# Alpha toxin and its C-terminal domain as vaccine candidates for *Clostridium perfringens*-associated bovine necro-haemorrhagic enteritis

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Voor mijn grootouders

# Abbreviations key

| | |
|---|---|
| ATP | adenosine-triphosphate |
| BB | Belgian Blue |
| BHI | brain heart infusion |
| BUVEC | bovine umbilical vein endothelial cells |
| C | carboxy-terminal |
| CBM | carbohydrate-binding modules |
| CDC | cholesterol-dependent cytolysin |
| Cpa | alpha toxin |
| CPE | *Clostridium perfringens* enterotoxin |
| CPB2 | Beta2 toxin |
| DAG | diacylglycerol |
| DNA | deoxyribonucleic acid |
| ELISA | enzyme-linked immunosorbent assay |
| GST | glutathione-S-transferase |
| GTP | guanosine-triphosphate |
| HA | hyaluronic acid |
| HBS | haemorrhagic bowel syndrome |
| HE | haematoxylin-eosine |
| HF | Holstein Friesian |
| HIS | histidine |
| ICAM-1 | intracellular adhesion molecule 1 |
| IL | interleukin |
| LPS | lipopolysaccharide |
| MALDI | matrix-assisted laser desorption/ionization |
| MHC | major histocompatibility complex |
| MMP | matrix metalloproteinase |
| N | ammino-terminal |

| | |
|---|---|
| NE | necrotic enteritis |
| NOD | nucleotide-binding oligomerization domain |
| NRU | neutral red uptake |
| OD | optical density |
| PAF | platelet-activating factor |
| PBS | phosphate buffered saline |
| PC | phosphatidylcholine |
| PCR | polymerase chain reaction |
| PFO | perfringolysin O |
| PKC | protein kinase C |
| $PLA_2$ | phospholipase $A_2$ |
| PLC | phospholipase C |
| RAPD | random amplified polymorphic DNA |
| ROS | reactive oxygen species |
| SDS-PAGE | sodium dodecyl sulphate polyacrylamide gel electrophoresis |
| SIDS | sudden infant death syndrome |
| SM | sphingomyelin |
| TGY | tryptone glucose yeast |
| TMB | 3,3',5,5'-Tetramethylbenzidine |
| TLR | toll-like receptor |
| TNF | tumor necrosis factor |
| TSA | tryptone soy agar |
| TSB | tryptone soy broth |

# PART I
# General introduction

# 1

# Bovine gastro-intestinal disorders associated with *C. perfringens* type A

## Bovine enterotoxaemia

Enterotoxaemia is a condition where toxins produced in the intestine are absorbed in the general circulation and cause toxic effects in other organs. According to this definition, an enterotoxaemia can be caused by multiple bacteria, including *Escherichia coli* and some pathogenic *Clostridium* species. However, the term is most frequently used to delineate the disease caused by *Clostridium perfringens*. Enterotoxaemia caused by *C. perfringens* can occur in different hosts, but the disease is most common in young, rapidly growing ruminants, although also foals and piglets are sometimes affected[96,138,181].

Bovine enterotoxaemia, also known as (necro-)haemorrhagic enteritis, is an acute or peracute syndrome classically reported as sudden death without previous symptoms. Sometimes preceding symptoms of abdominal pain or colic and neurological disorders are described[96,216]. Enterotoxaemia is less common in cattle than in small ruminants, often only affecting a single animal or a very limited number of animals in the same herd[96,110]. Although the morbidity is rather low, mortality is close to 100%, making it an economically important disease[96].

## Aetiology

Bovine enterotoxaemia is associated with an imbalance in the small intestinal microbiota composition, leading to uncontrolled *C. perfringens* multiplication and toxin production. This massive toxin production is believed to be responsible for both local and systemic effects, leading to rapid death. Based on the production of four major toxins (alpha, beta, epsilon and iota toxin) *C. perfringens* strains are classified into five toxinotypes (types A, B, C, D and E)[152].

Bovine enterotoxaemia has been linked to different *C. perfringens* toxinotypes[40,80,185]. However, the *C. perfringens* toxinotype most frequently associated with bovine enterotoxaemia, is type A[31,110,118,152]. *C. perfringens* is ubiquitous in the environment and is also a member of the normal gut microbiota of man and animals[181]. Therefore, it is generally accepted that predisposing factors or risk factors, contributing to an impaired homeostasis of the gut ecosystem, are required to cause disease. In the literature, stressful environmental conditions and sudden changes in the diet are the most commonly reported risk factors[151]. Stress may be caused by several factors, including regrouping or transporting the animals and medical interventions, and may have an effect on the intestinal microbiota by induction of paralytic ileus[96,235]. Also an increased consumption of a high-energy and protein diet is a plausible risk factor for enterotoxaemia. It is known in other animal species that high levels of dietary crude protein result in increased numbers of intestinal *C. perfringens*[37,106,107].

Bovine enterotoxaemia caused by *C. perfringens* type A affects mainly suckling and veal calves in good to excellent body condition up to eight months of age. In Wallonia, a mortality rate of 4.7% was described in 1999[108]. The majority of the affected animals (89%) were of the double muscled Belgian Blue beef cattle breed, suggesting a possible genetic influence for the susceptibility to enterotoxaemia. However, beef cattle breed calves in Wallonia are mainly raised as suckling calves, whereas dairy breed calves are separated from the cow as soon as possible. Hence, as the majority of the affected animals are suckling calves, dietary difference can also be responsible for the difference in disease susceptibility. Flanders is more specialized in veal production and less calves are raised as suckling calves than in Wallonia. Also in veal calves, predominantly beef cattle breeds are affected, accounting for 20% of total mortalities on average, compared to 4% in dairy and mixed breed veal calves between 2007 and 2009[96,151]. In addition to a possible breed influence, dietary differences between veal production systems are also here suspected to have a great effect as an eliciting factor[96,151,213]. Whereas dairy breed veal calves receive milk powders with very little animal protein, beef calves still receive a high amount of skimmed milk powder. An important risk period for bovine enterotoxaemia is situated at the end of the production cycle, where calves are fed high amounts of highly concentrated milk proteins[151]. Whereas dairy or traditional beef calves receive on average a maximum of 6 litres milk replacer per day at a concentration of 125 g/L, veal calves receive at the end of fattening as much of 16 litres daily, at a concentration ranging from 150 to 190 g/L[212]. The predisposition of these calves may be linked to their higher feed intake.

## Clinical symptoms and lesions

Bovine enterotoxaemia caused by *C. perfringens* type A is characterized by sudden death, mostly without premonitory symptoms. Post-mortem, a rapid putrefaction of the abdomen with gas production and a putrid smell is observed[96]. At necropsy, diffuse small intestinal haemorrhages with bloody intestinal content is observed[134] (Figure 1). These macroscopic lesions are mainly located in the small intestine, but sometimes also in the large intestine[110]. Microscopically, the intestinal lesions consist of haemorrhages and cell necrosis extending from the tip of the villi to the base of the crypts, and infiltration of neutrophils and lymphocytes[96,235] (Figure 2). In the intestinal lumen, clusters of *C. perfringens* bacteria can be found, localized in the necrotic areas. However, they are typically not found in the mucosa of the intestinal wall[110]. Lesions typical for toxaemia are not consistently present in internal organs[110,235].

Experimental reproduction in a calf intestinal loop model of necro-haemorrhagic lesions, comparable with the lesions seen in field cases, generated more insight into the sequence of histopathological events during lesion development[213]. The primary lesions in the pathogenesis of necro-haemorrhagic enteritis were epithelial cell detachment and congestion of the capillaries, followed shortly thereafter by necrosis of the intestinal villi and haemorrhages. When severe necrosis is present, a clear demarcation line can be observed, separating the necrotic tips of the villi from the underlying viable tissue.

Next to *C. perfringens* type A, bovine enterotoxaemia can also be associated with *C. perfringens* type B, C, D and E. Type C enterotoxaemia is characterized by sudden death in neonatal calves less than 10 days of age[178]. The intestinal lesions are similar to those described for type A enterotoxaemia with severe necrosis and haemorrhages in the small intestine and neutrophil infiltration[58,139]. *C. perfringens* type D enterotoxaemia is an important disease in sheep and goats[80,210]. In sheep, this disease is acute and fatal,

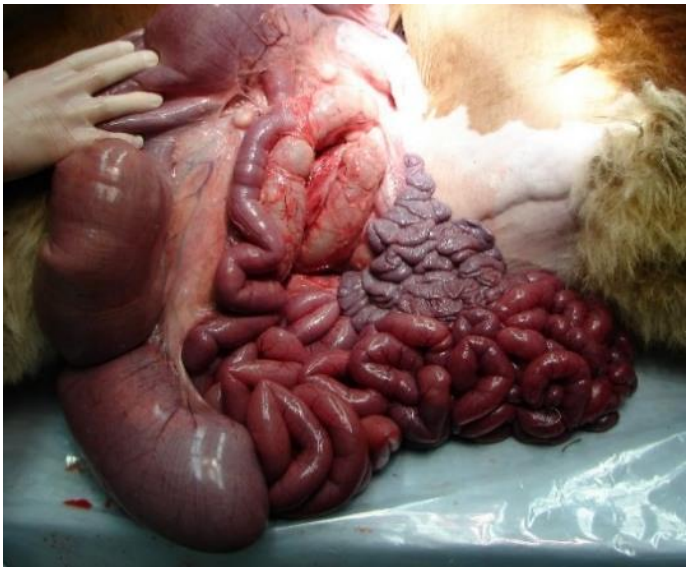

**Figure 1**

Diffuse haemorrhagic enteritis in the small intestine of a case of bovine enterotoxaemia.

characterized predominantly by neural signs with usually minor and inconsistent intestinal changes. In goats, the disease is most commonly an enterocolitis, though lung and brain oedema can occur as well. There are only a few reports about this condition in cattle, but it seems that the lesions are similar to those observed in sheep enterotoxaemia (e.g. focal symmetrical encephalomalacia in the brain)[80,120,129]. Despite the limited number of reports describing natural cases of bovine type D enterotoxaemia, the disease is extensively investigated[103,110,113]. Experimental reproduction of the disease in cattle has confirmed similar clinical and pathological characteristics as the disease in sheep[51]. The presence of neurological signs and absence of intestinal necrosis clearly indicate that type A and type D enterotoxaemia are two completely different diseases. Next to type A, C and D, bovine enterotoxaemia is sporadically associated with *C. perfringens* type B and E[40,58,156,185]. Only one report describing type B enterotoxaemia in cattle was found[40]. This report provides only limited information, but bloody diarrhoea, haemorrhagic enteritis and haemorrhages in all vital organs were described[40]. Type E enterotoxaemia is considered an infrequent cause of haemorrhagic enteritis and sudden death in neonatal calves[185], however, one report also describes type E enterotoxaemia in adult cows[156].

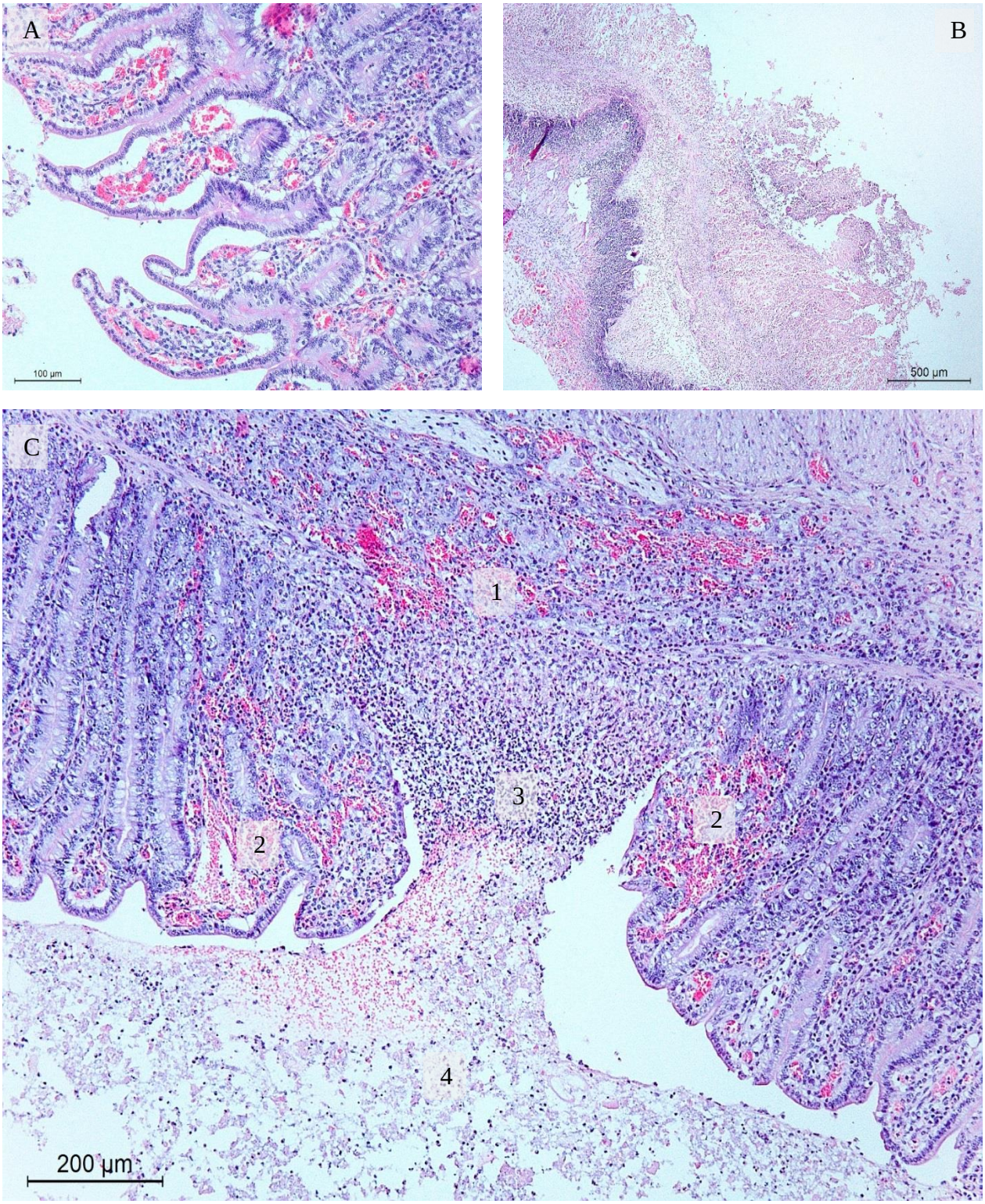


**Figure 2**

**Small intestine of a field case of bovine enterotoxaemia.**

The intestinal section showed multiple regions with necrosis. (A) The intestinal villi adjacent to the necrosis are congested with oedema of the lamina propria and epithelial cell detachment. (B) Segment of severe, diffuse necrosis involving the whole mucosa. (C) Focal, necro-haemorrhagic lesion with (1) submucosal and (2) mucosal haemorrhages, (3) infiltration of viable and dead neutrophils and (4) a pseudomembrane composed of fibrin, necrotic cells, cell debris and neutrophils in the intestinal lumen. (HE staining)

## Other gastro-intestinal *C. perfringens* type A-associated diseases

In this thesis, we focus on type A bovine enterotoxaemia, also described as (necro-)haemorrhagic enteritis. However, multiple other *C. perfringens* type A-associated bovine alimentary tract disorders are described in the literature. Some of these diseases show remarkable similarities in aetiology, clinical symptoms and histological findings and are briefly described below.

### Abomasitis in young calves

There is a thin line between clostridial abomasitis and type A bovine enterotoxaemia in young calves. Reports describing abomasitis are scant, but all describe sudden death, abomasal necrosis, haemorrhages and bloat[184]. The role of *C. perfringens* type A has been confirmed by experimental reproduction of the disease through intraruminal inoculation of *C. perfringens* type A cultures[160]. In naturally occurring cases, next to the abomasal lesions, duodenal oedema, necrosis and haemorrhages are often described[184,219]. As with bovine enterotoxaemia, dietary issues, such as overfeeding, or conditions which effect decreased gut motility probably contribute to the occurrence of the disease[184].

### Abomasal ulcerations

Abomasal ulcerations are regarded as an important economic concern in all types of calf-rearing systems[112]. Non-perforating ulcers with or without severe blood loss, as well as perforating ulcers with local and diffuse peritonitis can be observed[219]. Clinically, there are almost no signs or symptoms. Ulcers are a common finding at slaughter in veal calves, but also sudden deaths, due to perforating ulcers are reported (1.9% of total mortality)[112,151,220]. The underlying cause is unclear and it probably concerns a multifactorial disorder[214]. *C. perfringens* has often been suggested to be associated with bovine abomasal ulcerations, but no studies confirmed this relationship and other pathogens may be responsible for this disease as well[214,219]. On histology, abomasal ulcers may appear as demarcated mucosal areas of coagulative necrosis and haemorrhages, with moderate infiltration of neutrophils and macrophages[219]. As with abomasitis in young calves, also small intestinal necrosis and inflammation are reported[219]. In accordance with other gastro-intestinal *C. perfringens* type A-associated diseases, stress and dietary factors including overfeeding, are proposed as predisposing factors[112].

**Table 1**

**Description of the main characteristics of enterotoxaemia[96,110,130,134,213], haemorrhagic bowel syndrome[3,13,41,149,171], abomasitis[160,184,219] and abomasal ulcerations[112,214,219,220].**

| | Enterotoxaemia | Haemorrhagic bowel syndrome | Abomasitis | Abomasal ulcerations |
|---|---|---|---|---|
| **Alternative nomenclature** | Necrotic, Haemorrhagic or Necro-haemorrhagic enteritis | Jejunal haemorrhage syndrome,<br>Jejunal haematoma | | Clostridial abomasal disease |
| **Disease progress** | Sudden death | Rapid progress or sudden death | Sudden death | Mostly subclinical with almost no signs or symptoms, sometimes acute with sudden death |
| **Characteristics** | Lesions in the small intestine, mostly jejunum:<br>• Necrosis<br>• Haemorrhages<br>• Oedema<br>• Epithelial cell detachment<br>• Neutrophil influx | Lesions in the jejunum and sometimes duodenum:<br>• Necrosis<br>• Haemorrhages<br>• Oedema<br>• Epithelial cell detachment<br>• Neutrophil influx<br>• Intraluminal blood clots | Abomasal lesions:<br>• Necrosis<br>• Haemorrhages<br>Often with duodenal lesions:<br>• Necrosis<br>• Oedema<br>• Haemorrhages | Abomasal lesions:<br>• Necrosis<br>• Haemorrhages<br>• Neutrophil influx<br>Acute form often with small intestinal lesions:<br>• Necrosis<br>• Oedema<br>• Neutrophil influx |
| **Age** | 1-8 months of age | Adult cattle | Neonatal calves | Calves in the preruminant stage or in the transitional phase |
| **Aetiological agent** | *C. perfringens* type A | Strong association with *C. perfringens* type A | *C. perfringens* type A | Associated with *C. perfringens* type A |
| **Risk factors** | Increased consumption of high-energy diet,<br>Stressful conditions | Increased consumption of high-energy diet | Over-feeding,<br>Conditions which decrease gut motility | Over-feeding,<br>Stressful conditions,<br>Mechanical stress to abomasal mucosa |

## Haemorrhagic bowel syndrome

Haemorrhagic bowel syndrome (HBS) is a disorder affecting adult dairy cows. It is characterized by intraluminal and intramural small intestinal haemorrhages and necrosis with subsequent clot formation and intestinal obstruction[3,41]. Clinically, a sudden onset of colic or ileus can be observed, typically associated with blood in the faeces. This is the most typical presentation, but the animals can also be found dead without premonitory signs[149,171]. A case fatality rate exceeding 85% has been reported[37]. Although the cause of HBS is unknown, several investigators have revealed a strong association between HBS and the presence of

*C. perfringens* type A[41]. On histology, severe segmental submucosal haemorrhages and oedema of the small intestine is observed. This is often accompanied by a mixture of segmental necrosis, ulceration, or complete detachment of the epithelium with infiltration of neutrophils and the presence of Gram-positive rods[41]. Similar to other *Clostridium*-associated enteric diseases, increased consumption of a high-energy and protein diet seems to be the most plausible risk factor[13].

It should be noted that for abomasal ulcerations, the role of *C. perfringens* type A is questionable, whereas the involvement of *C. perfringens* type A in bovine enterotoxaemia, HBS and abomasitis is more clear. Up to now, it is not clear whether bovine enterotoxaemia, HBS and abomasitis are truly different diseases or whether they should be considered as clinical or pathological variants of the same disease. The same inconclusiveness is also reflected in the literature in the description of haemorrhagic bowel syndrome in calves[168] or enterotoxaemia in a three-year-old cow[83]. All three syndromes are associated with *C. perfringens* type A infection and are characterized by sudden death associated with haemorrhages, necrosis and predominantly neutrophil influx. The same risk factors predispose for all three syndromes. It is therefore not unlikely that a common pathological mechanism underlies these gastro-intestinal diseases (Table 1), and as a consequence, the results presented in this thesis may be of importance for all of these syndromes.

