runder lactoferrine (bLF) had een significant sterker inhibitorisch effect op de bacteriële groei dan menselijk lactoferrine (hLF). De niet-ijzergesatureerde vorm van runder lactoferrine (apo-LF) reduceerde de groei van *E. coli* O157:H7 significant sterker dan de ijzer-gesatureerde vorm. Bovendien waren alle vormen van lactoferrine, onafhankelijk van oorsprong of ijzersaturatie, in staat tot het reduceren van *E. coli* O157:H7 adhesie aan HEp-2 en Caco-2 cellen in dosissen die geen effect hadden op de bacteriële groei. Dit wijst erop dat factoren verschillend van simpelweg ijzeropname uit de omgeving en/of reductie van bacteriële groei een rol spelen bij het anti-adhesief effect van lactoferrine. In **hoofdstuk 4** werd aangetoond dat dit effect op zijn minst

gedeeltelijk te wijten is aan het katalytisch effect van lactoferrine op type III secretiesysteem (TTSS) eiwitten van *E. coli* O157:H7, nl. EspA en EspB, welke essentieel zijn voor kolonisatie van *E. coli* O157:H7. Het katalytisch effect van lactoferrine op het TTSS apparaat werd reeds aangetoond voor gelijkaardige bacteriën (EPEC). Tot besluit kunnen we stellen dat de resultaten bekomen in dit hoofdstuk het idee ondersteunen dat lactoferrine de kolonisatie van *E. coli* O157:H7 *in vivo* kan reduceren.

Om meer inzicht te bekomen in het effect van lactoferrine op kolonisatie van schapen met *E. coli* O157:H7, werd een schapendarmexplant model ontwikkeld in ons laboratorium. In **hoofdstuk 5** werden aan de hand van dit model volgende zaken onderzocht: 1) het weefseltropisme van *E. coli* O157:H7 infectie bij schapen, 2) de cytokinerespons van schapen darmweefsel na infectie met *E. coli* O157:H7, en 3) het effect van lactoferrine op kolonisatie van de explanten en de daaropvolgende cytokinerespons. De data weergegeven in dit hoofdstuk tonen aan dat *E. coli* O157:H7 sterker bindt aan het ileaal follikel-geassocieerd epitheel van schapen dan aan het absorptief epitheel van het ileum of het colonepitheel. Er werd ook aangetoond dat bacteriële adhesie de expressie van IL-8 en TNF-α mRNA verhoogt en dat het niveau van deze expressie gecorreleerd is met het aantal bacteriën dat vasthecht aan de intestinale explanten. Voorts wijzen de resultaten in dit hoofdstuk erop dat lactoferrine de *E. coli* O157:H7 adhesie inhibeert en o.a. daardoor de IL-8 en TNF-α mRNA expressie in de intestinale explanten reduceert.

In **hoofdstuk 6** werd het potentieel van oraal toegediend lactoferrine om *E. coli* O157:H7 excretie bij schapen te reduceren, nagegaan. Oraal lactoferrine werd gebruikt in 2 verschillende dosissen, nl. 1,5 of 0,15 g toegediend om de 12h gedurende 30 dagen. Toediening van beide dosissen reduceerde het aantal *E. coli* O157:H7 bacteriën in schapenfaeces alsook de duur van de faecale uitscheiding. Bovendien vertoonden schapen die de hoge dosis lactoferrine kregen (1,5 g elke 12h) een significant hogere serumantistoffenrespons tegen EspA en EspB in vergelijking met de controlegroep. Aangezien de piek van de antistoffenrespons samenviel met het stoppen van de *E. coli* O157:H7 uitscheiding, is het waarschijnlijk dat een mucosale immuunrespons een rol speelt in de reductie van de bacteriële excretie bij schapen die oraal behandeld werden met lactoferrine. Dit levert nieuwe mogelijkheden om *E. coli* O157:H7 infectie en excretie

in het bacterieel reservoir te reduceren en daaruitvolgend ook infecties bij de mens te voorkomen.

In het laatste hoofdstuk van het experimenteel deel van deze thesis werd het potentieel van een systemische vaccinatie tegen *E. coli* O157:H7 bij schapen onderzocht. Het experiment dat beschreven wordt in **hoofdstuk 7** was opgezet om de hypothese na te gaan of een vaccin gericht tegen het oppervlakteantigeen intimine en de TTSS eiwitten EspA en EspB de kolonisatie van *E. coli* O157:H7 bij schapen kan voorkomen of reduceren, zoals reeds aangetoond werd bij runderen. De belangrijkste bevinding van dit gedeelte van de thesis was dat vaccinatie tegen *E. coli* O157:H7 TTSS eiwitten en intimine het niveau van bacteriële uitscheiding in schapenfaeces significant verminderde. Deze resultaten zijn consistent met eerdere studies bij runderen. Hoewel de vaccinatie leidde tot sterk verhoogde serum IgG titers tegen intimine, EspA en EspB, is er meer onderzoek vereist om het mechanisme achter de geobserveerde reductie van *E. coli* O157:H7 in de faeces te achterhalen. Deze resultaten suggereren dat vaccinatie van schapen een belangrijke methode kan worden in de controle van *E. coli* O157:H7.

Het vierde deel van de thesis omvat de algemene discussie en het toekomstig onderzoek. Alhoewel in deze thesis belangrijke stappen gezet zijn naar nieuwe strategiën voor de bestrijding van *E. coli* O157:H7 blijven er meerdere belangrijke vragen te beantwoorden zoals: 1) Heeft *E. coli* O157:H7 een tropisme voor de rectale mucosa bij schapen en kan lactoferrine deze lokatie voldoende bereiken om de bacterie te verwijderen?; 2) Wat is het mechanisme voor de verhoogde antistoffenrespons gezien in de groep dieren die de hoge dosis lactoferrine kreeg?; 3) Is deze immuunrespons niet alleen aanwezig in het serum, maar ook mucosal ter hoogte van de darm?; 4) Kunnen we een toedieningsvorm ontwikkelen die makkelijk te gebruiken is in de praktijk en nog efficiënter werkt?; 5) Welk is het mechanisme voor de reductie in excretie na parenterale vaccinatie?; 6) Kunnen we de vaccinatie nog verbeteren door meer antigenen toe te voegen of door andere combinaties van antigenen en/of andere adjuvantia te gebruiken?; 7) Hoelang blijft het effect van een vaccinatie aanwezig?

Verder onderzoek moet zich vooral naar deze vragen richten. Voor sommige van deze vragen werden ondertussen reeds experimenten opgezet om ze te kunnen beantwoorden.

Curriculum vita

Maryam Atef Yekta was born on December 10th 1978 in Rasht (Iran). After obtaining the Diploma in Science at Mostashari high school in Iran in 1997, she started her study at the Faculty of Veterinary Medicine, Shahrekord University, Iran where she graduated in 2004. In May 2005, she started her PhD research on *E. coli* O157:H7 colonisation of sheep at the Lab of Immunology, Faculty of Veterinary Medicine, Ghent University. She was granted a scholarship (BOF, Ghent University) to continue her research. From September 2009, she worked on a joint project (Ghent University- Intercell Company, USA) to study *the* effect of LT on intestinal mucosa of pig.

Maryam is the author of scientific papers in peer-reviewed journals. She has attended national and international meetings and presented frequently her results as oral and poster presentations.

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Thank you

Bedankt

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