# 2

# *C. perfringens* strain specificity in relation to disease

Certain anaerobic bacteria are pathogenic to humans and animals. Their main virulence determinants consist of secreted toxins, which are often produced by species belonging to the *Clostridium* genus. The genus *Clostridium* consists of a diverse group of anaerobic, Gram-positive bacteria which have the ability to form heat-resistant endospores. More than 120 described species make up this genus, but few can induce disease. Pathogenic clostridia are non-invasive bacteria, but they secrete potent extracellular toxins that act at a distance from the bacteria and cause all the symptoms and lesions of the clostridial diseases. The clostridia produce more toxins than any other bacterial genus, and each type of toxin induces one or more specific syndromes[153].

*Clostridium perfringens* ranks amongst the most widespread bacteria, with an ubiquitous environmental distribution in soil, sewage, food, faeces, and the normal intestinal microbiota of humans and animals[181]. However, this Gram-positive, anaerobic spore former is also one of the most common pathogens, causing a spectrum of important human and animal diseases ranging from histotoxic to enteric infections[152,181]. Intestinal infections caused by *C. perfringens* are often generically called enterotoxaemias because toxins produced in the intestine may be absorbed into the general circulation. However, while this is true for many *C. perfringens* toxins, some toxins produced in the intestine act only locally, while others act both locally and systemically[186,208,211].

The virulence of *C. perfringens* is mediated in large part by its intimidating toxin arsenal. The organism lacks many enzymes for amino acid biosynthesis and therefore must obtain various essential materials from its host by producing several toxins and degradative enzymes[174]. As a species, *C. perfringens* produces at least 16 toxins and extracellular enzymes[72,89,152]. However, no single strain produces this entire toxin panoply, resulting in considerable variation in the

repertoire of toxins produced by different strains of this bacterium. These strain-to-strain differences in toxin production permits the classification of *C. perfringens* isolates into five toxinotypes, based on the presence of genes encoding for four so-called major toxins: alpha, beta, epsilon and iota toxin (Table 2). Besides expressing one or more of the typing toxins, *C. perfringens* strains can produce additional toxins including, but not limited to enterotoxin (CPE) or necrotic enteritis B-like toxin (NetB), which are also very important in certain diseases[89,167].

**Table 2**

**Classification of *Clostridium perfringens* based on the presence of the genes encoding for the four major toxins[152,206].**

| Toxin | *C. perfringens* toxinotype | | | | | Cellular target (mode of action) |
|---|---|---|---|---|---|---|
| | A | B | C | D | E | |
| **Alpha** | + | + | + | + | + | Membrane (phospholipid destruction) |
| **Beta** | | + | + | | | Membrane (pore formation) |
| **Epsilon** | | + | | + | | Membrane (pore formation) |
| **Iota** | | | | | + | Actin (cytoskeleton destruction) |

The different toxinotypes of *C. perfringens* are associated with particular human or animal diseases, indicating that variations in toxin production profoundly influence the virulence properties of *C. perfringens* isolates (Table 3)[152]. These isolate-to-isolate differences in toxin production also help explain the pathogenic versatility of *C. perfringens*. Strains of type B to E are mostly associated with disease and only sporadically isolated as a member of the normal intestinal microbiota. Type A strains are also associated with disease, but can equally well be commensal in the gastro-intestinal tract of humans and animals[72,181]. Rather than being the result of a single toxin, virulence of different *C. perfringens* isolates is considered as a multifactorial trait, with different determinants contributing to adaptation of the organism to its niche and to the induction of pathology[195]. Below a description is given of the function and role of the different known *C. perfringens* toxins in disease, starting with the plasmid-encoded toxins used for toxinotyping (beta, iota, epsilon) and other plasmid-encoded toxins, followed by the 2 toxins that are present in all *C. perfringens* strains, i.e. the alpha toxin and perfringolysin O, and some enzymes.

**Table 3**

**Main diseases associated with *C. perfringens* in humans and animals[99,152,211].**

| Type | Toxins produced | Human disease(s) | Animal disease(s) |
|---|---|---|---|
| **A** | Alpha toxin* | Gas gangrene (myonecrosis) | Gas gangrene (myonecrosis)<br>Yellow lamb disease in sheep<br>Enteritis in piglets<br>Enterotoxaemia (necro-haemorrhagic enteritis), abomasitis and haemorrhagic bowel disease in cattle |
| | Alpha toxin, enterotoxin | Food poisoning,<br>Non-foodborne gastrointestinal diseases | Enteritis in dogs, pigs, horses, foals, and goats |
| | Alpha toxin, NetB | Not reported | Necrotic enteritis in chickens |
| | Alpha toxin, beta2 toxin | Not reported | Possible enteritis in pigs<br>Possible enterocolitis in horses |
| **B** | Alpha toxin, beta toxin, epsilon toxin | Not reported | Necro-haemorrhagic enteritis of sheep (lamb dysentery) |
| **C** | Alpha toxin, beta toxin | Human necrotic enteritis (enteritis necroticans, pigbel, darmbrand) | Necrotic enteritis of neonatal individuals of several animal species (horse, cattle, pigs)<br>Enterotoxaemia in sheep (struck) |
| **D** | Alpha toxin, epsilon toxin* | Not reported | Enterotoxaemia in lambs, sheep, calves and goats |
| **E** | Alpha toxin, iota toxin | Not reported | Suspected, but not confirmed association with gastrointestinal disease of cattle, sheep and rabbits. |

* Critical toxin(s) for virulence. Perfringolysin O is not directly responsible for disease, but also contributes to the virulence during gas gangrene and bovine enterotoxaemia, and has a possible synergism with epsilon toxin in enterotoxaemia.

## Plasmid-encoded toxins produced by *C. perfringens* type A

It is commonly accepted that, when causing enteritis and enterotoxaemia, *C. perfringens* often relies upon specific toxins other than those shared with all *C. perfringens* isolates (alpha toxin, perfringolysin O, …). Those toxins involved in most intestinal infections are encoded by genes located on extrachromosomal elements or in variable regions of the chromosome. The array of toxins produced by a single strain can greatly vary and is characteristic for the diseases it can induce. The role of the major toxins from *C. perfringens* type B-E (beta, iota, epsilon toxin) in intestinal disease is well documented. Also certain subtypes of type A strains produce specific,

disease-associated toxins, namely *C. perfringens* enterotoxin (CPE), Beta2 toxin (CPB2), necrotic enteritis B-like toxin (NetB) and NetF, among others.

**Beta toxin** is a necrotizing, pore-forming toxin that is considered to be responsible for the fatal intestinal necrosis seen in type C infections in several animal species (e.g. piglets) and humans. Beta toxin is highly sensitive to the action of proteolytic enzymes such as trypsin. As a consequence of the trypsin inhibiting effect of colostrum to prevent proteolytic degradation of immunoglobulins during the first days of life, also beta toxin is protected. Therefore neonates are highly susceptible to *C. perfringens* type C infections. In addition type C disease also occurs occasionally in adult animals and humans ingesting significant amounts of food with trypsin inhibitors, such as sweet potatoes and soy bean, and also in patients with reduced pancreatic function[206,208]. Although type C enterotoxaemia in neonatal calves is commonly described in textbooks, only minimal evidence concerning the role of beta toxin (and type C strains) is available[58].

**Epsilon toxin** is a pore-forming toxin responsible for the neurologic signs caused by type D strains. It is produced as a relatively inactive protoxin that needs proteolytic activation to obtain its full functionality. Epsilon toxin reaches the blood circulation by absorption through the gut mucosa and causes widespread vascular damage and increased vascular permeability in the brain and intestine[80,113]. The toxin rapidly crosses the blood-brain barrier, binds to neuronal cells and causes mortality[52,191]. Type D enterotoxaemia is an important disease in sheep and goats. However, epsilon toxin differentially affects sheep and goats, as the former have more overt brain lesions, where the latter are more affected in the gut[191]. Less frequently, type D enterotoxaemia is also described in calves, with clinical signs similar to those in sheep[46,191].

**Iota toxin** is a binary toxin composed of a cell-binding component (Ib) and a complementary enzyme component (Ia). Ib binds to the receptor on the target cells and translocates Ia into the cytosol of the cells. Ia ADP-ribosylates actin, thereby blocking the polymerization of actin, eventually leading to cell rounding and death[165]. Type E strains are the only *C. perfringens* strains producing iota toxin. Type E is a putatively uncommon cause of enterotoxaemia in lambs, calves and rabbits[93]. However, Songer reported the isolation of type E strains from neonatal calves with haemorrhagic enteritis[185].

***C. perfringens* enterotoxin** can be produced by type A, C, D and E strains, but production has not been shown for any known type B strain[99,209]. This toxin is important for *C. perfringens* type A food poisoning in humans as well as many cases of non-food-borne human

gastrointestinal diseases, such as antibiotic-associated diarrhoea[159]. Enterotoxigenic type A strains are also implicated in diarrheal conditions in pigs, dogs and goats[118,182]. Unlike the other toxins, enterotoxin is produced during sporulation[206]. The toxin interacts with epithelial tight junction proteins and induces leakage of water and ions through pores or channels in the plasma membrane of host cells[115,177]. Most, but not all, *C. perfringens* type A food poisoning strains carry their enterotoxin gene (*cpe*) on the chromosome. In contrast, the *cpe* gene of type A non-food-borne human disease strains and strains isolated from veterinary cases is plasmid-borne[27,29].

**Beta2 toxin** is a pore-forming toxin that is associated with enteritis in neonatal pigs[92,229] and gentamicin-associated diarrhoea in horses[12,74,225]. The *cpb2* gene coding for this toxin can be present in a consensus or atypical allele and is found in both type A and type C strains of *C. perfringens*[60]. Beta2 toxin positive *C. perfringens* strains are widespread and can be isolated from various wild and domestic animals and humans, but also from food, soil and sludge[78,81,155,217]. A possible role of beta2 toxin in bovine enterotoxaemia was suggested in 2002 when Manteca *et al.* induced necrotic lesions with a beta2 producing type A strain in a ligated loop experiment[109]. However, this was only tested in one intestinal loop in one calf, and the strain also produced large amounts of alpha toxin. In addition, no isogenic strain deficient in beta2 toxin was used as a control. Therefore, effects of other factors cannot be excluded. More recently, Morris *et al.* (2011) and Valgaeren *et al.* (2013) were able to induce necrotic lesions in an intestinal loop model by inoculation of type A strains not producing the beta2 toxin[130,213]. Taking these results together with the observation that there is also no correlation between the isolation of *cpb2* positive strains and the occurrence of enterotoxaemia[217], the role of beta2 toxin in bovine enterotoxaemia seems rather limited. The actual role in other enteric diseases is also not clear and recent papers suggest a limited role of beta2 toxin in disease[4] and that beta2 toxin positive *C. perfringens* type A strains merely reflect the normal intestinal microbiota[95].

**NetB** is a pore-forming toxin essential for the development of necrotic enteritis in broiler chickens[89]. NetB is expressed by virulent type A strains and causes necrosis of the small intestine in the chicken. The toxin interacts with cholesterol and pore formation is enhanced by its presence in bilayers[169]. The action of NetB seems to be species-specific and not all cell lines are equally sensitive for the toxin action of NetB, suggesting the involvement of a still unidentified protein receptor[88,169].

**NetF** is a novel pore-forming toxin in *C. perfringens* type A strains, associated with two distinct severe enteric diseases, canine haemorrhagic gastroenteritis and foal necrotizing enteritis[119]. The toxin is highly cytotoxic for an equine cell line, while cell lines from other animal hosts are less susceptible, suggesting a specific receptor for the toxin[119]. Because of the novelty of this toxin, it is only described in one paper and the importance of NetF in the pathogenesis still needs to be clarified. In a previous paper from the same research group, *C. perfringens* type A strains isolated from foals and adult horses with acute enterocolitis were not cytotoxic for the equine cell line, strongly suggesting the absence of NetF in those isolates[63].

## The controversial role of *C. perfringens* type A strains in disease

Although the role of other toxinotypes of *C. perfringens* in diseases originating in the intestine is well documented, the involvement of *C. perfringens* type A strains is rather controversial and often questioned. For many years, the involvement of *C. perfringens* type A strains in intestinal disorders was commonly accepted[99,152,199,211]. More recently, this idea has shifted towards underrating the role of type A strains in enteric disease, leading to the postulation that type A strains are only important for histotoxic infections (gas gangrene) and that enteric diseases are generally caused by type B-E or type A subtypes, producing specific toxins such as enterotoxin and NetB[206,209]. The idea prevails that common virulence factors inherent to all *C. perfringens* strains (and as a result thereof, present in the healthy intestinal tract) are not sufficient for induction of intestinal diseases. The controversy around type A strains in intestinal disease should not come as a surprise. As type A strains can be present in the normal microbiota, isolation of this toxinotype is not indicative of disease. Also detection of its major toxin, alpha toxin, has little diagnostic value, as it can be present in the faeces of healthy animals[36]. Therefore, diagnosis of enteric type A disease is not straightforward and experimental reproduction of disease is needed to confirm the role of type A in disease. By making the statement that type A strains are not involved in gastro-intestinal disorders, numerous reports describing type A-associated enteric diseases in both humans and animals[63,110,175,179,183,210], as well as experimental data underlining the involvement of type A strains are ignored[31,47,129,130,213]. Because of this reduced focus on type A strains in gastro-intestinal diseases, the importance of the common *C. perfringens* toxins (alpha toxin, perfringolysin O) and virulence factors (e.g. enzymes) is poorly documented.

For bovine type A enterotoxaemia, as for the majority of enteric diseases thought to be evoked by type A strains, no unique, disease-specific toxin is known. The disease cannot be reproduced experimentally after oral or intraduodenal inoculation of *C. perfringens* type A strains[140]. However, necro-haemorrhagic lesions, comparable to those seen in natural cases, can be induced in calf intestinal loop models by *C. perfringens* strains from healthy and enterotoxaemic cattle as well as from other host species, confirming the importance of type A strains in the pathogenesis[109,130,213]. Because of the observation that strains from both bovine and non-bovine origin can induce necro-haemorrhagic lesions, it is likely that, in contrast to most *C. perfringens*-associated enteric diseases in other animals, no disease-specific toxin is involved in the pathogenesis of bovine enterotoxaemia, and that the causative toxin(s) is (are) produced by all *C. perfringens* type A strains. A possible role of alpha toxin in the pathogenesis of bovine enterotoxaemia was demonstrated in a calf intestinal loop model. Exposure of the small intestine to alpha toxin resulted in epithelial cell detachment, villus tip blunting, erosion, mild inflammation and haemorrhages of the lamina propria. However, no necrosis could be observed, suggesting that alpha toxin is not the only common virulence factor involved[129]. Recently, the involvement of alpha toxin in the induction of necro-haemorrhagic lesions has been shown by inoculation of mutant strains in a calf intestinal loop model[222]. Although the ability to induce necro-haemorrhagic intestinal lesions was significantly reduced in alpha toxin-deficient strains, these strains were not unable to induce lesions. Therefore, it cannot be ruled out that other commonly produced *C. perfringens* virulence factors may be involved in the pathogenesis of bovine enterotoxaemia. A review of these potential virulence factors is given in the following sections, followed by a hypothesis on the key events in *C. perfringens* type A-induced intestinal necrosis (Figure 7).

## Alpha toxin

### *Molecular architecture of alpha toxin*

Alpha toxin is a zinc-dependent phospholipase C (PLC) enzyme produced by all *C. perfringens* strains, although type A strains usually produce higher amounts than other toxinotypes[138]. The toxin is secreted by means of a signal peptide (28 first amino acids), resulting in a mature protein of 370 amino acids (43 kDa). Alpha toxin is composed of two domains, an α-helical N-terminal domain (residues 1-246), and a β-sandwich C-terminal domain (residues 256-370), which are joined by a short flexible linker region (residues 247-255)[198]. The N-terminal domain harbours

the active site, which is located within a cleft and contains two to three zinc ions, essential for the catalytic activity. Two flexible loops (residues 55-93 and 132-149) are located on either side of the active cleft and undergo a change of conformation between the open (active) and closed (inactive) form of the toxin (Figure 3)[39]. In the open conformation, the active site is accessible for substrate binding, whereas in the closed form, the two loops obstruct the active site, rendering the toxin inactive. The first loop domain (residues 55-93) has a ganglioside-binding site and may play an important role in tethering of alpha toxin to the membrane[147]. The C-terminal domain of alpha toxin is essential for membrane-binding and is structurally similar to eukaryotic phospholipid-binding C2 domains[199]. The C-terminal domain directs alpha toxin to, and interacts with, the membrane, thereby correctly positioning the catalytic domain for its activity[66]. Calcium-ions are essential for the phospholipid binding by conferring a positive charge on the polar head groups of membrane phospholipids, thereby favouring interactions with the negatively charged toxin domain[116,127] and allowing insertion of portions of the C-terminal domain into the bilayer[56]. Membrane binding probably induces a conformational change in the N-terminal domain from the closed to open configuration, thereby uncovering the active site[39,153].

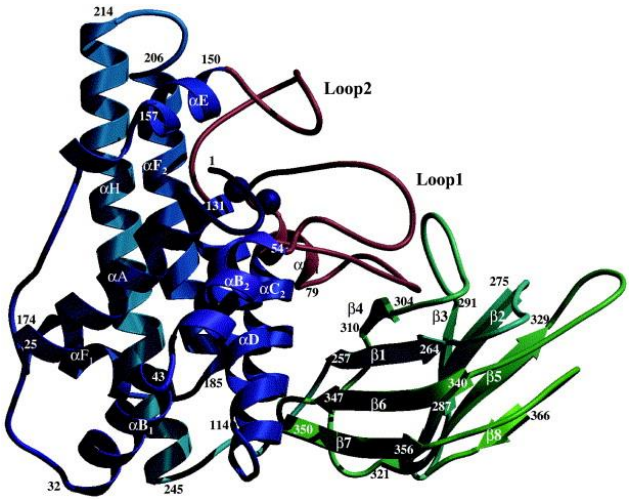


**Figure 3**

**Ribbon diagram of the closed structure of alpha toxin.**

In blue is the N-terminal domain. In green is the C-terminal domain. A short linker region exists between the two domains. In brick red are the two loop regions, which differ between the closed and open structures. The active site zinc ions are shown in dark blue[198].

## *Biological activity and mode of action*

Alpha toxin causes membrane damage to a variety of erythrocytes and cultured mammalian cells and is known to be haemolytic, cytotoxic, myotoxic and lethal. Alpha toxin is preferentially active towards phosphatidylcholine (PC or lecithin) and sphingomyelin (SM), two major components of the outer leaflet of eukaryotic cell membranes. The N-terminal

domain possesses full activity towards phosphatidylcholine but lacks the sphingomyelinase activity and is not haemolytic or cytotoxic[198]. The C-terminal domain is devoid of enzymatic activity, but interaction between the N- and C-terminal domains is essential to confer sphingomyelinase activity, haemolytic activity and cytotoxicity to the toxin[56]. At high concentrations, alpha toxin induces massive degradation of PC and SM, followed by membrane disruption and cell lysis[164]. Although alpha toxin is a potent haemolysin, the lysis of erythrocytes is only seen after intravenous administration of toxin in experimental animals or in cases of clostridial septicaemia[198]. Therefore, it is believed that alpha toxin preferentially acts in a more subtle way, perturbing the metabolism of the host cells to the advantage of the pathogen and the disadvantage of the host[198].

The sensitivity to membrane damage of different cells varies markedly depending on the cellular capacity to up-regulate PC synthesis as well as the proportion of phospholipids, cholesterol and gangliosides in the cell membrane[56,203]. Membrane damage by alpha toxin reflects the balance between the phospholipid degradation caused by the toxin and the synthesis of the phospholipids by the cell. In order to perform its action, alpha toxin must first bind to the plasma membrane of the target cell and acquire its active conformation[56]. The composition of the outer leaflet of the plasma membrane can influence both the binding of alpha toxin and its proper positioning towards the substrates[199]. The presence of cholesterol increases alpha toxin binding and its activity in liposomes[135,203,204]. The stimulatory effects of cholesterol can be largely explained in terms of its negative intrinsic curvature, which facilitates insertion of alpha toxin in the membrane[204]. Furthermore, gangliosides, particularly those rich in sialic acid, exert a protective effect on cultured cells against alpha toxin, whereas other cell surface glycoconjugates do not[55,56]. The exact mechanism behind this protective effect is not yet fully elucidated, but might be explained by the bulky head groups of gangliosides which protrude from the interface and create a polar and hydrated microenvironment. This alters the availability of the phospholipid substrates in ganglioside-containing membranes due to either steric effects or to the electrostatic changes induced at the interface[55]. Because the upregulation of PC synthesis to compensate for phospholipid degradation imposes an increased consumption of ATP, the cellular susceptibility to membrane disruption by alpha toxin also depends on the energy metabolism of the cell. Metabolic changes in cells of ischaemic tissues sensitize them to the cytotoxic effect of alpha toxin[56].

As mentioned before, cells exposed to alpha toxin do not always show membrane disruption and more subtle effects may be of greater importance in disease. At sublytic concentrations,

alpha toxin causes limited hydrolysis of PC and SM, generating the second messengers diacylglycerol (DAG) and ceramide respectively. This triggers various intracellular signal transduction pathways leading to uncontrolled production of several intracellular mediators[198]. The intracellular pathways induced by alpha toxin are still subject of debate and may depend on cell types. Ceramide production can activate protein phosphatases as well as various protein kinases involved in signal transduction pathways associated with cell death and cell cycle arrest[56]. DAG production can activate the arachidonic acid metabolism, resulting in the formation of thromboxanes, leukotrienes and prostaglandins, which contribute to local inflammation and vasoconstriction[56]. DAG may be formed as result of the enzymatic action of alpha toxin itself, or by activation of endogenous phospholipases through binding of alpha toxin with a GTP-binding protein or a TrkA receptor on the plasma membrane[146,147,194]. DAG triggers the activation of protein kinase C (PKC), which in turn can activate various signalling cascades[87,126,164]. It has been demonstrated that alpha toxin can become internalized in cells and that endocytosis is required for cytotoxicity in multiple cell lines *in vitro*[125]. In endothelial cells, alpha toxin induces the production of two vasoactive lipids, platelet-activating factor (PAF) and prostacyclin, and the neutrophil chemoattractant, interleukin-8 (IL-8)[19,20]. The synthesis of PAF likely contributes to increased vascular permeability and neutrophil adhesion to endothelial cells[20]. Furthermore, alpha toxin also induces the upregulation of adhesion molecules, both in endothelial cells and neutrophils, thus altering the processes of neutrophil adherence and extravasation[19,20]. In neutrophils, alpha toxin also induces the generation of superoxide ($O_2^-$), which can cause oxidative stress through the production of further reactive oxygen species (ROS) and may contribute to its cytotoxic and myotoxic effects[124,144]. Additionally, alpha toxin induces the aggregation of platelets and the formation of aggregates between platelets and neutrophils, which cause vascular occlusion[18]. Indirectly, alpha toxin contributes to shock by stimulating the production of endogenous mediators such as TNF-α and platelet-activating factor (PAF)[19,145,187]. Furthermore, the lethal effect of alpha toxin is closely related to the release of TNF-α from mononuclear cells into the bloodstream[145].

### *The role of alpha toxin in the pathogenesis of disease*

Up till now, little is known about the role of alpha toxin in *C. perfringens* type A-associated enteric diseases such as bovine enterotoxaemia. Most of the research about alpha toxin is focused on gas gangrene, giving a detailed view on the role of alpha toxin in histotoxic

infections, while many questions regarding the role of alpha toxin in enteric disorders remain unanswered.

Alpha toxin is the major virulence factor in gas gangrene, an acute and devastating infection, most frequently caused by *C. perfringens* that affects mammalian and avian species worldwide[176]. This histotoxic disease is characterized by fever, sudden onset of prominent pain, massive local oedema, severe myonecrosis, and the accumulation of gas at the site of infection. Histopathologically, the infected muscle tissue is characterized by the marked absence of inflammatory cells infiltrating the myonecrotic tissue, together with their accumulation at the borders of the necrotic area, and significant thrombosis[54,187,198]. The role of alpha toxin in gas gangrene was proven using different approaches. Firstly, when injected intramuscularly in mice, recombinant alpha toxin causes myonecrosis and reproduces many of the histopathological features of gas gangrene[20]. Furthermore, an alpha toxin mutant strain was attenuated in a mouse model for gas gangrene, whereas immunization with a recombinant alpha toxoid protected mice from experimental gas gangrene[9,198,232].

One of the hallmarks of gas gangrene is the absence of neutrophils in the infected tissue. This is largely due to the alpha toxin-induced upregulation of adhesion molecules and IL-8 expression in endothelial cells and leukocytes as described above, which inhibit the influx of neutrophils to the infected tissue and promote thrombotic events, hereby enhancing the conditions for anaerobic growth[9,10,190]. Another remarkable characteristic of gas gangrene is the severe myonecrosis. Experimental injection of alpha toxin in guinea pigs has shown that alpha toxin is responsible for the extensive damage to muscle fibres during gas gangrene[193]. This high susceptibility to alpha toxin might be related to the low concentration of complex gangliosides on the muscle cell plasma membrane[55,56]. In addition, the susceptibility of muscle cells under ischemia could increase even further by the fact that ganglioside synthesis is down regulated in cells exposed to low oxygen tension *in vitro*[56].

Alpha toxin might play a role in enteric diseases in both humans and animals. However, for virtually all these diseases no conclusive evidence for the role of alpha toxin is provided[198]. In the ‘90s, alpha toxin has been associated with sudden infant death syndrome (SIDS), Crohn’s disease and rheumatoid arthritis in humans[132,198]. More recently, several cases of adult necrotizing enterocolitis have been associated with *C. perfringens* type A strains, suggesting a possible role of alpha toxin in disease[133,179]. In animals alpha toxin has frequently been associated with type A enteritis in piglets[183,186,198], enterotoxaemia in calves[96,130] and

haemorrhagic bowel syndrome in adult cattle[41,149]. Although alpha toxin is not essential for the induction of necrotic enteritis in chickens, a possible role in disease cannot be ignored[91,157,158]. Histopathologically all these intestinal disorders are characterized by damage to the tips of the villi or epithelial cell detachment, congestion of the capillaries, mucosal oedema and necrosis. In most cases, also haemorrhages and mucosal inflammation with concomitant influx of inflammatory cells is reported[41,96,149,180,196]. For some of these pathological findings, there is indirect evidence that alpha toxin is responsible. In small intestine explants of rabbits incubated with alpha toxin, this toxin causes **detachment of the epithelial cells** at the tip of the villi[84]. Epithelial sloughing was also observed when alpha toxin was inoculated in bovine intestinal loops[129]. Alpha toxin is able to upregulate the matrix metalloproteinase (MMP) expression of the host as seen *in vitro*. This increased host MMP activity may be related to derangement of normal epithelial growth and increased degradation of subepithelial matrix, possibly explaining the observed epithelial detachment[53]. Additionally it has been shown that intraperitoneal or systemic administration of TNF-α to mice or intraduodenal TNF-α injection in rats induces pathological intestinal cell shedding and that dysregulated TNF-α production is highly important in driving epithelial damage as shown in mice[67,231]. Alpha toxin induces the production of TNF-α by mononuclear cells, indicating a possible role of this pathway in epithelial sloughing. However, the effect of alpha toxin on TNF-α production by epithelial cells still needs to be investigated. Another characteristic of *C. perfringens* type A-associated intestinal disorders that can be a result of the alpha toxin activity is the **influx of inflammatory cells**. Neutrophilic inflammation of the small intestine has been observed after intragastric administration of alpha toxin to neonatal piglets and when alpha toxin was injected in small intestinal loops of rats, sheep and calves[47,129,148]. This trafficking of inflammatory cells to the infected tissues seems contradictory to the image in gas gangrene, where the leukocytes are trapped within the blood vessels. However, this difference may be related to the concentration of alpha toxin in the tissue. In gas gangrene, alpha toxin is produced in the tissue, leading to high toxin concentrations at the site of infection. This is in contrast to intestinal infections, where alpha toxin is produced by *C. perfringens* in the intestinal lumen and enters the mucosa through a currently unknown mechanism. Little is known about the permeability of the intestine to alpha toxin, but it is likely that lower concentrations will be present in the intestinal mucosa than in the infected muscle tissue during gas gangrene[181]. As described above, alpha toxin causes upregulation of adhesion molecules and IL-8 expression in endothelial cells and leukocytes. When present in abnormal high concentrations as observed in mouse models for

gas gangrene, these intercellular mediators alter the processes of leukocyte adherence and extravasation, resulting in impaired movement of inflammatory cells to the infected tissue[187]. However, physiological levels of upregulation lead to trafficking of neutrophils into the tissue space, as observed in intestinal *C. perfringens* type A infections. Furthermore, Otamiri *et al.* have shown that this neutrophil influx was caused by alpha toxin-induced activation of endogenous phospholipase $A_2$ in the rat intestinal mucosa[148]. Activation of endogenous phospholipase $A_2$ can result in the generation of highly pro-inflammatory lysophosphatidic acid which can damage the mucosa. The mucosal damage can be aggravated by granulocyte-derived oxidants, proteolytic enzymes and cytotoxic proteins originating from the infiltrated neutrophils, and may be associated with **increased mucosal permeability**. Indeed, an alpha toxin-triggered increase in intestinal permeability was shown in rats, rabbits, sheep and chickens[47,84,148,158]. Additionally, alpha toxin induces the production of PAF and TNF-α by endothelial and mononuclear cells respectively, which likely contributes to the **increased vascular permeability** and **oedema** during *C. perfringens* infections[189,190]. This increased vascular permeability may explain the **haemorrhages** observed after *C. perfringens* type A infections in some species. Indeed, haemorrhages of the lamina propria were reproduced after alpha toxin inoculation in ligated loops of the bovine small intestine[129]. Another effect of alpha toxin observed in experimental animals is the contraction of isolated ileum of rats[163]. The significance of this finding in natural disease however, is not clear. It should be noted that, although alpha toxin is described as a necrotizing toxin, no intestinal **necrosis** could be observed in any of the experimental models mentioned before. However, a mutant strain devoid of alpha toxin caused less necrosis than its isogenic wild type strain when injected in bovine intestinal loops[222]. Together, these findings suggest that alpha toxin is involved, but most likely a synergism with other factors is needed to cause intestinal necrosis.

## Perfringolysin O

### *Molecular architecture and mode of action of perfringolysin O*

Perfringolysin O (also called theta toxin) is a cholesterol-dependent cytolysin (CDC) that has the ability to lyse red blood cells. It is produced by nearly all *C. perfringens* strains, except the enterotoxin-producing food poisoning strains[34]. The toxin binds to membrane cholesterol, and then forms oligomeric pores causing membrane damage[226]. Perfringolysin O is secreted by means of a signal peptide (27 amino acids), resulting in a mature peptide of 472 amino acids

long (53 kDa)[202]. Structurally, perfringolysin O is a rod-shaped molecule comprised of three discontinuous domains (domains 1-3) and a compact C-terminal β-sandwich (domain 4) (Figure 4)[162]. The C-terminal domain is essential for cholesterol recognition and binding with the plasma membrane, resulting in a conformational change in domain 3[173]. This conformational change allows perfringolysin O to oligomerize and to form a prepore complex, after which the domain 3 loops can be inserted into the lipid bilayer, leading to a large, transmembrane pore and subsequent lysis of the cell[30,221]. It has been suggested that the ability of perfringolysin O to perforate the membrane of target cells is dictated by how much free cholesterol molecules are present[122].

### *The role of perfringolysin O in the pathogenesis of disease*

Perfringolysin O is not considered essential for disease, but seems to have an important accessory role in some disorders. As for alpha toxin, the involvement of perfringolysin O is best characterized for gas gangrene. Studies using a perfringolysin O-deficient strain have shown that perfringolysin O affects the host inflammatory response and is, at least partially, involved in tissue destruction in a mouse model for gas gangrene. However, these effects were less pronounced than those elicited by alpha toxin and a synergistic effect between both toxins has been shown[9,10,190]. The most likely mechanism underlying the concerted action of alpha toxin and perfringolysin O is that the phospholipid hydrolysis caused by alpha toxin increases the availability of cholesterol molecules in the membrane, thereby facilitating perfringolysin O binding and cytolysis on membranes that contain low cholesterol levels (Figure 5)[122]. *In vitro* perfringolysin O enhances the expression of pro-adhesive molecules on leukocytes, as well as PAF and ICAM-1 (intracellular adhesion molecule 1) on endothelial cells[17,19,230]. This may lead to

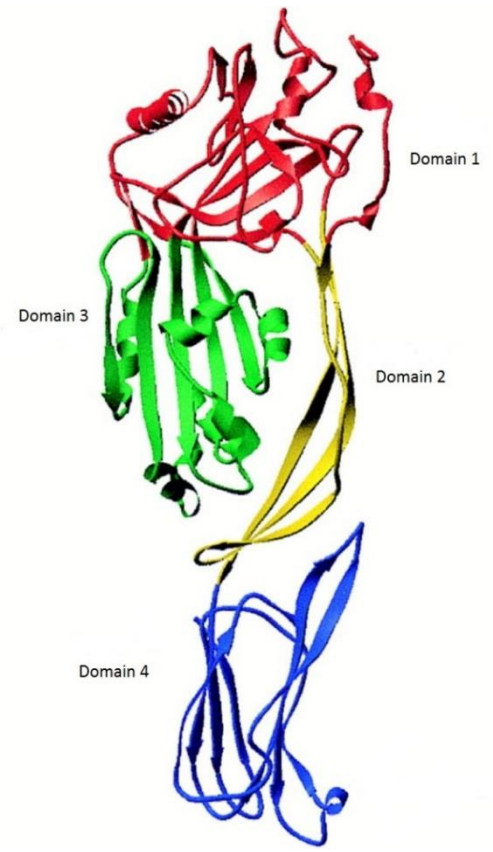


**Figure 4**

Ribbon diagram of the structure of perfringolysin O, with each domain designated by a different colour[202].

hyperadhesion of leukocytes to endothelial surfaces, explaining the leukocyte accumulation within the vasculature during gas gangrene[190].

The role of perfringolysin O in intestinal diseases caused by *C. perfringens* is still subject of debate. There are some indications that perfringolysin O can act in synergy with both alpha toxin, as seen *in vitro*, and epsilon toxin, as shown in a mouse model[45,219].

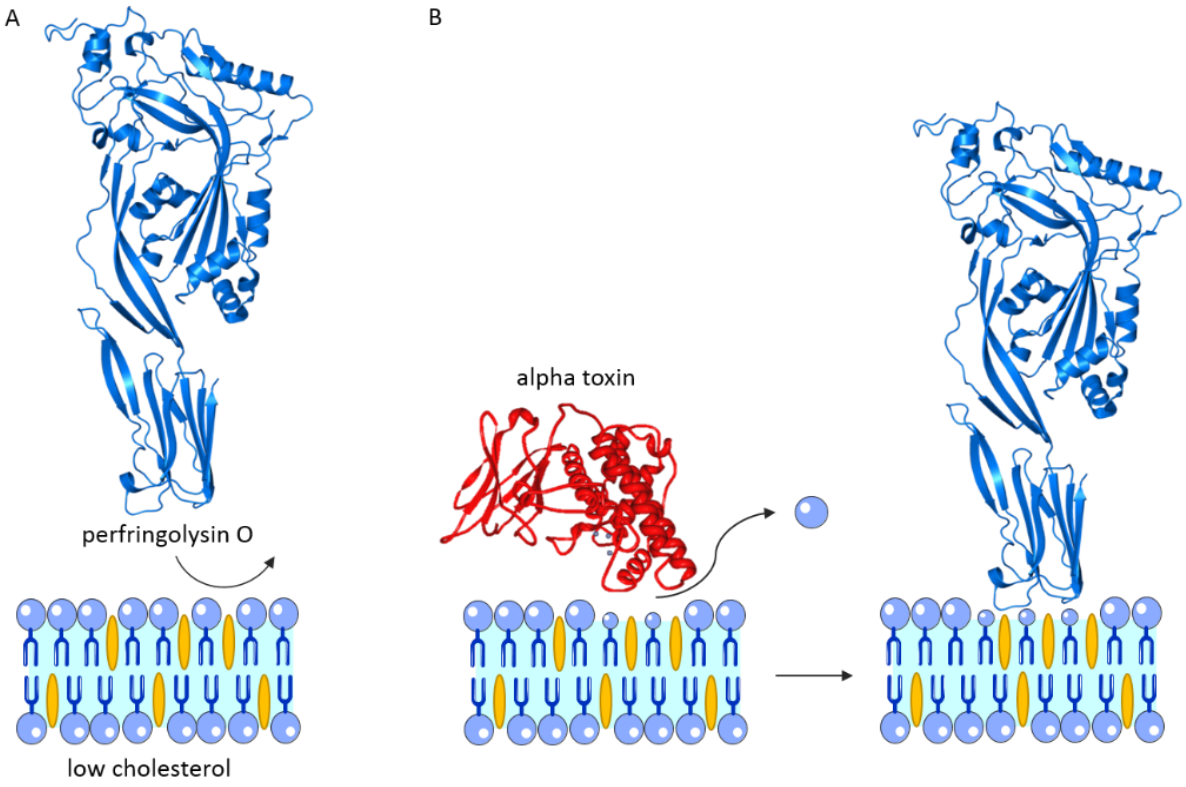


**Figure 5**

**A schematic model for the *C. perfringens* alpha toxin effect on perfringolysin O binding.**

(A) Perfringolysin O does not bind to membranes containing low cholesterol content, presumably because no free cholesterol molecules are present in the membrane. (B) *C. perfringens* alpha toxin hydrolyzes phosphatidylcholine releasing the phosphocholine headgroup and generating diacylglycerol (DAG). The appearance of free cholesterol molecules triggers domain 4-mediated perfringolysin O binding. The phosphocholine headgroup is represented as a large blue circle. The glycerol moiety of DAG is shown as a small blue circle. Cholesterol is shown as an orange oval. (Adapted from Moe *et al.*, 2010[122])

However, more research is needed to elucidate the role of perfringolysin O in *in vivo* situations[45,222]. Verherstraeten *et al.* recently demonstrated a synergistic cytotoxic effect between alpha toxin and perfringolysin O to bovine endothelial cells. However, a perfringolysin O-deficient strain was still able to cause necrosis in calf ligated intestinal loops and in this

model no synergy between alpha toxin and perfringolysin O could be observed[222]. In accordance with these observations, a perfringolysin O-deficient type C strain retained full virulence in a rabbit intestinal loop model, suggesting no synergistic effect of perfringolysin O and beta toxin for the induction of type C enteritis[170]. We should note that these findings do not exclude a possible accessory role of perfringolysin O in intestinal disease. Based on the knowledge obtained from gas gangrene, the effect of perfringolysin O is expected to be more subtle and further research should be focused on the host inflammatory response and more specific the neutrophil and macrophage influx into the lesions.

In contrast to the contribution of perfringolysin O in intestinal lesions, the potential of perfringolysin O to enhance the lethal effects of epsilon toxin during enterotoxaemia was demonstrated in a mouse model for type D enterotoxaemia. However, in this study purified toxins were used and it is unknown whether the same amount of toxin, in the same ratio, will be present during natural disease. In order to confirm the potential contribution of perfringolysin O in type D enterotoxaemia, experiments using knockout mutants are needed[45].

In conclusion, although less pronounced than the effect seen in gas gangrene, perfringolysin O may also exert an accessory role in *C. perfringens*-associated enteritis and enterotoxaemia in some species.

## Proteolytic and carbohydrate-active enzymes

### *Mucin-degrading enzymes (mucinases)*

Mucus is a complex fluid that is rich in mucin glycoproteins and a diverse range of antimicrobial molecules. It is the major barrier separating the epithelial cells and underlying host tissues from the commensal microbiota and is the first line of host defence against invading pathogens by forming a biophysical barrier to infection[117,123]. Mucin consists of a peptide backbone with O-linked glycosylated regions comprising 70-80% of the polymer. *N*-acetylglucosamine, *N*-acetylgalactosamine, fucose and galactose are the 4 primary mucin monosaccharides. Mucin oligosaccharide chains are often terminated with sialic acid or sulphate groups[35]. The large, complex structure of mucins makes them targets for many proteases, glycosidases and sulphatases. Enzymatic digestion of the mucus layer provides access to readily available sources of carbon and enables bacteria to reach the epithelial surface[35]. For *C. perfringens*, many glycosidases are described which have varying catalytic specificities that reflect the breakdown of a diversity in host glycans. These enzymes cleave sugars from mucin

oligosaccharides and may act in conjunction with each other to promote complete degradation of the glycoproteins. Among the glycosidases employed by *C. perfringens* to cope with the mucosal surface are sialidases (neuraminidases)[15,137], α-L-fucosidases[44], exo- and endo-β-*N*-acetylglucosaminidases[1,49] and exo- and endo-α-*N*-acetylgalactosaminidases[8,50,57]. For most of these enzymes, more research is needed to elucidate their function in the pathogenesis. However, it should be noted that most clostridial glycosidases are extracellular soluble enzymes, which may penetrate the mucin layer and reach the surface of the epithelial cells. Thus, these enzymes could damage intestinal mucus. This is in contrast to bifidobacterial glycosidases (from probiotic bacteria), which are membrane or cell-wall anchored and are not liberated from bacterial cells[8].

### *Sialidases (neuraminidases)*

Many of the studies concerning enzymes that are capable of degrading mucins were carried out specifically on sialidases. These enzymes cleave terminal sialic acids from sugar chains of glycoproteins, glycolipids, oligosaccharides, gangliosides and other sialoglycoconjugates. Sialic acids are especially abundant in the intestinal tract, where they are a major constituent of mucins. *C. perfringens* produces enzymes that release sialic acid from sialoglycoconjugates, transport it into the cell, and degrade it as a source of nutrients (Figure 6)[227]. Furthermore, the release of sialic acid is an initial step in the sequential degradation of mucins, since the terminal location of sialic acid residues in the mucin oligosaccharide chains may prevent the action of other glycosidases[82]. In *C. perfringens* three sialidase enzymes have been reported, the large exo-sialidases NanI and NanJ, and a smaller NanH enzyme. Genome sequencing showed that the majority of strains carry all three sialidase-encoding genes[71]. However, the NanI sialidase is absent in type A food poisoning strains, as is also the case for perfringolysin O[100]. The small NanH sialidase is synthesized in a 43 kDa (382 amino acids) polypeptide that lacks a signal peptide and is located in the cytoplasm. Its cellular location suggests that NanH is involved in the cleavage of short oligosaccharides that enter the cell and are subsequently broken down for nutritional purposes (Figure 6)[23,161,227].

In contrast to the small NanH sialidase, both large sialidases are secreted enzymes. Secretion of these enzymes results in a mature 77 kDa NanI sialidase and a 129 kDa NanJ sialidase[101]. The large sialidases may also play a role in nutrition, releasing sialic acid from higher order gangliosides and glycoproteins for subsequent transport into the cell. As a result of their extracellular location the large sialidases may also interact with the extracellular environment

of the host during infection[23]. They might facilitate the degradation of tissues to allow for more efficient cell-to-cell spread of the bacteria[223].

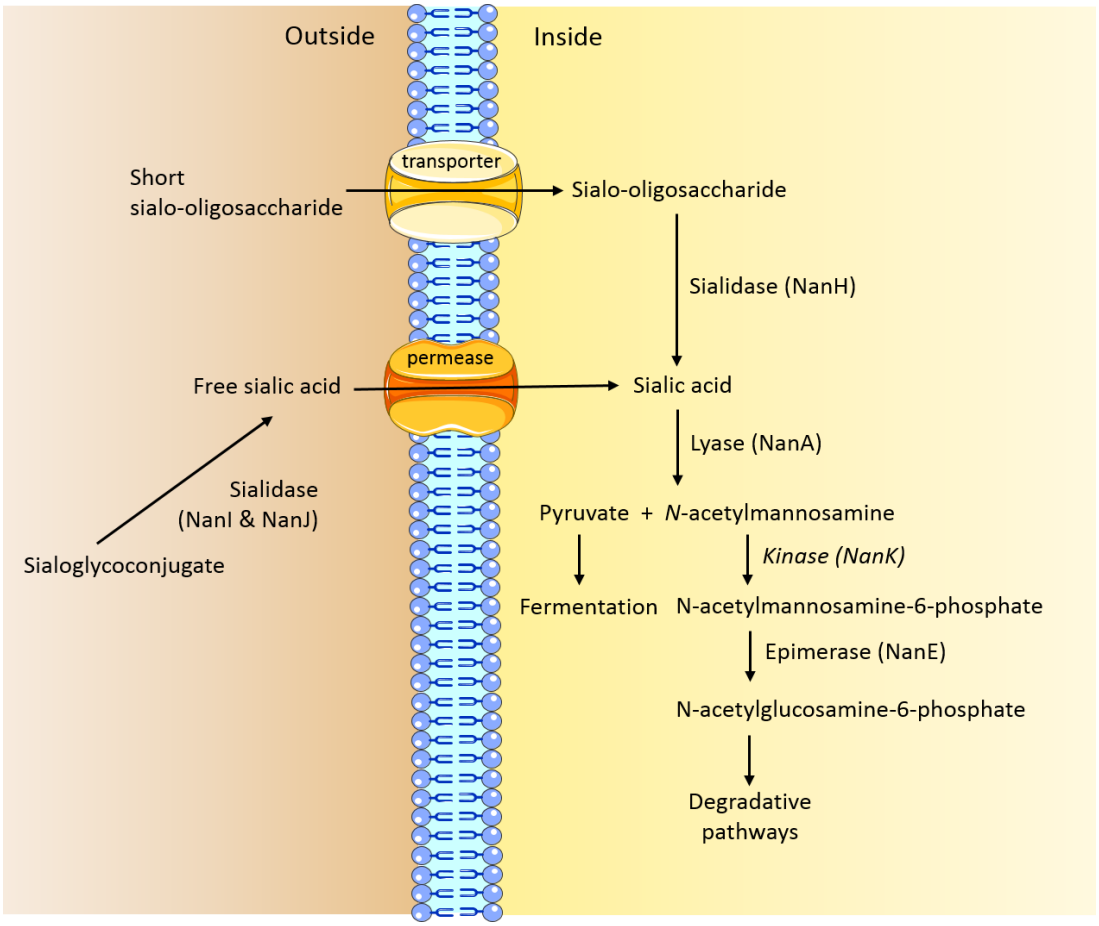


**Figure 6**

**Schematic diagram illustrating the proposed pathway of sialic acid metabolism in *C. perfringens*.**

The genes encoding each enzyme except the transporter, permease, and kinase (NanK) have been detected in *C. perfringens.* (Adapted from Walters *et al.*, 1999[227])

In contrast with gut commensals, which appear to use sialidases primarily for nutrient acquisition, some pathogens, such as *C. perfringens*, also use sialidases to decrypt adhesins or toxin-binding sites[82]. Indeed, in studies on gas gangrene a synergy between alpha toxin and the NanI sialidase was observed[23,55]. In these experiments alpha toxin had greater pathological effects on cultured cells that had been pretreated with NanI. Intramuscular injection of both alpha toxin and NanI in mice confirmed this synergy *in vivo*[55]. This enhancement of alpha toxin activity by NanI is dependent on the presence of gangliosides on the surface of the cell. Cleavage of sialic acid from these gangliosides, which protrude from the cell surface, most

likely allows better interaction of alpha toxin with its substrates at the cellular surface[23,55]. However, the use of either a *nanI* mutant or *nanJ* mutant strain showed that large sialidases are not essential for virulence in a mouse model for gas gangrene[23]. This, however, does not exclude the possibility that sialidases are involved in the pathogenesis of gas gangrene, because subtle effects that might be mediated by the sialidases are masked in this model[23,206].

In addition to a possible role in gas gangrene, the large sialidases may also be of importance during intestinal infections. Recent research suggests that NanI sialidase may contribute to intestinal attachment and colonization. This conclusion was based on the observation that NanI sialidase promotes the adherence of a *C. perfringens* type A, type C and type D strain to enterocyte-like host cells *in vitro*[100,101]. Furthermore, pretreatment of sensitive cells with NanI sialidase enhanced the subsequent binding and cytotoxic effects of epsilon toxin, suggesting that the large sialidases of *C. perfringens* can act in synergy with this toxin during type D enterotoxaemia[101]. To further unravel the role of large *C. perfringens* sialidases in intestinal diseases, *in vivo* confirmation is needed.

### *Collagenases*

Collagen is widely distributed throughout the body and is an integral component of the connective tissue and the basement membrane. Collagen disruption by bacterial collagenases may result in the loss of tissue integrity and subsequent tissue necrosis[70]. Bacterial collagenases possess broad substrate specificities and degrade both native and denatured collagens (gelatin), while vertebrate collagenases preferentially cleave the native form at a specific site[228]. *C. perfringens* can produce various gelatinolytic enzymes with molecular masses ranging from ≈ 80 to ≈ 120 kDa[114,154]. Historically, research was focused on the 80 kDa collagenase, which was designated as kappa toxin[85,114,233]. Up till now, this enzyme is not fully characterized and the corresponding DNA sequence is still unknown. It is a heat-labile protein, which is destroyed completely at temperatures above 60°C[85]. This 80 kDa collagenase was lethal for mice after intravenous injection. Haemorrhagic activity was proven after intracutaneous administration in rabbits and subcutaneous injection in guinea pigs. Furthermore, this enzyme showed local dermonecrotic activity in rabbits following intracutaneous administration[85]. Subcutaneous injection in guinea pigs resulted in extensive destruction of connective tissue. However, no visible changes in the muscle layer were observed and only direct injection into the muscle tissue lead to muscle destruction[85]. These results led to the assumption that the 80 kDa collagenase may be important in the pathogenesis of gas gangrene and that the primary site of

attack in the muscle tissue may be the connective tissue supporting the muscle fibres[85]. Further research revealed that this collagenase is a chemoattractant for human polymorphonuclear leukocytes and is able to degrade the human complement component C1q[233]. However, a positive correlation was not always found between the virulence of *C. perfringens* and the ability to produce collagenase, and anti-collagenase was not effective in preventing experimental gas gangrene in guinea pigs, nor did it enhance the protective properties of anti-alpha toxin[43].

From 1994 onwards, research has switched from the 80 to the 120 kDa collagenase and the term kappa toxin is used to describe the 120 kDa protein[114,142,143]. This enzyme is encoded by the *colA* gene and is synthesized in a 126 kDa (1104 amino acids) precursor form (preprocollagenase), with a stretch of 86 amino acids containing a putative signal sequence and pro region[114]. This pro region also contains a collagenase target sequence, suggesting that self-processing is involved in maturation of the collagenase enzyme[114,237]. The signal peptide and pro region are removed to produce a mature 116 kDa extracellular collagenase which is closely related to the collagenase from *C. histolyticum*[161]. The mature collagenase protein is a zinc metalloprotease, which contains a consensus zinc-binding sequence (HEXXH) at the center of the active site[114]. In analogy with the collagenase from *C. histolyticum*, it is suggested that the 80 kDa collagenase can be generated from the 120 kDa protein[11,237]. However, no experimental evidence exists to support this hypothesis.

As described above collagenases could play a role in clostridial virulence in terms of spreading toxins and bacterial cells to host tissue, and in tissue necrosis. The use of a *colA* mutant *C. perfringens* strain revealed that its gene product is not essential for disease in a mouse model for gas gangrene[11]. However, studies using this model are limited given that the mouse gas gangrene model does not enable conclusions to be drawn about the early stages of the infection[206].

### *Hyaluronidases*

Hyaluronan (hyaluronic acid, HA) is a glycosaminoglycan composed of alternating units of glucuronic acid and *N*-acetylglucosamine, but unlike other glycosaminoglycans, it lacks a covalently linked peptide[59,75,94]. Hyaluronan can form highly viscous solutions and is a major constituent of the extracellular matrix, especially in soft connective tissue[94]. The viscous consistency usually provides resistance to penetration of infectious agents and their extracellular products. However, some bacteria have adapted ways to weaken the restraints of

connective tissue. Bacterial hyaluronidases act as endo-*N*-acetylhexosaminidases and are produced by a number of bacteria that initiate infections at mucosal surfaces[75]. Hyaluronidase-mediated degradation of HA decreases the viscosity, which results in increased permeability of the connective tissues and potentially increased spread of microorganisms and toxins through the connective tissues[61,75]. Alternatively, hyaluronidase may degrade HA cell coatings, thereby allowing direct contact between the pathogens and the host cell surfaces. Furthermore, the end products of hyaluronidase degradation are disaccharides which can be used as nutrients by the pathogen[75].

In *C. perfringens* 5 hyaluronidase genes are described (*nagH*, *nagI*, *nagJ*, *nagK* and *nagL*)[174]. The enzymes encoded by these genes differ in length (1001 – 1628 amino acids), but all the N-terminal amino acid sequences are similar and have putative signal sequences, suggesting that they encode secreted enzymes[174]. Not much research has been done on the *C. perfringens* hyaluronidases. The best studied enzyme is mu toxin or NagH (encoded by the *nagH* gene), a ≈ 182 kDa enzyme composed of an N-terminal region, harbouring the active site and a C-terminal putative calcium-binding dockerin fold[21,24]. By itself, mu toxin is a non-lethal toxin but it is thought to contribute to the pathogenesis of *C. perfringens* through the degradation of mucins and connective tissue[48]. Furthermore it facilitates the spread of alpha toxin, thereby potentiating its activity[21]. Because the *C. perfringens* hyaluronidases are not as well studied as the other *C. perfringens* toxins, no experimental evidence exists about the actual role of these enzymes in either gas gangrene or intestinal infections.

### *The concerted action of* C. perfringens *carbohydrate-active enzymes*

*C. perfringens* produces a large number of carbohydrate-active enzymes, including the hyaluronidases and sialidases. These enzymes are involved in degradation of complex glycans, including the intestinal mucosal layer, which comprises a group of highly hydrated glycoproteins and glycosaminoglycans. The carbohydrate-active toxins are among the largest and most modular enzymes produced by *C. perfringens*. Next to the catalytic domain, these enzymes can also contain various non-catalytic conserved modules, which may play a role in their function. For example, carbohydrate-binding modules (CBM) are present in many of these enzymes and are shown to mediate their attachment to host glycan targets[15,48]. Other modules present in *C. perfringens* glycoside hydrolases are a cohesion-like X82 module, present in amongst others the NanJ sialidase[26], and a dockerin-like sequence present in the NagH hyaluronidase (mu toxin)[24,25]. These X82 and dockerin modules, and by extension their

associated enzymes, can form noncovalent complexes through ultra-high-affinity interactions[2,24]. In this way, NanJ sialidase and NagH hyaluronidase can form dockerin/X82-mediated enzyme complexes which act simultaneously and in concert to exert their pathogenic effects. The observation that multiple carbohydrate-active enzymes contain such non-catalytical domains suggests a unique property of these clostridial toxins to associate into large, noncovalent, multitoxin complexes. The formation of such complexes might allow a highly efficient degradation of host glycans by combining complementary toxin activities[2].

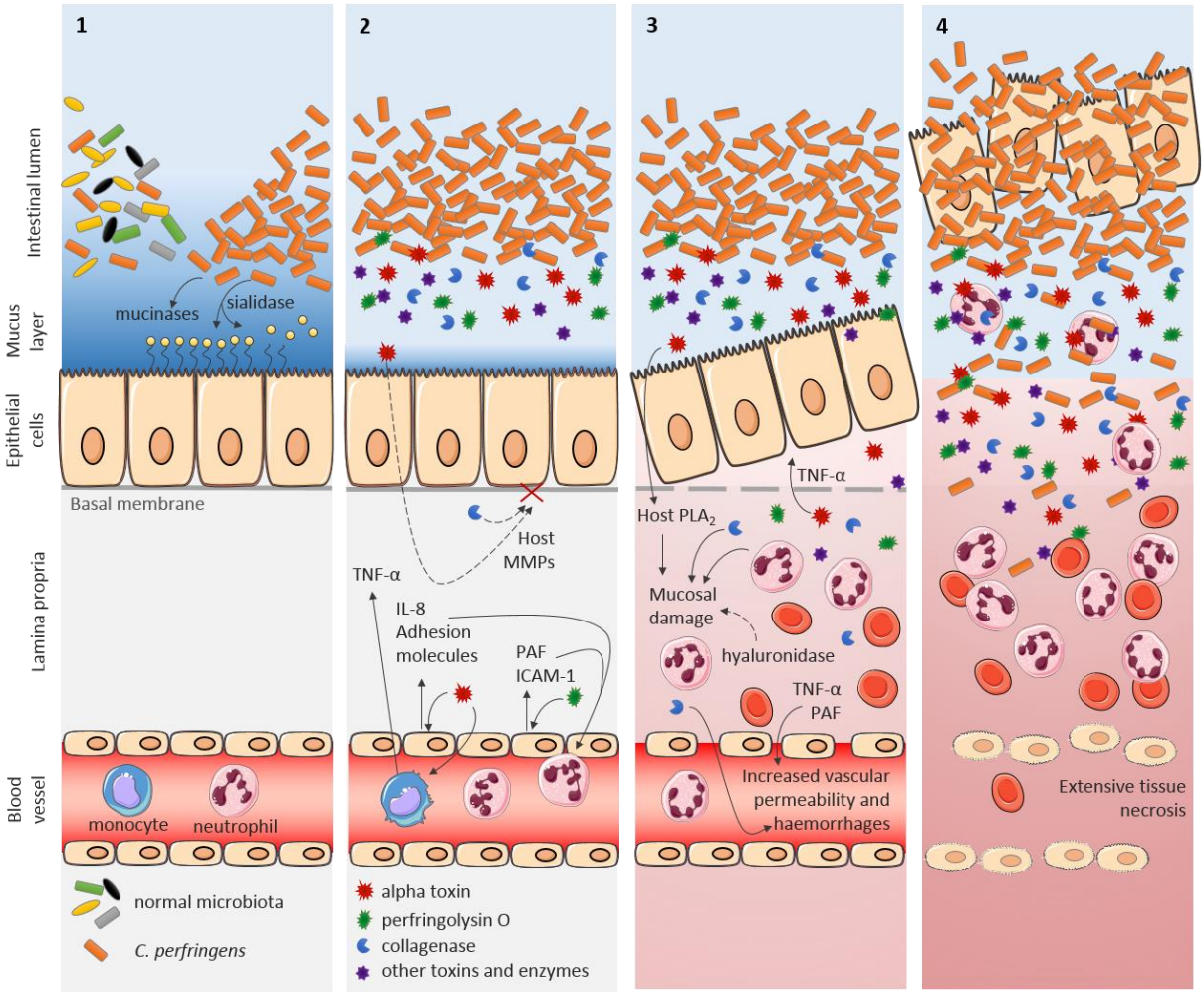


**Figure 7**

**Hypothesis on key events in *C. perfringens* type A-induced intestinal necrosis.**

*C. perfringens* type A strains can cause gastro-intestinal diseases in different animal species. The onset of disease most likely results from a coordinated interplay between different events and factors. The key risk factors for the development of *C. perfringens*-associated enteric disease is an intestinal environment that favours growth of *C. perfringens* and/or induces initial epithelial damage. Amongst the predisposing factors, a high protein diet and stressful conditions are of major importance. (1) The disease starts with rapid proliferation of *C. perfringens* type A. The production of a variety of mucin degrading enzymes leads to breakdown of the protective mucus layer

and sialidases remove sialic acid residues from the cells, making the epithelial cells easier to reach and unmasking potential binding sites for other *C. perfringens* toxins and enzymes. Furthermore, the free sialic acid and mucin fragments provide a source of carbon and nitrogen, favouring further clostridial growth and toxin production. (2) *C. perfringens* produces alpha toxin, perfringolysin O, collagenase as well as multiple other toxins and enzymes. Alpha toxin and perfringolysin O stimulate endothelial cells for the production of IL-8, adhesion molecules (by alpha toxin), ICAM-1 and PAF (by perfringolysin O), leading to trafficking of neutrophils into the tissue space. Furthermore alpha toxin induces TNF-α production by monocytes and may have an effect on host matrix metalloproteinases (MMPs). (3) The earliest histopathological changes observed are epithelial cell detachment and congestion of the capillaries evolving to haemorrhages. Interestingly, the epithelial lining appears intact at this stage. Alpha toxin induces epithelial sloughing, probably through TNF-α and through host MMP activity on the basal membrane. Furthermore TNF-α and PAF likely contribute to the increased vascular permeability. *C. perfringens* collagenase has haemorrhagic activities and may be involved in the breakdown of the basal membrane and further destruction of the connective tissue. This mucosal damage is a result from various factors, including the alpha toxin-induced activation of endogenous phospholipase $A_2$ ($PLA_2$) and neutrophil-derived oxidants, proteolytic enzymes and cytotoxic proteins. (4) All these events eventually lead to fulminant intestinal necrosis and allow the absorption of inflammatory cytokines (such as TNF-α) and toxins from the intestinal lumen into the systemic circulation, leading to rapid death.

# 3

# *C. perfringens*-associated immune response

## Host immune response to *C. perfringens* infection

### Innate immune response to *C. perfringens*

The mechanisms of innate immune response against *C. perfringens* and its toxins remain unclear. *C. perfringens* is a non-invading pathogen and innate immune cells as well as the mucus layer and epithelial cells form a first barrier to infection. In general, peptidoglycan, the main cell wall component of Gram-positive bacteria, activates the innate immune response via pattern recognition receptors on epithelial cells, especially the membrane-bound Toll-like receptor (TLR) 2 and cytoplasmic nucleotide-binding oligomerization domain (NOD) receptors 1 and 2[102,131,236]. TLR activation triggers subsequent inflammatory responses through various signalling pathways and finally leads to release of pro-inflammatory cytokines[38]. In chickens, TLR2 is strongly involved in the host response to *C. perfringens* challenge in the intestine[38,104] and also NOD1 seems to be involved in the recognition of *C. perfringens* peptidoglycan, as shown *in vitro*[68]. No studies concerning the involvement of pattern recognition receptors in *C. perfringens* infections in other species are described. Both the *C. perfringens* bacterial cell itself and its secreted toxins can trigger innate immunity and lead to intense production of inflammatory cytokines[68]. Furthermore, membrane vesicles released from *C. perfringens* also stimulated production of inflammatory cytokines[77]. Together with the *C. perfringens* toxins and enzymes, the excessive and long-term production of pro-inflammatory cytokines may lead to gut damage. Once the epithelial barrier has been breached, *C. perfringens* toxins and enzymes come in contact with submucosal innate immune cells (macrophages, monocytes, dendritic cells) and trigger further release of pro-inflammatory cytokines. *C. perfringens* can resist macrophage-mediated killing through the action of alpha toxin and perfringolysin O[141]. It has

been demonstrated that macrophages are able to kill *C. perfringens*, but it has also been reported that *C. perfringens* cells survive in the presence of macrophages, are cytotoxic to macrophages and can escape from the phagosome of macrophages after phagocytosis[141]. This discrepancy in macrophage mediated killing of *C. perfringens* was suggested to be a result of the amount of alpha toxin and perfringolysin O, with high concentrations resulting in the survival of *C. perfringens*[141].

## Adaptive immune responses to *C. perfringens* infection

Little research has focused on the natural acquired immunity towards *C. perfringens*. In general, antibodies to *C. perfringens* toxins are present in the serum of adult, healthy, non-vaccinated individuals[14,32,65,73,79,97,111,224]. This implies that part of the toxins produced during subclinical infection or produced by the normal microbiota (for *C. perfringens* type A strains) may enter the general circulation and be processed by the immune system without any clinical symptoms[96,224]. The prevalence of serum antibodies as well as the levels of serum antibody tend to increase with the age of the individuals and the infection pressure in the environment[79,97,224]. A more profound study of the host response to *C. perfringens* infections in broilers strongly suggests that both cell-mediated and antibody-mediated immune responses via major histocompatibility complex (MHC) class I and II systems are actively involved[239]. More research is needed to further elucidate the molecular regulation of the host immune response in both chickens and other species.

Maternal antibodies are transferred to the neonate via either the placenta (e.g. humans), the egg or for most species via the colostrum[200,215]. This passive maternal immunity protects the neonate during the first weeks of life. Passive immunity then gradually declines after which the animals must develop their own active immunity. In the ideal situation a fluent transition from passive maternal to active immunity occurs. Only limited reports describe the evolution of serum antibodies during the first period of life. Active development of serum antibodies to *C. perfringens* epsilon toxin can appear as early as 6 weeks after birth in young goats[224]. The development of serum antibodies to *C. perfringens* alpha toxin in calves seems to be influenced by the diet. Under certain circumstances a fluent transition of active immunity with the decline in maternal antibodies is achieved, whereas under other conditions a complete absence of active immunity until the age of 26 weeks is demonstrated[215]. This immunity gap may render the animals susceptible for *C. perfringens* type A related infections such as abomasitis and enterotoxaemia[215].

# Vaccination against *C. perfringens* infections

## Currently available clostridial vaccines

Clostridial diseases are common and usually cause sudden death with little time for treatment, so vaccination is usually the only achievable medical intervention. All current clostridial vaccines contain inactivated toxoids. These toxoid-vaccines are made from culture supernatants which are inactivated, mostly using formalin. These toxoids are subjected to an ultrafiltration purification process, which removes the bacterial cells and concentrates the desired clostridial antigens. Most available clostridial vaccines are combination vaccines against several clostridial species, often including toxoids derived from multiple toxinotypes of *C. perfringens*. Amongst the *C. perfringens* toxinotypes, type C and type D toxoids are almost always included in clostridial vaccines, whereas the other *C. perfringens* toxinotype toxoids are not always all included. In addition, toxoids from several other clostridial species are usually present in the vaccines: *Clostridium chauvoei, Clostridium novyi, Clostridium sordellii, Clostridium septicum* and *Clostridium tetani*. A few vaccines also include other bacteria such as *Mannheimia haemolytica* or enterotoxigenic *Escherichia coli*[96]. To prevent botulism a vaccine containing toxoid of *Clostridium botulinum* type C and D is available. *C. chauvoei* vaccines also require the inclusion of some cellular material[98]. The resulting vaccines still contain a lot of undesired, uncharacterized proteins which may disturb the immune response against the toxins of interest[96]. Moreover, inactivation, especially formalin inactivation, can induce changes in the tertiary protein structures of relevant toxins, influencing the immunogenicity. Although the protection afforded by these vaccines against bovine type A enterotoxaemia is still a subject of debate, for most clostridial diseases the formaldehyde inactivation seems to have little effect on the efficacy of the vaccines[69,86,98,181,197]. Covexin® (Zoetis, Lovain-la-Neuve, Belgium), a commonly used commercial vaccine in Belgium, was developed over 50 years ago for immunization of sheep. It is claimed that the vaccine was extensively tested in large-scale field trials in sheep[7]. Unfortunately, no scientific reports on either the antibody development, nor on the efficacy of these vaccines were published. However, the use of this vaccine in practice has empirically proven its efficacy in protection of sheep against clostridial diseases. In contrast, despite being licensed, there are no published reports of vaccination studies in cattle, nor indications that this was tested by the manufacturer. Furthermore, empirical data from the field lead to the presumption that the vaccine is not adapted to protect cattle against *C. perfringens* intestinal diseases. The same observations were made with other *C. perfringens* vaccines

registered in Belgium. As a consequence, more research is needed to evaluate the efficacy of current commercial vaccines against *C. perfringens*-associated diseases in cattle. Most likely, the development of novel vaccines, using modern techniques will be necessary. Possible strategies can be, amongst others, the development of subunit vaccines in which immunogenic protein regions are used instead of culture filtrate, the use of alternative methods for vaccine delivery and/or the use of novel adjuvants that induce both systemic and mucosal immunity.

## Immune response to vaccination against *C. perfringens*-associated diseases

In accordance with the scarce literature about the natural immunity to *C. perfringens*, the molecular mechanisms underlying the development of protective immunity after vaccination still remains unclear. The current knowledge about the immune response to vaccination against *C. perfringens* is mainly focused on the development of circulating antibodies. In this section the immune response to current clostridial vaccines (all containing formaldehyde inactivated toxoids) is described, after which a brief introduction to the immunity to recombinant toxoids is given.

As is also the case for other clostridia, *C. perfringens* toxins are highly antigenic. However, the antibody titers and the rate of decline in antibody levels differ depending on the host species and the *C. perfringens* toxins. One example is the use of vaccines containing *C. perfringens* epsilon toxoid against enterotoxaemia caused by *C. perfringens* type D, a common disease of sheep and goats. There is evidence that sheep can be fully protected against this disease by vaccination and remain protected for a year when a booster dose is applied between 4 to 6 weeks after the initial vaccination[14,121,207]. In goats, vaccination with *C. perfringens* type D epsilon toxoid also provides protection against experimental enterotoxaemia[207]. However, the antibody levels are lower than in sheep and of short duration and therefore goats should be vaccinated every 3 to 4 months following an initial double immunization[121,205]. Also in cattle vaccination against *C. perfringens* type C or D results in an antibody response, however the duration of the elevated antibody levels is unknown[200,201]. For *C. perfringens* type A, different opinions exist concerning its implication in clostridial enteric infections and thus also the necessity of inclusion in vaccines is controversial[31,62,96]. Only one report was found describing the use of a commercial vaccine against *C. perfringens* type A in cattle, which suggests that antibodies are developed after vaccination, but booster injections every six months are necessary to maintain high antibody levels[111]. The majority of vaccination studies against *C. perfringens* type A are

focussed on gas gangrene. In most cases the immune response obtained with these vaccines was determined in terms of serum antitoxin levels, but there are also reports in which the vaccinated animals were challenged to measure protection[16]. Experimental studies in mice, guinea pigs, rabbits, pigeons, dogs and humans showed that *C. perfringens* type A toxoids were effective in producing a measurable and protective antibody response[6,42]. However, serious problems are reported concerning the reproducibility of such vaccination experiments. *C. perfringens* toxoids can vary in antigenicity and in protective capacity to quite a large degree. Preparations of *C. perfringens* type A toxoid can not only differ markedly in antigenicity from one batch to another, but the same batch might also induce different antibody responses in different groups of animals[5,105]. Furthermore, there seems to be no correlation between the serum antitoxin titres and the degree of protection in mice[6]. These observations raised doubt about the immunogenicity of alpha toxin, the main *C. perfringens* type A virulence factor, which might be easily destroyed by formaldehyde inactivation. It was therefore questioned whether any protective effects of *C. perfringens* type A toxoid might reside in residual undenatured alpha toxin[76,105]. Because of these problems with reproducibility, up till now, no commercial vaccines against gas gangrene are available. A major problem in the evaluation of the different vaccines against *C. perfringens*-associated cattle enterotoxaemia is the absence of an experimental model. Furthermore, diagnosis of adult cattle enterotoxaemia is not always straightforward and deaths caused by enterotoxaemia are frequently classified under sudden death syndrome, a term that describes unexpected deaths without observed premonitory signs of illness[62]. Possible causes of sudden death syndrome include clostridial infection, anaphylaxis, pneumonia, acidosis and endotoxaemia[33]. As a result of the lack of experimental model and the difficult diagnosis, the few published reports concerning the efficacy of clostridial vaccines in cattle focused on the reduction of the sudden death syndrome mortality rate in the field. To the best of our knowledge, no reports describe the efficacy of *C. perfringens* type A containing vaccines in protection of cattle against enterotoxaemia. Some evidence suggests that the use of *C. perfringens* type C and D vaccines may reduce performance but has no significant effect on losses due to sudden death when administered during the finishing period[33,62]. The possible negative effects of clostridial vaccines in cattle are frequently linked to injection site reactions. In the majority of the vaccinated animals, the use of multivalent clostridial vaccines results in intense local reactions at the site of injection[64,192,218,234]. The development of injection-site lesions seems to be associated with enhanced antibody titers[201], but also with a decrease in feed consumption which may impact animal growth and productivity[64,192,234]. This negative impact

on feed consumption presumably results from the tissue injury and inflammatory response caused by injection of the vaccines[192].

To eliminate the undesired effects of multicomponent vaccines, recent research focused on the development of subunit vaccines which only contain the main virulent toxins rather than the numerous molecules present in the current clostridial vaccines[90,128,172]. Such subunit vaccines may eliminate the irrelevant components of the crude toxin vaccines, which should result in a stronger protective immunity with a minimal inflammatory response at the site of injection. Nowadays, the most frequently used approach for the development of subunit vaccines against *C. perfringens*-associated diseases in various animal species, is the use of recombinant toxoids. This research is focused on the expression of either non-toxic fragments of the toxin or the use of non-toxic forms of the whole toxin as immunogen, thereby avoiding the need to detoxify the vaccines by e.g. formaldehyde[103,166,197,232,238]. Recombinant vaccines against many types of *C. perfringens* toxins have been widely studied and were able to induce protective responses, particularly against alpha toxin[28,136,172,188,232] and epsilon toxin[22,150]. However, the majority of these studies didn't test the vaccines in the host species for which they are intended. Furthermore, evidence concerning the efficacy of these vaccines in protecting the host against disease (either natural or experimental) is largely absent. Taking into account the above mentioned discrepancy between the antibody titers and the actual protection against a *C. perfringens* type A challenge, future research should focus on the actual protection afforded by these vaccines in the host species.

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# PART II
# Scientific Aims

# Scientific aims

Bovine enterotoxaemia, also known as (necro-)haemorrhagic enteritis, is an intestinal disease that mainly affects suckling and veal calves. *C. perfringens* type A, which produces alpha toxin, a phospholipase C, as major toxin, is considered to be the causative agent. However, the role of alpha toxin in intestinal diseases is controversial and heavily debated. Despite the economic importance of bovine enterotoxaemia, at the start of this PhD thesis, the pathogenesis was not yet clear and no efficient measures to control the disease were available.

The **general aim** of this thesis was therefore **to obtain more insight in the virulence properties of *C. perfringens* strains isolated from bovine enterotoxaemia cases, to evaluate the role of *C. perfringens* alpha toxin in disease, and to evaluate the use of *C. perfringens* toxins for future vaccine development.**

*C. perfringens* type A is associated with bovine enterotoxaemia, but can also be present in the intestinal tract of healthy animals. Furthermore, all investigated *C. perfringens* strains, independent of their origin, are capable of inducing necro-haemorrhagic intestinal lesions in a calf intestinal loop model. It is hitherto unclear whether *C. perfringens* strains isolated from enterotoxaemia cases have specific virulence properties as compared to other type A strains. Therefore, the **first specific aim** of this work was to analyse the production of virulence factors that are potentially involved in enterotoxaemia. These virulence factors can include the main toxins of *C. perfringens* type A strains (alpha toxin and perfringolysin O), as well as proteolytic and carbohydrate-active factors that degrade the protective mucus layer or extracellular matrix components. To approach this, a collection of strains isolated from enterotoxaemia cases was compared to bovine strains originating from healthy animals and to strains isolated from other animal species (Chapter 4).

Because alpha toxin is the major virulence factor of *C. perfringens* type A strains, the **second aim** of this thesis was to evaluated the role of this toxin in the pathogenesis of bovine enterotoxaemia. This was done by examining the ability of an alpha toxin-mutant strain to induce necro-haemorrhagic lesions in a calf intestinal loop model (Chapter 6).

Given the importance of alpha toxin and the potential importance of other toxins, the **third aim** of this thesis was to explore the use of *C. perfringens* toxins for future vaccine development. First, the use of a mixture of *C. perfringens* toxins as possible vaccine antigens was evaluated (Chapter 5). Therefore, calves were vaccinated with *C. perfringens* toxins, either as native

toxins or after formaldehyde vaccination. Subsequently, the use of *C. perfringens* alpha toxin as a possible vaccine candidate was evaluated (Chapter 6). In both studies, the antibody production was monitored and the ability of the antisera to neutralize the activity of *C. perfringens* toxins *in vitro* was evaluated. Furthermore, it was determined whether the antisera were able to neutralize *C. perfringens*-induced endothelial cytotoxicity and to protect against *C. perfringens* challenge in an intestinal loop model for bovine enterotoxaemia.

# PART III
# Experimental studies

# 4

# *Clostridium perfringens* strains from bovine enterotoxaemia cases are not superior in *in vitro* production of alpha toxin, perfringolysin O and proteolytic enzymes

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# Abstract

## Background

Bovine enterotoxaemia is a major cause of mortality in veal calves. Predominantly veal calves of beef cattle breeds are affected and losses due to enterotoxaemia may account for up to 20% of total mortality. *Clostridium perfringens* type A is considered to be the causative agent. Recently, alpha toxin and perfringolysin O have been proposed to play an essential role in the development of disease. However, other potential virulence factors also may play a role in the pathogenesis of bovine enterotoxaemia. The aim of this study was to evaluate whether strains originating from bovine enterotoxaemia cases were superior in *in vitro* production of virulence factors (alpha toxin, perfringolysin O, mucinase, collagenase) that are potentially involved in enterotoxaemia. To approach this, a collection of strains originating from enterotoxaemia cases was compared to bovine strains isolated from healthy animals and to strains isolated from other animal species.

## Results

Strains originating from bovine enterotoxaemia cases produced variable levels of alpha toxin and perfringolysin O that were not significantly different from levels produced by strains isolated from healthy calves and other animal species. All tested strains exhibited similar mucinolytic activity independent of the isolation source. A high variability in collagenase activity between strains could be observed, and no higher collagenase levels were produced *in vitro* by strains isolated from enterotoxaemia cases.

## Conclusions

Bovine enterotoxaemia strains do not produce higher levels of alpha toxin, perfringolysin O, mucinase and collagenase, as compared to strains derived from healthy calves and other animal species *in vitro*.

## Background

Bovine enterotoxaemia caused by *Clostridium perfringens*, is a sudden death syndrome with necro-hemorrhagic lesions in the small intestine, which mainly affects suckling calves and veal calves[8,12]. In veal calves, predominantly beef cattle breeds are affected. The syndrome accounts for approximately 20% of the mortalities in these calves, compared to 4% in dairy and mixed breed veal calves[8,13]. *C. perfringens* is an anaerobic Gram-positive spore-forming bacterium, which is a commensal of the gastrointestinal tract of both humans and animals, and is also ubiquitous in soil and sewage[8-10]. Strains of *C. perfringens* are classified into five toxinotypes (A-E) based on the presence of four major toxin genes (alpha, beta, iota and epsilon)[18]. Mainly strains of type A are recovered from enterotoxaemia cases but also from the intestine of healthy cattle and other warm-blooded animals as well as from the environment[18]. *C. perfringens* type A has the ability to produce numerous extracellular toxins and enzymes, of which alpha toxin is the most toxic[15]. Recently alpha toxin, a phospholipase C, and perfringolysin O, a pore forming cytolysin, have been proposed as essential factors for induction of enterotoxaemia[24]. In addition to these toxins, other potential virulence factors might have a role in the pathology of enterotoxaemia. Possible virulence traits can be proteolytic factors that degrade the protective mucus layer and extracellular matrix components or intra-species inhibitory antibacterial substances that confer a selective advantage to the producing strain, as suggested for necrotic enteritis strains in broilers[20].

It is hitherto unclear whether the *C. perfringens* type A strains isolated from enterotoxaemia cases are more virulent than other type A strains. In a calf intestinal loop model, it has been shown that *C. perfringens* strains isolated from healthy and enterotoxaemic cattle as well as from other host species are all capable of inducing necro-hemorrhagic intestinal lesions[23].

The purpose of this study was to analyze the expression of virulence factors that are potentially involved in enterotoxaemia. To approach this, the alpha toxin and perfringolysin O production, the mucinolytic and gelatinolytic activity as well as the intra-species inhibitory activity of a collection of strains originating from enterotoxaemia cases was compared to bovine strains isolated from healthy animals and to strains isolated from other animal species.

# Methods

## Bacterial strains and culture conditions

The 46 *C. perfringens* strains used in this study are listed in Table 1. One isolate per animal was used. Eight strains were isolated from enterotoxaemic calves, 23 were from healthy calves. Also five strains from ruminating cattle were included. In addition five strains isolated from chicken and five ovine strains were included. This study describes the *in vitro* characterization of *C. perfringens* strains and thus does not need approval of an ethical committee.

Bacteria were isolated on Columbia agar (Oxoid, Basingstoke, UK) supplemented with 5% defibrinated sheep blood, 12 mg/l kanamycin sulphate and 30 000 IU/l polymixin B sulphate. The toxinotype of the *C. perfringens* strains was determined by a multiplex polymerase chain reaction (PCR), as described by Yoo *et al*.[26], while the presence of the enterotoxin, NetB and the consensus and atypical beta2 toxin genes were detected with previously described single PCR reactions[6,7,11]. The strains were cultured anaerobically for 24h at 37°C in TGY broth (3% tryptone, 2% yeast extract, 0.1% glucose and 0.1% L-cysteine) for the toxin assays, in BHI broth (VWR, Leuven, Belgium) supplemented with 0.4% (w/v) glucose for growth-inhibition assays and measurements of gelatinolytic activity and in tryptone soy broth (TSB) (Oxoid) for quantification of the mucinolytic activity. Cell-free supernatants from the *C. perfringens* cultures were obtained by centrifugation followed by filtration of the supernatants through a 0.2 µm filter and stored at -20°C.

**Table 1**
**Origins and toxinotypes of *C. perfringens* strains**

BB = Belgian Blue, HF = Holstein Friesian

| Strain | Origin | Toxin genes | Reference |
|---|---|---|---|
| **Bovine enterotoxaemia** | | | |
| BCP62 | BB calf, haemorrhagic gut | *cpa*$^{+}$ | 10 |
| BCP134 | HF calf, haemorrhagic gut | *cpa*$^{+}$ | 10 |
| BCP256 | BB calf, haemorrhagic gut | *cpa*$^{+}$ *cpb2*$^{con+}$ | 10 |
| BCP472 | BB calf, haemorrhagic gut | *cpa*$^{+}$ | This study |
| BCP510 | BB calf, haemorrhagic gut | *cpa*$^{+}$ | 10 |
| BCP544 | BB calf, haemorrhagic gut | *cpa*$^{+}$ | 10 |
| BCP588 | BB calf, haemorrhagic gut | *cpa*$^{+}$ | This study |
| BCP730 | BB calf, haemorrhagic gut | *cpa*$^{+}$ | This study |
| **Healthy calves** | | | |
| BCP20 | HF calf, abomasal ulcer | *cpa*$^{+}$ | 25 |
| BCP311 | BB calf, rectal swab | *cpa*$^{+}$ | This study |
| BCP334 | BB calf, rectal swab | *cpa*$^{+}$ | 10 |
| BCP447 | BB calf, healthy gut | *cpa*$^{+}$ | 10 |
| BCP506 | BB calf, rectal swab | *cpa*$^{+}$ | 10 |
| BCP513 | BB calf, rectal swab | *cpa*$^{+}$ | This study |
| BCP740 | HF calf, healthy gut | *cpa*$^{+}$ | This study |
| BCP747 | BB calf, healthy gut | *cpa*$^{+}$ | This study |
| BCP795 | BB calf, rectal swab | *cpa*$^{+}$ | This study |
| BCP796 | BB calf, rectal swab | *cpa*$^{+}$ | This study |
| BCP797 | HF calf, rectal swab | *cpa*$^{+}$ | This study |
| BCP799 | HF calf, rectal swab | *cpa*$^{+}$ | This study |
| BCP806 | BB calf, rectal swab | *cpa*$^{+}$ | This study |
| BCP808 | BB calf, rectal swab | *cpa*$^{+}$ | This study |
| BCP812 | BB calf, rectal swab | *cpa*$^{+}$ | This study |
| BCP821 | BB calf, rectal swab | *cpa*$^{+}$ | This study |
| BCP822 | HF calf, rectal swab | *cpa*$^{+}$ | This study |
| BCP823 | HF calf, rectal swab | *cpa*$^{+}$ | This study |
| BCP824 | HF calf, rectal swab | *cpa*$^{+}$ | This study |
| BCP825 | HF calf, rectal swab | *cpa*$^{+}$ | This study |
| BCP828 | BB calf, rectal swab | *cpa*$^{+}$ | This study |
| BCP836 | HF calf, rectal swab | *cpa*$^{+}$ | This study |
| BCP837 | HF calf, rectal swab | *cpa*$^{+}$ | This study |
| **Ruminating cattle** | | | |
| BCP783 | HF bull, rectal swab | *cpa*$^{+}$ | This study |
| BCP815 | BB cow, rectal swab | *cpa*$^{+}$ | This study |
| BCP820 | BB cow, rectal swab | *cpa*$^{+}$ | This study |
| L2660 | HF cow, rectal swab | *cpa*$^{+}$ | This study |
| L2664 | BB cow | *cpa*$^{+}$ | This study |
| **Sheep** | | | |
| SCP1 | Rectal swab | *cpa*$^{+}$ | This study |
| SCP2 | Rectal swab | *cpa*$^{+}$ | This study |
| SCP3 | Rectal swab | *cpa*$^{+}$ | This study |
| SCP4 | Rectal swab | *cpa*$^{+}$ | This study |
| SCP5 | Rectal swab | *cpa*$^{+}$ | This study |
| **Chicken** | | | |
| CP17 | Healthy | *cpa*$^{+}$ | 24 |
| CP23 | Healthy | *cpa*$^{+}$ *netB*$^{+}$ | 24 |
| CP24 | Healthy | *cpa*$^{+}$ | 24 |
| CP56 | Necrotic enteritis | *cpa*$^{+}$ *netB*$^{+}$ | 24 |
| NE18 | Necrotic enteritis | *cpa*$^{+}$ *netB*$^{+}$ | 26 |

## Detection of toxin activity

To determine the alpha toxin activity in the supernatants, the lecithinase activity was assayed in an egg yolk agar well diffusion assay[14]. Therefore 7 mm diameter holes were punched out in Columbia agar (Oxoid) supplemented with 2% (vol/vol) egg yolk with the back of a 20-200µl pipette tip and 20 µl of the tested supernatants was added to each hole. Pure alpha toxin (Sigma-Aldrich, St-Louis, USA) was used as standard. Plates were incubated at 37°C for 24 hours and scanned with a GS-800 calibrated densitometer (Bio-Rad Laboratories, Hercules, CA). The diameters of the zones of opacity were measured using Quantity One software (Bio-Rad Laboratories).

Perfringolysin O (PFO) activity in the supernatants was determined by measuring the haemolysis of horse erythrocytes using a doubling dilution assay as previously described[1]. The PFO titre is the reciprocal of the last dilution which showed complete hemolysis. The unit of activity was expressed on a logarithmic scale as a log2 value (titer), and consequently each difference in titre of one unit represents a twofold difference in perfringolysin O activity.

Both assays were performed in triplicate, with supernatants of two independent biological replicates of *C. perfringens* cultures grown in TGY.

## Detection and measurement of proteolytic activity

To determine the mucinolytic activity of the different *C. perfringens* strains, 7 mm diameter holes were punched out TSA plates supplemented with 0.5% (w/v) type II gastric mucin (Sigma-Aldrich) with the back of a 20-200µl pipette tip. Twenty µl of each overnight culture was added to each hole (3 wells per overnight culture). The plates were anaerobically incubated for 16 hours at 37°C and subsequently stained for 30 minutes with amido black staining solution (Sigma-Aldrich). Plates were destained with destaining solution (25% isopropanol and 10% acetic acid) and scanned with a GS-800 calibrated densitometer (Bio-Rad Laboratories). Lysis of mucin was observed as a halo of clearing around the wells. The diameters of the zones of mucin lysis were measured using Quantity One software (Bio-Rad Laboratories).

Detection of gelatinolytic activity with an EnzChek Gelatinase/Collagenase Assay kit was carried out according to the recommendations of the manufacturer (Molecular Probes). Briefly, filter-sterilized supernatant of BHI overnight cultures was incubated with 12.5 µg/ml fluorescein-labelled gelatin substrate for 3 hours at 37°C (2 wells per overnight culture).

Gelatinolytic activity was measured as an increase in fluorescence (excitation 495 nm, emission 515 nm) by a Fluoroskan Ascent Fluorometer and Luminometer (Thermo Fisher Scientific Inc., Waltham, USA). Both assays were performed with two independent biological replicates of *C. perfringens* cultures.

## *In vitro* growth-inhibition assay

All 46 *C. perfringens* strains were used in a checkerboard test for intra-species growth-inhibition as previously described[20]. Each strain was cultured anaerobically in BHI broth for 24h at 37°C. The overnight cultures were diluted 1/1000 in 10 ml BHI agar and poured on the whole surface of BHI agar plates to obtain a bacterial lawn. With a sterile toothpick each *C. perfringens* isolate was transferred from the overnight culture to the agar plates seeded with the different *C. perfringens* strains. Absence of growth of the bacterial lawn around a colony results in an inhibition zone. After overnight incubation under anaerobic conditions, inhibition was evaluated. The test was performed in duplicate.

## Statistical analysis

All tests were performed in duplicate and data were analysed using a Kruskal-Wallis test followed by a Dunn's multiple comparison test. Statistical analyses were performed using GraphPad Prism 5 software.

# Results

## *Clostridium perfringens* strains from enterotoxaemia cases are not superior alpha toxin and perfringolysin O producers

To determine whether alpha toxin and perfringolysin O levels differ between bovine enterotoxaemia strains and strains from other sources, the culture supernatants of various type A strains were tested (Figure 1 A). Lecithin breakdown was used as a measure of alpha toxin activity. The supernatants of the different strains showed high variability in alpha toxin activity, independent of their origin. In eight strains, the alpha toxin activity was below the detection limit of 15.6 $10^{-3}$ U/ml (three strains originating from enterotoxaemia cases and five strains from healthy calves). Perfringolysin O activity was determined by measuring the haemolysis of horse erythrocytes. Supernatants of strains originating from bovine enterotoxaemia cases showed a high variability in haemolytic activity. The mean erythrocyte hydrolysis by strains from bovine enterotoxaemia was not significantly different from other strains, independent of the origin (Figure 1 B). For both alpha toxin and perfringolysin O, no significant differences between enterotoxaemia strains and other *C. perfringens* strains could be observed.

## Mucinolytic and gelatinolytic activity is not higher in *Clostridium perfringens* strains from bovine enterotoxaemia cases

The thickness of the mucin layer reflects an equilibrium between synthesis by the host and bacterial degradation by the intestinal microbiota. We tested the mucinolytic activity of *C. perfringens* strains from enterotoxaemia cases and from other sources to evaluate whether enterotoxaemia strains have higher potential to degrade the protective mucus layer. All strains exhibited similar mucinolytic activity (Figure 1 C). To elucidate whether *C. perfringens* proteases might have the potential to contribute to the pathology of enterotoxaemia, gelatinolytic activity was investigated as a measure for degrading extracellular matrix components within the gut. Gelatin was used as a specific proteolytic substrate to screen for clostridial protease activity that might contribute to intestinal host matrix degradation. A high variability of gelatinolytic activity was seen. The mean gelatin breakdown by strains from bovine enterotoxaemia was not different from other strains, independent of the origin (Figure 1 D). No significant difference between the groups could be observed.

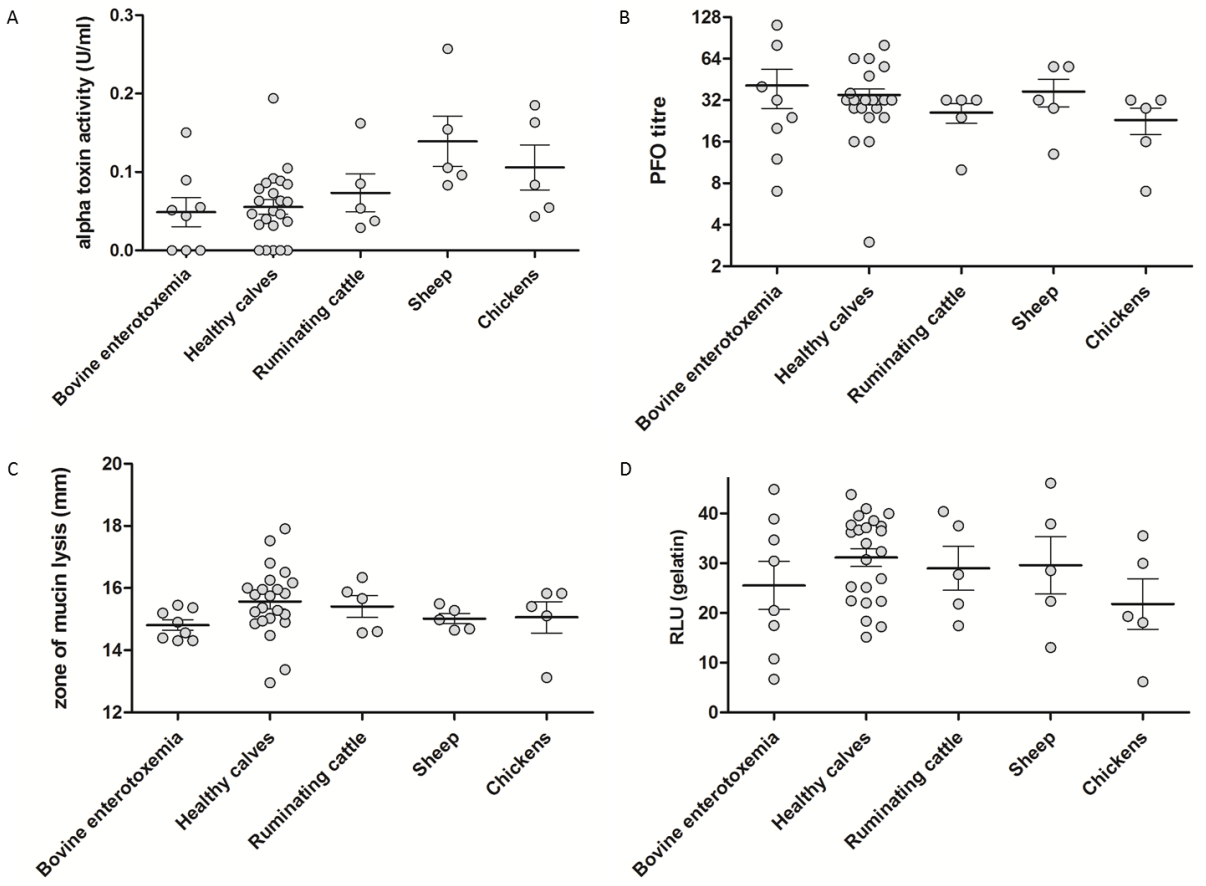


**Figure 1**

**Quantification of putative virulence factor activities of *C. perfringens* strains derived from cattle, sheep and chickens.**

The lines represent the mean with the standard error of the means. (A) The alpha toxin activity in the supernatant of anaerobically grown *C. perfringens* strains was determined by measuring the lecithinase activity in an egg yolk agar well diffusion assay. (B) The perfringolysin O activity in the supernatant of anaerobically grown *C. perfringens* strains was determined by measuring the haemolysis of horse erythrocytes. The PFO titre is the reciprocal of the last dilution which showed complete haemolysis. Each difference in titre of one unit represents a twofold difference in perfringolysin O activity. (C) The mucinolytic activity of *C. perfringens* strains was assayed by adding cultures of strains to wells in TSA-mucin plates and quantification of zones of mucin lysis (in mm). (D) The potential to degrade the extracellular matrix was examined by measuring the breakdown of fluorescent labelled gelatin by supernatant of anaerobically grown *C. perfringens* strains. RLU = relative light units.

## Enterotoxaemia strains have no increased intra-species growth-inhibitory activity

The intra-species growth-inhibitory activity is shown in Figure 2. Of all tested strains, sixty-one percent was able to inhibit growth of at least one other *C. perfringens* strain. There was a lot of variation between the inhibitory spectra of the tested strains. Some strains inhibited only a limited number of strains, while others had a broad inhibitory spectrum. Five out of eight enterotoxaemia strains, eight out of twenty-three strains from healthy calves, four out of five ruminating bovine strains and one out of five sheep strains were unable to inhibit growth of any other strain. No significant differences between the groups could be observed.

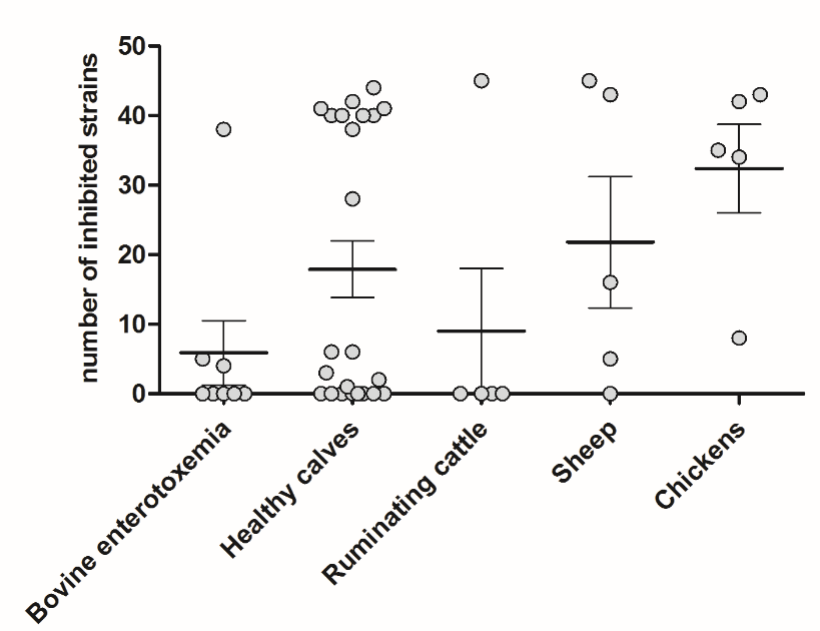


**Figure 2**

**Intra-species growth-inhibition.**

Number of *C. perfringens* strains (n=46) inhibited by the individual strains originating from bovine enterotoxaemia cases, healthy calves, ruminating cattle, sheep and chickens. The lines represent the mean with the standard error of the means.

## Discussion

*Clostridium perfringens* type A strains isolated from bovine enterotoxaemia cases showed no difference in *in vitro* expression of a selection of potential virulence factors compared to strains from healthy cattle as well as various other animal species. This is in accordance with the capacity of lesion induction in a calf intestinal loop model, in which all tested strains are capable of inducing necro-haemorrhagic intestinal lesions, independent of the origin of the isolate[23].

No increased activity of the alpha toxin and perfringolysin O was detected in bovine enterotoxaemia strains. In a recent study carried out with alpha toxin and perfringolysin O mutants we demonstrated an essential role of both toxins in bovine enterotoxaemia[24]. The alpha toxin is the most toxic enzyme produced by *C. perfringens* type A strains and hydrolyses two major constituents of the eukaryotic membrane (phosphatidylcholine and sphingomyelin) causing membrane disruption and cell lysis[1,21]. In sublytic concentrations, alpha toxin leads to activation of signal transduction pathways and uncontrolled production of intracellular mediators[17,21,22]. Perfringolysin O is a pore-forming cytolysin which has the ability to kill eukaryotic cells by punching holes in their membranes[16]. As shown *in vitro*, alpha toxin and perfringolysin O seem to have a synergistic action in bovine enterotoxaemia[24]. Considering this essential role of alpha toxin and perfringolysin O in bovine enterotoxaemia, *C. perfringens* type A strains with higher activity of these toxins might be more virulent. Despite the importance of alpha toxin and perfringolysin O in enterotoxaemia, strains originating form diseased animals showed no higher activities of these toxins *in vitro*.

No increased collagen degrading and mucinolytic potential was detected in strains from enterotoxaemia cases. Collagen is widely distributed throughout the body and is an integral component of the connective tissue. Collagen disruption by bacterial collagenases may result in the loss of tissue integrity and subsequent tissue necrosis in the infected host and allow penetration of bacterial toxins to deeper tissues[5]. Therefore, the ability to produce collagenase may play an important role in the tissue destruction observed in bovine enterotoxaemia. This *in vitro* study showed no difference in collagen destroying potential between *C. perfringens* strains from bovine enterotoxaemia cases compared to strains from healthy cattle, sheep and chickens.

In addition to the collagen degradation, the mucinolytic activity of the *C. perfringens* strains may also contribute to the pathology of bovine enterotoxaemia. The gastrointestinal tract represents a large surface of the host that interacts with the external world. A protective mucus

layer covers the epithelial surface, forming a barrier between the lumen and the intestinal epithelium. It is a potential binding site for both commensal and pathogenic organisms[2,3]. *C. perfringens* type A strains with stronger mucinolytic activity may have an advantage in colonizing and degrading the protective mucus layer, which may lead to a compromised barrier function. Enterotoxaemia strains, however were not different from the other strains with respect to their mucinolytic activity *in vitro*.

Another possible virulence trait that was explored is the ability to cause intra-species growth-inhibition. It is known that many *C. perfringens* strains are able to produce antibacterial proteins capable of lysing other *C. perfringens* strains, called bacteriocins[19,25]. The potential of a pathogenic strain to suppress the growth of other *C. perfringens* strains has been shown in necrotic enteritis in broiler chickens, leading to single clone dominance in a broiler flock suffering from necrotic enteritis[4,20]. In contrast to the situation in broiler chickens, our results showed no difference in intra-species growth-inhibition between the enterotoxaemia strains and other *C. perfringens* strains. This suggests that intra-species growth-inhibition probably does not play a role in the pathogenesis of bovine enterotoxaemia.

None of the possible virulence traits examined in this study were distinctive for *C. perfringens* type A strains isolated from bovine enterotoxaemia cases *in vitro*. This is in accordance with a recent observation from our laboratory that, when the conditions are favourable, strains from different origin are capable to provoke necro-haemorrhagic lesions in an intestinal loop model[23]. It should be noted that the medium in which the bacteria are grown will have an influence on the production of the investigated virulence traits. In the current study only one *C. perfringens* isolate per bovine enterotoxaemia case was used and we cannot say whether or not this was the causative strain. However, most of these strains were tested in bovine ligated loops and all tested strains could cause lesions. In fact, it could well be that any intestinal strain, even a mixture of strains present at that moment, can cause disease when the conditions are right. Furthermore, it cannot be ruled out that other possible virulence factors may be involved in disease. This was the case for necrotic enteritis in broiler chickens, an enteric disease caused by *C. perfringens* type A strains. The essential virulence factor for causing disease remained unclear until the NetB toxin, a previously unknown toxin specific for necrotic enteritis, was found[7]. Although the presence of such an undiscovered toxin in the pathology of bovine enterotoxaemia cannot be excluded, it is unlikely because type A strains originating from bovine enterotoxaemia cases as well as from healthy cattle and other animal species are capable of inducing lesions in an intestinal loop model[23].

## Conclusions

Strains from bovine enterotoxaemia cases did not have a higher alpha toxin, perfringolysin O, mucinolytic or gelatinolytic activity in comparison to strains isolated from healthy cattle and other animal species. Also production of intra-species inhibitory substances was not higher in bovine enterotoxaemia strains. This could indicate that yet another, hitherto unknown, *C. perfringens* virulence factor might be involved in the pathogenesis of calf enterotoxaemia. Taking these results together with our previous observations that strains from various origin can induce the typical lesions in an intestinal loop model[23], it seems however more plausible that the primary trigger in bovine enterotoxaemia is not *C. perfringens* and that *C. perfringens* is merely responsible for propagating and exacerbating the intestinal damage to the point that it becomes haemorrhagic and necrotic.

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# 5

# Toxin-neutralizing antibodies protect against *Clostridium perfringens*-induced necrosis in an intestinal loop model for bovine necro-haemorrhagic enteritis

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# Abstract

## Background

Bovine necro-haemorrhagic enteritis is caused by *Clostridium perfringens* type A. Due to the rapid progress and fatal outcome of the disease, vaccination would be of high value. In this study, *C. perfringens* toxins, either as native toxins or after formaldehyde inactivation, were evaluated as possible vaccine antigens. We determined whether antisera raised in calves against these toxins were able to protect against *C. perfringens* challenge in an intestinal loop model for bovine enterotoxaemia.

## Results

Alpha toxin and perfringolysin O were identified as the most immunogenic proteins in the vaccine preparations. All vaccines evoked a high antibody response against the causative toxins, alpha toxin and perfringolysin O, as detected by ELISA. All antibodies were able to inhibit the activity of alpha toxin and perfringolysin O *in vitro*. However, the antibodies raised against the native toxins were more inhibitory to the *C. perfringens*-induced cytotoxicity (as tested on bovine endothelial cells) and only these antibodies protected against *C. perfringens* challenge in the intestinal loop model.

## Conclusion

Although immunization of calves with both native and formaldehyde inactivated toxins resulted in high antibody titres against alpha toxin and perfringolysin O, only antibodies raised against native toxins protect against *C. perfringens* challenge in an intestinal loop model for bovine necro-haemorrhagic enteritis.

## Background

The ubiquitous, spore forming, Gram-positive bacterium *Clostridium perfringens* is considered to be the most widespread pathogenic bacterium in the world[15,18,21,23]. It can cause a wide range of diseases including, amongst others, gas gangrene in man and necro-haemorrhagic enteritis in suckling and veal calves[10,14,16,28]. Most of these diseases follow a very rapid, often fatal course. Therefore, curative treatment is difficult and control must rely on preventive measures, including vaccination. Virulence properties of different *C. perfringens* strains are largely determined by their ability to secrete a variety of proteinaceous toxins and enzymes, which can cause different forms of tissue damage[18,21,23,24]. Alpha toxin and perfringolysin O have been identified as the principal toxins involved in the pathogenesis of both *C. perfringens*-induced gas gangrene and bovine necro-haemorrhagic enteritis[3,33]. These toxins exert different effects in both diseases. Bovine necro-haemorrhagic enteritis is characterized by congestion of the capillaries, haemorrhages and inflammation. This is in contrast to gas gangrene, where these toxins lead to tissue necrosis, thrombosis and lack of leukocyte infiltration at the site of infection[3,26,33]. It is well known that humoral antibodies against secreted proteinaceous virulence factors of *C. perfringens* can be protective, as shown in different animal models. As the enzymes and toxins of *C. perfringens* are highly destructive to tissues, vaccines against a variety of clostridial diseases have been developed using the denatured proteins[5,11,35]. Despite the usefulness of formaldehyde toxoids for other *C. perfringens*-associated diseases, there is controversy about the efficacy of such vaccines for gas gangrene, as opposed to crude toxin preparations[1,4,13,28]. In addition, multivalent clostridial vaccines based on formaldehyde inactivated exotoxins derived from culture supernatant are commercially available for domestic livestock, including bovines, but no studies on their efficacy for necro-haemorrhagic enteritis in calves are available.

The objective of the present study was to evaluate whether antibodies against *C. perfringens* toxins could protect against the development of necrotic lesions in the intestine. Therefore, calves were immunized with native *C. perfringens* toxins. To evaluate whether we could eliminate the undesired toxin activity, but conserve the immune-protective potential, a previously described, modified formaldehyde treatment was also tested[8]. Also a commercial formaldehyde inactivated multivalent clostridial vaccine was used. As necro-haemorrhagic enteritis in veal calves is an unpredictable event and experimental reproduction of the disease is difficult, the neutralizing activity of the antibodies was evaluated in a previously developed

intestinal loop model[31]. To further unravel the mechanism of protection, the inhibitory effect of the evoked antibodies on *C. perfringens*-induced cytotoxicity on bovine endothelial cells was evaluated and the toxin-neutralizing capacity against alpha toxin and perfringolysin O was analysed.

## Methods

All experimental protocols were approved by the ethical committee of the Faculty of Veterinary Medicine, Ghent University (EC2013/187 and EC2013/38). The animal experiments were conducted in accordance with the approved protocols.

### Vaccine preparation and immunization

*C. perfringens* toxin preparation (P4039, Sigma-Aldrich, Bornem, Belgium) was either used as native toxin or treated with formaldehyde to generate a formaldehyde toxoid. Inactivation was obtained by adding a combination of 0.4% formaldehyde solution (Sigma-Aldrich) and 0.05M L-lysine (Sigma-Aldrich) and incubation at 37°C for two days. The addition of 0.05M L-lysine has previously been shown to preserve the antigenicity of alpha toxin during toxoid formation[8]. Inactivation of alpha toxin was confirmed by spotting 5µl drops on 2% egg yolk Columbia agar plates (Oxoid, Wesel, Germany), followed by incubation for 16h at 37°C[20]. Native and formaldehyde inactivated toxins were formulated with the adjuvant Quil A (Brenntag Biosector, Frederikssund, Denmark) at a final concentration of 350µg antigen and 750µg Quil A in 1.5ml phosphate buffered saline (PBS) per animal and filter-sterilized using a 0.2 µm filter. A standard formalin inactivated multivalent commercial vaccine was used according to the manufacturer's instructions (Covexin 10®, Zoetis, Louvain-la-Neuve, Belgium).

For immunization six 2-months old male Holstein Friesian calves were used. They were housed on straw and received water and hay at libitum, and concentrates adjusted to the body weight.

For each antigen, two calves were immunized subcutaneously in the neck. The calves received a primer vaccination at the age of two months, with booster immunizations 14 and 28 days later. No strong adverse reactions were observed. Although no fever (> 39.5°C) was induced, all calves experienced a mild hyperthermia for two days following the vaccination. As described in the drug information leaflet of the commercial vaccine, localized swelling occurred at the site of injection. This effect was more pronounced in the calves vaccinated with the commercial formaldehyde inactivated clostridial vaccine (7-10 cm diameter) as compared to the calves

vaccinated with either native toxins or the L-lysine protected, formaldehyde inactivated toxins (0-6 cm diameter). Blood samples were taken before primer vaccination and two weeks after the final booster vaccination.

## SDS-PAGE and Western Blot

The proteins present in the toxin preparation were visualized on a 12% SDS-PAGE followed by Coomassie Briliant Blue staining (Sigma-Aldrich). For the Western Blot analysis, 16 µl of cell-free supernatants of the *C. perfringens* strain JIR325 (10x concentrated using Vivaspin, Sartorium Stedim Biotech GmbH, Goettingen, Germany) or 6 µg of the *C. perfringens* toxin preparation were loaded on a 12% SDS-PAGE. The proteins from the gel were transferred to nitrocellulose membranes of 0.45 µm pore size. Non-specific binding to the blots was blocked with 5% skimmed milk powder in PBS, followed by overnight incubation at 4°C with a 1/500 dilution of the immune sera collected two weeks after the final booster immunization. For this incubation step, the sera of the 2 animals that were vaccinated with a given vaccine preparation were pooled. Blots were washed with 0.1% tween 20 in PBS and incubated for 1h at room temperature with horseradish peroxidase-labelled rabbit-anti-bovine IgG (Sigma-Aldrich). Blots were developed with CN/DAB substrate kit (Thermo Fisher Scientific, Rockford, USA). The test was performed in triplicate. The specific immunoreactive protein bands were identified in the parallel-run Coomassie stained gel followed by MALDI analysis.

## Enzyme-linked immunosorbent assay

The immune response following vaccination was also measured by ELISA using serum samples two weeks after the final booster immunization.

Alpha toxin-specific antibody levels were determined by the end-point dilution method using a blocking ELISA (*Clostridium perfringens* alpha toxin serological ELISA kit, Bio-X Diagnostics, Jemelle, Belgium). For each ELISA, sera were used at a dilution 1:50 and assays were performed in duplicate. The specific antibody level of the immune serum was expressed as the percent inhibition (% inhib) by means of the following formula: % inhib = [(OD neg – OD sample)/OD neg]*100

Perfringolysin O-specific antibody levels were measured using an indirect ELISA. Briefly, 96-well microtitre plates (Nunc MaxiSorp, Thermo Fisher Scientific) were coated with 20 µg recombinant perfringolysin O [29]. Non-specific binding was blocked with 1% (w/v) bovine

serum albumin (Sigma-Aldrich) in PBS. Two-fold dilutions of the sera ranging from a dilution of 1:50 to 1:51200 were added to the plates (100µl of each dilution/well; in duplicate) and incubated for 2 h at 37°C. Plates were washed with 0.1% (v/v) Tween 20 in PBS and incubated for 1 h 30 min at 37°C with horseradish peroxidase-labelled rabbit-anti-bovine IgG (Sigma-Aldrich). Bound conjugate was detected using the substrate 3,3′,5,5′-Tetramethylbenzidine (TMB) (Sigma-Aldrich). The reaction was blocked with $H_2SO_4$ and the absorbance was measured at 450 nm using a microplate reader (Multiscan MS, Thermo Labsystems, Helsinki, Finland). The end-point titer is expressed as the reciprocal of the last dilution that gave a reading of 0.1U above background (precolostral neonatal bovine serum).

## Intestinal loop model

To study the protection against *C. perfringens*-induced necrosis provided by the antisera from calves vaccinated with the vaccine preparations, four intestinal loop experiments were performed. Intestinal loop experiments were performed according to a previously described protocol using 4 healthy male Holstein Friesian calves[31]. Briefly, the calves were anesthetized and the small intestine was exteriorized. Per calf 80 intestinal loops of approximately 10 cm were ligated in the jejunum and a 5 cm space was left between the loops. Only half of the loops were injected, thus each time leaving one intervening loop to avoid leakage between sampled loops. For each vaccine preparation individual pre- and post-vaccination sera of 2 calves were used in two intestinal loop experiments. Intestinal loops were inoculated with 20 ml of a wild-type strain (JIR325) in combination with 10 ml of 25% commercial milk replacer suspended in sterile NaCl solution, resulting in a total volume of 30 ml which was the same across all treatments and control loops. Prior to inoculation pre- or post-immune serum derived from calves immunized with the different vaccine preparations was added to the NaCl solution containing milk replacer, to obtain a final concentration of 6% serum (v/v). In each calf five intestinal loops per test serum were injected. Also an equal number of control loops without addition of serum were injected either with *C. perfringens* (positive control) or with sterile bacterial growth medium (negative control). After injection of the loops, the abdomen was closed and the calves were maintained under anaesthesia. At 5-h post-inoculation the animals were euthanized and samples were taken. Samples were fixed in 4% phosphate buffered formaldehyde. They were embedded in paraffin wax, sectioned and stained with haematoxylin-eosin. The sections were evaluated in a blinded manner by a board certified pathologist for presence of tissue necrosis (0 = absence of necrosis, 1 = necrotic lesions present).

## Neutralization of alpha toxin activity on egg yolk lipoproteins *in vitro*

The alpha toxin activity was determined by its effect on egg yolk lipoproteins as previously described[12]. Therefore, fresh egg yolk was centrifuged (10,000 x g for 20 min at 4°C) and diluted 1:10 in PBS. The ability of the sera to neutralize the alpha toxin activity was assessed by pre-incubating a two-fold dilution series of the sera (two wells per dilution) with a constant amount of alpha toxin (10µg/ml recombinant alpha toxin in PBS solution) for 30 minutes at 37°C prior to the addition of 10% egg yolk emulsion. Recombinant alpha toxin was expressed in *E. coli* using the pBAD TOPO® TA Expression Kit (Invitrogen, Paisley, UK) followed by purification onto a Ni-sepharose column (His Gravitrap, GE Healthcare, Buckinghamshire, UK). After incubation of the plates at 37°C for 1 hour, the $A_{620}$ was determined. Alpha toxin activity was indicated by the development of turbidity which results in an increase in absorbance. The inhibitory capacity of the antiserum was determined by applying a Hill function to the concentration-response data (GraphPad Prism 5, GraphPad Software, San Diego, CA, USA) and expressed as the dilution that inhibited 50% of the alpha toxin activity. The test was performed in duplicate.

## Neutralization of perfringolysin O activity *in vitro*

Perfringolysin O (PFO) activity was determined by measuring the haemolysis of horse erythrocytes using a doubling dilution assay as previously described[2]. The PFO titre is the reciprocal of the last dilution which showed complete haemolysis. Similar to the inhibition of the alpha toxin activities, the ability of sera to neutralize the PFO activity was assessed by pre-incubating a two-fold dilution series of the sera (two wells per dilution) with a constant amount of perfringolysin O (2 µg/ml recombinant perfringolysin O). Recombinant perfringolysin O was produced as previously described[32]. The inhibitory capacity of the antiserum was expressed as the highest dilution that inhibited perfringolysin O induced haemolysis. The test was performed in duplicate.

## Endothelial cell cytotoxicity assay

Primary bovine umbilical vein endothelial cells (BUVEC) were isolated from umbilical cord veins by an adaptation of the method of Jaffe et al. as performed previously[9,33]. The toxicity of *C. perfringens* supernatant towards cultured bovine endothelial cells has been reported previously[33]. The ability of the antisera to neutralize the *C. perfringens* cytotoxicity towards

BUVECs was determined using a Neutral Red Uptake assay (NRU)[19]. Therefore, a two-fold dilution series of the sera (100% - 0.4%) prepared in serum free cell culture medium was pre-incubated for 30 minutes at 37°C with an equal amount of *C. perfringens* supernatant. Cells were treated with 100 µl of these supernatant-serum mixtures. The inhibitory capacity of the antiserum was expressed as the highest dilution that yielded 80% cell viability. As a positive control, cells were treated with *C. perfringens* supernatant which was pre-incubated for 30 minutes with serum free cell culture medium. Untreated cells, incubated with serum free cell culture medium served as a negative control. The test was performed in duplicate.

## Statistical analysis

The 20 loops tested for each condition provided enough statistical power to detect a 40% reduction in the development of necrotic lesions in the intestinal loop assay (95% confidence, 80% power) (Winepiscope 2.0).

The protective effect of the antisera in the intestinal loop assay as compared to the pre-immune sera and the untreated control loops were determined by a Fisher's exact test (GraphPad Prism 5 software). Differences between groups were considered significant at $p < 0.05$.

# Results

## Western blot analysis

The proteins in the *C. perfringens* toxin preparation were visualized by SDS-PAGE (Figure 1A). In the vaccinated calves, the production of circulating antibodies against *C. perfringens* supernatant and the *C. perfringens* toxin preparation was analysed by western blot in three separate experiments (Figure 1). No immune reaction was detected in the sera before immunization (data not shown). Sera obtained from calves six weeks after initial vaccination with either native toxins or the L-lysine protected, formaldehyde inactivated toxins, revealed immunoreactivity towards two proteins. Immune sera from calves vaccinated with the commercial formaldehyde inactivated clostridial vaccine showed immunoreactivity towards more proteins. The two proteins that were immunoreactive with antisera raised against all vaccine preparations were further identified as alpha toxin and perfringolysin O by MALDI analysis.

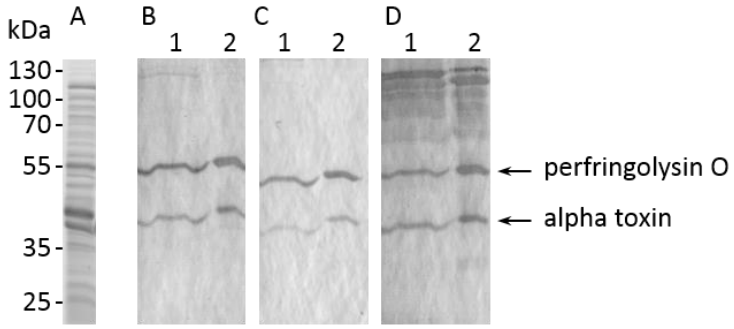


**Figure 1**

**Western blot analysis of the immune sera.**

(A) SDS-PAGE of the *C. perfringens* toxin preparation after Coomassie staining. (B-D) Representative Western blots showing the immunoreactivity towards crude *C. perfringens* supernatants (lane 1) and the *C. perfringens* toxin preparation (lane 2). The immune sera of calves vaccinated with native toxins (B) or formaldehyde inactivated, L-lysine protected *C. perfringens* toxins (C) detect only two proteins, whereas the immune sera of calves vaccinated with commercial formaldehyde inactivated multivalent clostridial vaccine (D) reacts with multiple proteins. The blots shown are representative pictures of one out of three experiments.

## ELISA

In the vaccinated calves, the production of circulating antibodies directed against alpha toxin and perfringolysin O was also monitored by ELISA. No antibodies against alpha toxin or perfringolysin O were detected in the sera before immunization. In all calves a strong antibody response against both alpha toxin and perfringolysin O was detected 6 weeks after initial

immunization. Calves vaccinated with the native *C. perfringens* toxins showed the highest antibody titres, whereas vaccination with formaldehyde inactivated toxins (either L-lysine protected or commercial inactivation) resulted in a more variable immune response (Table 1).

**Table 1**

**Antibody development towards alpha toxin and perfringolysin O.**

Calves were immunized with either a *C. perfringens* toxin preparation (native toxins), L-lysine protected, formaldehyde inactivated *C. perfringens* toxins (L-lysine/formaldehyde toxoid) or a commercial multivalent formaldehyde inactivated clostridial vaccine. The anti-alpha toxin and perfringolysin O response was measured by ELISA. The data represent antibody titres (mean ± standard error of the means), six weeks after initial immunization.

| Vaccine | Anti-alpha toxin titre | Anti-perfringolysin O titre |
|---|---|---|
| **Native toxins** | 64.44 ± 0.22 | 25600 ± 0 |
| **L-lysine/formaldehyde toxoid** | 24.26 ± 2.96 | 16000 ± 9600 |
| **Commercial formaldehyde vaccine** | 45.14 ± 20.42 | 4800 ± 1600 |

## Protective effect of antisera against *C. perfringens*-induced necrosis in an intestinal loop model

The potential of the antisera, derived after vaccination of calves with the respective vaccines, to inhibit *C. perfringens*-induced necrosis, was evaluated in an intestinal loop assay.

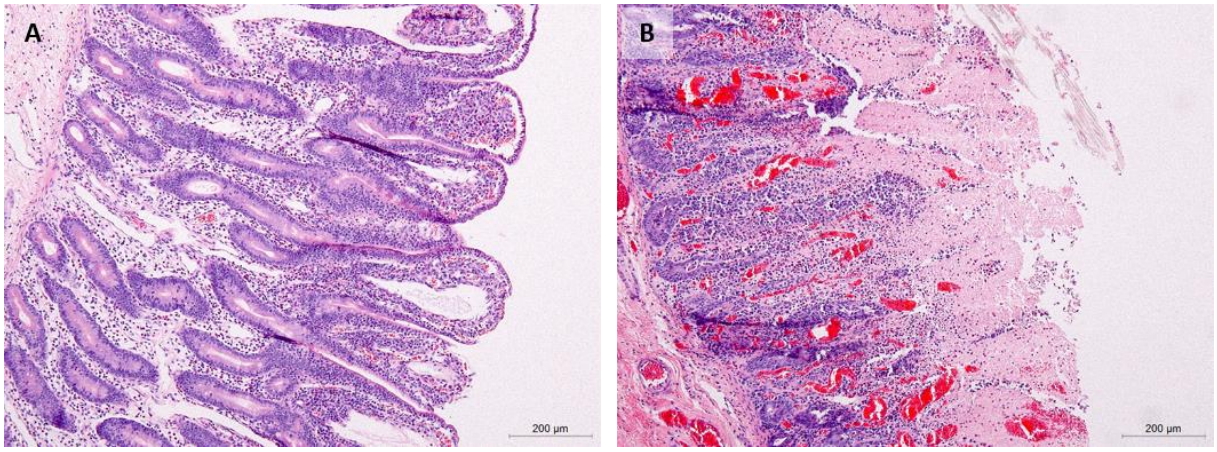


**Figure 2**

***C. perfringens*-induced necrosis in experimentally infected intestinal loops in calves.**

(A) Representative histological section from an intestinal loop without necrotic lesions. This loop was injected with *C. perfringens* in combination with antiserum to native *C. perfringens* toxins. (B) Representative section from an intestinal loop from the same calf, showing haemorrhage and extensive necrosis of the villi. This loops was injected with *C. perfringens* in combination with naive immune serum.

All positive control loops inoculated with *C. perfringens* developed necrosis. Injection of loops with *C. perfringens* together with sera from naive calves (pre-immune sera) also resulted in a high percentage of necrotic loops. Injection of *C. perfringens* together with antisera raised against native toxins resulted in significantly fewer necrotic loops as compared to the positive control loops ($p < 0.001$) and the loops injected with the pre-immune sera ($p < 0.01$). Antisera raised against formaldehyde inactivated toxoid (either L-lysine protected or commercial) were unable to significantly neutralize the necrosis-inducing activity of *C. perfringens* (Figures 2, 3).

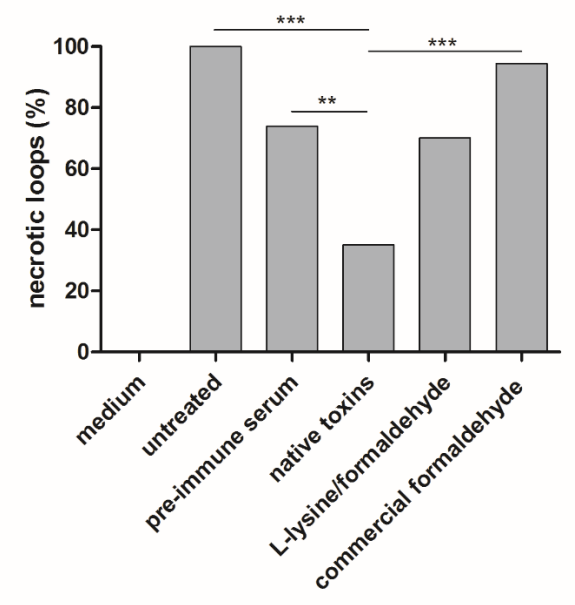


**Figure 3**

**Neutralization of the lesion-inducing potential of *C. perfringens*.**

The graph represents the percentage of ligated intestinal loops in which necrotic lesions were present after 5h of incubation with sterile culture medium (n=20), *C. perfringens* alone (untreated, n=20) or *C. perfringens* in combination with naive sera (pre-immune serum, n=60), antiserum to *C. perfringens* toxins (native toxins, n=20), formaldehyde inactivated, L-lysine protected *C. perfringens* toxins (L-lysine/ formaldehyde, n=20) and commercial formaldehyde inactivated multivalent clostridial vaccine (commercial formaldehyde, n=20).

** $0.001 \leq p < 0.01$ or *** $p < 0.001$

## Neutralization of alpha toxin and perfringolysin O activity *in vitro*

The inhibitory capacity of the sera towards alpha toxin and perfringolysin O activities was further examined using recombinant toxins. All antisera decreased the activity of alpha toxin *in vitro* (Table 2). Up to a final dilution of 409.8 the antisera against the native toxins neutralized 50% of the alpha toxin activity. To the contrary, in order to obtain the same inhibition of alpha toxin activity, antisera against L-lysine protected, formaldehyde inactivated toxoid or against the commercial formaldehyde inactivated clostridial vaccine could only be diluted up to final dilutions of 80.47 or 22.39, respectively.

The haemolytic activity of perfringolysin O towards equine erythrocytes *in vitro* was decreased by all antisera (Table 2). Up to a final dilution of 18 either antiserum inhibited the perfringolysin O activity completely. This neutralizing ability of the sera was observed up to a final dilution of 48 for the anti-native toxins antisera, a final dilution of 72 for the anti-L-lysine protected,

formaldehyde inactivated toxoid or a final dilution of 18 when the antiserum obtained after vaccination with the commercial vaccine was used. The pre-immune sera had no effect on the alpha toxin or perfringolysin O activity *in vitro*.

## Neutralization of *C. perfringens* cytotoxicity on bovine endothelial cells

To determine whether the cytotoxic activity of *C. perfringens* could be inhibited by the antisera to the vaccines, *C. perfringens* supernatants were incubated with serial dilutions of the antisera. Exposure of the endothelial cells to untreated *C. perfringens* supernatant resulted in 100% cell death. More than 80% cell viability could be measured by pre-incubation of the *C. perfringens* supernatant with a 32-fold dilution of the native toxins antiserum. At this concentration, neither the antisera raised against L-lysine protected, formaldehyde inactivated toxins nor the antisera raised against the commercial formaldehyde inactivated clostridial vaccine had an effect on the cytotoxicity (Table 2). The pre-immune sera had no effect on the *C. perfringens* cytotoxicity.

**Table 2**

***In vitro* neutralization of biological activities of alpha toxin and perfringolysin O and the *C. perfringens* cytotoxicity on bovine endothelial cells.**

Calves were immunized with either a *C. perfringens* toxin preparation (native toxins), L-lysine protected, formaldehyde inactivated *C. perfringens* toxins (L-lysine/formaldehyde toxoid) or a commercial multivalent formaldehyde inactivated clostridial vaccine. Alpha toxin activity was determined by measuring its lecithinase activity on egg yolk lipoproteins. Perfringolysin O (PFO) activity was determined by measuring the hemolysis of horse erythrocytes. The cytotoxicity of *C. perfringens* supernatant to primary bovine endothelial cells was measured using a neutral red uptake (NRU) assay.

| Antiserum | Inhibitory capacity (Mean ± SEM) | | |
|---|---|---|---|
| | Alpha toxin activity[a] | PFO activity[b] | *C. perfringens* cytotoxicity[c] |
| **Native toxins** | 409.8 ± 5.75 | 48.0 ± 0.0 | 32.00 ± 0.0 |
| **L-lysine/formaldehyde toxoid** | 80.47 ± 46.93 | 72.0 ± 24.0 | 9.00 ± 7.0 |
| **Commercial formaldehyde vaccine** | 22.39 ± 2.17 | 18.0 ± 6.0 | 4.00 ± 0.0 |

[a]Neutralization of 10 µg/ml alpha toxin. The inhibitory capacity of the antiserum is expressed as the dilution that gives 50% inhibition of the alpha toxin activity.
[b]Neutralization of 2 µg/ml perfringolysin O. The inhibitory capacity of the antiserum is expressed as the highest dilution that inhibited perfringolysin O-induced hemolysis.
[c]The inhibitory capacity of the antiserum is expressed as the highest dilution that yields 80% cell viability.

## Discussion

Necro-haemorrhagic enteritis caused by *C. perfringens* in suckling and veal calves is characterized by sudden death. Due to the very rapid course of the disease, curative treatment is not possible and therefore, protection by vaccination would be of high value. The virulence of *C. perfringens* is due to the many extracellular toxins it produces. In this study we showed that toxin neutralizing antibodies protect against *C. perfringens*-induced necrotic lesions in an intestinal loop assay and are able to prevent endothelial damage. Western blot analysis revealed antibodies towards alpha toxin and perfringolysin O as the most abundant antibodies in the immune sera from calves vaccinated against *C. perfringens* toxins.

We previously reported congestion and leakage of the capillaries as an early event in the pathogenesis of necro-haemorrhagic enteritis as shown in an intestinal loop assay[31]. Furthermore we showed that alpha toxin and perfringolysin O may exert their effect by directly targeting the endothelial cells[33]. This points towards endothelial damage as a key event in the pathogenesis of bovine necro-haemorrhagic enteritis. Indeed, in the present study antisera which protected against *C. perfringens*-induced cytotoxicity on bovine endothelial cells also offered protection against *C. perfringens*-associated necrosis in an intestinal loop assay. Moreover, the protective antisera were shown to inhibit the activity of alpha toxin and perfringolysin O, which further underscores the roles of these toxins in the pathogenesis of bovine necro-haemorrhagic enteritis. It can, however, not be ruled out that antibodies induced against other substances present in the vaccines also played a role in the protection observed in the intestinal loop model.

Formaldehyde inactivation of *C. perfringens* toxins diminished their capacity to induce protective antibodies. Antisera raised against L-lysine protected, formaldehyde inactivated C. perfringens toxins were also not protective in the intestinal loop model. This result is in disagreement with previous studies showing high antigenicity, low toxicity, and protection in mice that were immunized with L-lysine protected, formaldehyde inactivated toxoid and subsequently challenged with lethal doses of *C. perfringens*[8,25]. In the present study we demonstrated that vaccinations with *C. perfringens* toxins, either in their native forms or as formaldehyde inactivated toxoids, all resulted in high antibody responses as detected by ELISA. However, only serum derived from animals immunized with the native toxins offered protection against necrosis in an intestinal loop assay. There is thus a discrepancy between the antibody titres against formaldehyde inactivated *C. perfringens* toxins measured by ELISA and the

protective capacity of these antibodies in the intestinal loop model. Nevertheless, the value of vaccines based on formaldehyde inactivated *C. perfringens* toxins has been demonstrated for diseases associated with toxins other than alpha toxin and perfringolysin O[6,7,27,30]. This suggests that the protective immunogenicity of other *C. perfringens* toxins, such as, amongst others, NetB and epsilon toxin, is not affected by formaldehyde inactivation.

Although the use of *C. perfringens* native toxins represents an efficient strategy for vaccine development, active toxins cannot be regarded as safe. Therefore methods for the development of toxoids other than formaldehyde inactivation are needed. Possible strategies include the use of genetically modified toxoids based on site-directed mutants with reduced toxic activity or the use of immunologically active fragments of the essential toxins. Immunization with the carboxy-terminal domain of alpha toxin has previously been shown to provide protection in a mouse model against *C. perfringens* gas gangrene and may be a good candidate for development of a vaccine against bovine necro-haemorrhagic enteritis[25,34]. The identification of the structural elements responsible for membrane interaction of perfringolysin O provides opportunities for the development of non-toxic site-directed mutants as alternatives for native perfringolysin O[22].

In order to obtain the ultimate evidence that vaccination against *C. perfringens* toxins protects against bovine necro-haemorrhagic enteritis, field trials need to be performed. However, since necro-haemorrhagic enteritis is a low incidence disease, this would be a huge cost and more evidence concerning the immune-protective potential of the antisera is needed before considering this type of trial. Unfortunately, no *in vivo* model to validate the protective immune-potential of the candidate vaccines against bovine necro-haemorrhagic enteritis is available. Niilo and colleagues were able to induce a mild diarrhoea in cattle inoculated intraduodenally or *per os* with *C. perfringens* type A cultures, but no necro-haemorrhagic enteritis was established[17]. Also we were unable to develop a reliable model of bovine necro-haemorrhagic enteritis after *per os* or intraduodenal administration of *C. perfringens* type A cultures (unpublished results).

## Conclusion

This study showed that toxin-neutralizing antibodies protect against *C. perfringens* challenge in an intestinal loop model for bovine necro-haemorrhagic enteritis. Immunization of calves with either native or formaldehyde inactivated toxins resulted in a strong immune response

against alpha toxin and perfringolysin O, but only antibodies raised against native toxins were protective in the intestinal loop model. Therefore it seems that, at least for alpha toxin mediated diseases, antibody titres detected by ELISA are not a guarantee for protection, even if protection against the disease is antibody mediated.

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# 6

# The C-terminal domain of *Clostridium perfringens* alpha toxin as a vaccine candidate against bovine necro-haemorrhagic enteritis

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## Abstract

Bovine necro-haemorrhagic enteritis is caused by *Clostridium perfringens* and leads to sudden death. Alpha toxin, together with perfringolysin O, has been identified as the principal toxin involved in the pathogenesis. We assessed the potential of alpha toxin as a vaccine antigen. Using an intestinal loop model in calves, we investigated the protection afforded by antisera raised against native alpha toxin or its non-toxic C-terminal fragment against *C. perfringens*-induced intestinal necrosis. Immunization of calves with either of the vaccine preparations induced a strong antibody response. The resulting antisera were able to neutralize the alpha toxin activity and the *C. perfringens*-induced endothelial cytotoxicity *in vitro*. The antisera raised against the native toxin had a stronger neutralizing activity than those against the C-terminal fragment. However, antibodies against alpha toxin alone were not sufficient to completely neutralize the *C. perfringens*-induced necrosis in the intestinal loop model. The development of a multivalent vaccine combining the C-terminal fragment of alpha toxin with other *C. perfringens* virulence factors might be necessary for complete protection against bovine necro-haemorrhagic enteritis.

## Introduction

*Clostridium perfringens* is a Gram-positive, spore-forming, anaerobic bacterium. It is a normal component of the intestinal microbiota of animals, including humans. It secretes several toxins and enzymes that cause different forms of tissue damage[27,30,33]. Consequently, it can cause a variety of diseases in various vertebrates[30]. The differences in virulence properties between *C. perfringens* isolates are largely due to differences in toxin production. Alpha toxin and perfringolysin O have been identified as the principal toxins involved in gas gangrene caused by *C. perfringens* as well as in bovine necro-haemorrhagic enteritis[39]. Gas gangrene is a frequently lethal histotoxic infection of humans and animals characterized by rapid tissue destruction and impaired immune response[21,35]. Bovine necro-haemorrhagic enteritis (bovine enterotoxaemia) is an enteric disease of veal calves and beef type suckling calves and is characterized by haemorrhagic to necrotizing enteritis. Calves often die without premonitory signs[17,22,23,39].

We recently showed that vaccination of calves with a mixture of native toxins from *C. perfringens* induces antibodies that protect against *C. perfringens* challenge in an intestinal loop model of bovine necro-haemorrhagic enteritis (Chapter 5). Although both alpha toxin and perfringolysin O are involved in the pathogenesis of gas gangrene, immunization against alpha toxin alone provides good protection against experimental gas gangrene[32,34,35]. Moreover, Evans showed that antiserum raised against alpha toxin was highly effective in protecting guinea pigs against experimental gas gangrene, whereas antiserum to perfringolysin O was not protective against *C. perfringens* type A infection, and it did not enhance the protective action of alpha toxin antiserum[4]. Studies on gas gangrene cannot be directly extrapolated to bovine necro-haemorrhagic enteritis, but these findings indicate that alpha toxin vaccines could provide protection against diseases in which alpha toxin is critically important.

Here, we tested vaccine preparations based on alpha toxin, the major toxin produced by *C. perfringens* type A. Since native toxins are not safe, we used the enzymatically inactive C-terminal domain of alpha toxin ($Cpa_{247-370}$). This component is non-toxic and has been shown to provide protection against *C. perfringens* type A gas gangrene in a mouse model, and it is known to elicit protective immunity against a broad range of clostridial phospholipase C toxins[25,34,41]. In addition, mice vaccinated with $Cpa_{247-370}$ were protected against challenge with alpha toxin derived from a calf necro-haemorrhagic enteritis isolate[7].

The aim of this study was to evaluate whether the non-toxic C-terminal fragment of alpha toxin could be a candidate for effective vaccination of calves against bovine necro-haemorrhagic enteritis.

## Materials and methods

All experimental protocols were approved by the ethics committee of the Faculty of Veterinary Medicine, Ghent University (EC2011/024, EC2012/056, EC2013/38, EC2013/39 and EC2013/187). All animal experiments were carried out in accordance with the approved guidelines.

### Bacterial strains

The *C. perfringens* strains were wild-type strain JIR325, the *plc* mutant JIR4107 (Δ*plc*), and the *C. perfringens* JIR4107 derivatives carrying either the *plc*$^{+}$ plasmid (complemented strain JIR4121) or the empty shuttle vector (complementation control JIR4120) (Table 1)[1,20].

**Table 1**

***C. perfringens* strains used in the study.**

| Strain[a] | Strain number | Phenotype | Origin | Toxin genes | Alpha toxin (*$10^{-3}$ U/ml) mean ± SEM | Ref. |
|---|---|---|---|---|---|---|
| Wild-type | JIR325 | Wild-type | Strain 13[b] | *plc* | 31.392 ± 0.079 | 16 |
| Δ*plc* | JIR4107 | Alpha toxin-deficient | JIR325 Δ*plc* | | < 0.8 | 17 |
| Δ*plc* (shuttle vector) | JIR4120 | Alpha toxin-deficient with shuttle vector | JIR4107(pJIR418) | | < 0.8 | 17 |
| complemented | JIR4121 | Alpha toxin-complemented | JIR4107(pJIR443) | *plc* | 28.32 ± 0.38 | 17 |

[a]*plc*: alpha toxin gene

[b]JIR325: a rifampicin and nalidixic acid-resistant derivative of strain 13, a *C. perfringens* strain originally isolated from soil.

The strains were cultured anaerobically at 37 °C in Brain Heart Infusion broth (BHI, Oxoid, Basingstoke, UK) containing 0.375% glucose. To culture JIR4120 (Δ*plc*; (shuttle vector)) and JIR4121 (complemented strain), the medium was supplemented with chloramphenicol (30 µg/mL). The logarithmic phase cultures used in intestinal loop experiments did not contain

antibiotics. To determine the alpha toxin concentration in the culture supernatant, cell-free supernatants were obtained by centrifugation followed by filtration of the supernatants through a 0.22-µm filter. The alpha toxin concentration in the bacterial supernatants was measured using the Bio-X α-toxin ELISA kit (Bio-X Diagnostics, Jemelle, Belgium) and twofold serial dilutions of the alpha toxin standard ($220 \times 10^{-3}$-$0.8 \times 10^{-3}$ U/mL of phospholipase C type I; Sigma-Aldrich, St Louis, MO, USA) as previously described[42].

## The role of *C. perfringens* alpha toxin in the induction of necrotic lesions in an intestinal loop model

To confirm the role of alpha toxin in the induction of necrotic lesions in an intestinal loop model, seven intestinal loop experiments were conducted using the wild-type *C. perfringens* strain JIR325 and the alpha toxin-deficient strain *C. perfringens* JIR4107. In two of the experiments, the *C. perfringens* JIR4107 derivatives carrying the empty shuttle vector (JIR4120) or the *plc*$^{+}$ plasmid (JIR4121) were also included. The number of loops injected with each strain is shown in Table 2. In each calf, an equal number of control loops were injected with sterile bacterial growth medium supplemented with milk replacer. The experiments were performed according to a published protocol using seven healthy male Holstein Friesian veal calves aged 3-5 months[38]. Briefly, the calves were anesthetized and the small intestine was exteriorized. The loops were ligated and injected with logarithmic phase cultures combined with 25% commercial milk replacer (Vitaspray, Nuscience Drongen, Belgium) in sterile 0.9% NaCl solution, as described[38]. The animals were kept under anaesthesia for five hours after inoculation, after which they were euthanized and samples were taken. Intestinal loop tissue samples were submerged in 4% (w/v) phosphate buffered formaldehyde. After fixation for 24 h, the samples were processed routinely, embedded in paraffin wax, sectioned, and stained with haematoxylin and eosin. Sections were evaluated in a blinded manner by a board certified pathologist for the presence of tissue necrosis (0 = absence of necrosis, 1 = necrotic lesions present).

## Preparation of recombinant alpha toxin

Alpha toxin was expressed in *Escherichia coli* using the pBAD TOPO® TA Expression Kit (Invitrogen, Paisley, UK). A fragment encoding the *C. perfringens* alpha toxin (*plc* gene; GenBank accession number BAB79742) was amplified from the DNA of *C. perfringens* JIR325 by PCR using a DNA polymerase with proofreading activity (Accuzyme, Bioline, Randolph,

MA, USA). The forward primer (5'- G **TGA** GAG GAG GAT ATA AAA **ATG** AAA AGA AAG ATT TGT AAG GCG -3') contained an in-frame stop codon and translation re-initiation sequence to remove the N-terminal leader and allow native protein expression. The reverse primer (5'- G TTT CTT TTT TAT ATT ATA AGT TGA ATT TCC TGA AAT CCA CTC -3') excluded the native *plc* gene stop codon and included the C-terminal V5 epitope and polyhistidine region for affinity purification. The resulting PCR product was incubated with *Taq* polymerase for 10 min at 72 °C (5 U; Promega, Madison, WI, USA) to add 3' A-overhangs, cloned into the pBAD-TOPO expression vector, and transformed into One Shot TOP10® *E. coli*. The correct orientation of the alpha toxin insert was verified by Sanger sequencing.

*E. coli* carrying the pBAD-alpha toxin vector was grown at 37 °C to an $OD_{600}$ of 0.4–0.5 in Terrific Broth supplemented with 100 µg/mL ampicillin. Expression of recombinant *C. perfringens* alpha toxin was induced for 4 h by adding L-arabinose to a final concentration of 0.002% (w/v). Bacteria were harvested by centrifugation and lysed enzymatically using BugBuster (Invitrogen). Alpha toxin was purified on a Ni-sepharose column (His Gravitrap, GE Healthcare Bio-Sciences AB, Uppsala, Sweden) according to the manufacturer's instructions. Subsequently, the protein was dialyzed against PBS, purity was analysed using SDS-PAGE, and protein concentration was measured using BCA protein assay (Thermo Fisher Scientific, Waltham, MA, USA).

**Table 2**

**The number of loops inoculated with each strain in the intestinal loop experiments to evaluate the role of *C. perfringens* alpha toxin in the induction of necrotic lesions.**

Seven intestinal loop experiments were conducted. The number of intestinal loops that were injected per animal are shown.

| Calf | Replicate loops/strain[a] | | | | |
|---|---|---|---|---|---|
| | JIR325 | JIR4107 | JIR4120 | JIR4121 | BHI |
| **1** | 3 | 3 | / | / | 3 |
| **2** | 3 | 3 | / | / | 3 |
| **3** | 3 | 3 | / | / | 3 |
| **4** | 5 | 5 | / | / | 5 |
| **5** | 5 | 5 | / | / | 5 |
| **6** | 5 | 5 | 5 | 5 | 5 |
| **7** | 5 | 5 | 5 | 5 | 5 |

[a]JIR325: wild-type *C. perfringens*; JIR4107: alpha toxin-deficient; JIR4120: alpha toxin-deficient strain carrying the empty shuttle vector; JIR4121: alpha toxin-complemented strain; BHI: sterile bacterial growth medium.

## Vaccine preparation and immunization

The recombinant carboxy-terminal domain of alpha toxin fused to glutathione-S-transferase (GST) was kindly provided by Prof. Titball, University of Exeter, UK. This $Cpa_{247-370}$ was produced in *E. coli* and was therefore devoid of any other *C. perfringens* proteins [37]. Recombinant native alpha toxin (rCpa) and $Cpa_{247-370}$ were formulated with the adjuvant QuilA (Brenntag Biosector, Frederikssund, Denmark) in PBS. Each animal was injected with 1.5 mL of the filter-sterilized (0.2 µm) formulation containing 350 µg antigen and 750 µg QuilA. Control animals received 750 µg QuilA in 1.5 mL PBS.

Six male Holstein Friesian calves aged two months were used. They were housed on straw and received water and hay *at libitum*, and concentrates adjusted to the body weight.

For each antigen (rCpa, $Cpa_{247-370}$ or QuilA control), two calves were immunized subcutaneously in the neck. The calves received a primer vaccination at the age of two months, and booster immunizations 14 and 28 days later.

## Enzyme-linked immunosorbent assay

The immune response following vaccination was measured using serum samples obtained two weeks after the final booster immunization. Alpha toxin-specific antibody levels were determined by the end-point dilution method using a blocking ELISA (*C. perfringens* alpha toxin serological ELISA kit, Bio-X Diagnostics). Sera were used at a dilution 1:50 and assays were performed in duplicate. The specific antibody level was expressed as percent inhibition according to the following formula: % inhibition = [(OD negative – OD sample)/OD negative] × 100.

## Neutralization of the haemolytic activity of wild-type *C. perfringens* JIR325 alpha toxin on blood agar plates in vitro

Incubation of cell-free supernatants of the wild-type strain JIR325 (concentrated tenfold using Vivaspin, Sartorius Stedim Biotech GmbH, Göttingen, Germany) on sheep blood agar at 37 °C overnight results in an inner, complete zone of haemolysis caused by perfringolysin O and a less complete outer zone caused by alpha toxin. The sera's ability to neutralize alpha toxin activity was assessed by incubating the JIR325 supernatant with an equal volume of the pooled sera from the two animals that were vaccinated with either a given vaccine or the adjuvant

QuilA for 30 min at 37 °C. Ten-microliter drops of these mixtures were spotted on sheep blood agar and haemolysis was assessed after overnight incubation. The test was performed in triplicate using supernatants of *C. perfringens* JIR325 from three independent biological replicates.

## Neutralization of alpha toxin activity on egg yolk lipoproteins

Alpha toxin activity was determined in duplicate in a 96-well microtitre plate by evaluating its effect on egg yolk lipoproteins as previously described [19]. The neutralizing ability of sera was assessed by pre-incubating a twofold dilution series of the sera (two wells per dilution) with a constant amount of alpha toxin (10 µg/mL in PBS) for 30 min at 37 °C before adding 10% egg yolk emulsion. To prepare the yolk emulsion, fresh egg yolk was centrifuged (10 000 × *g* for 20 min at 4 °C) and diluted 1:10 in PBS. After incubation of the 96-well plates at 37 °C for 1 h, absorbance was measured at 650 nm. Alpha toxin activity was indicated by the development of turbidity, which increases absorbance. The inhibitory capacity of the antiserum was expressed as the serum dilution that inhibited 50% of the alpha toxin activity. This was determined by applying a Hill function to the concentration-response data (GraphPad Prism 5, GraphPad Software, San Diego, CA, USA). The test was performed in duplicate.

## Neutralization of *C. perfringens* cytotoxicity to bovine endothelial cells

Primary bovine umbilical vein endothelial cells (BUVEC) were isolated from umbilical cord veins by an adapted procedure[39] based on the method of Jaffe et al.[10]. The toxicity of *C. perfringens* supernatant to cultured bovine endothelial cells has been reported[39]. The ability of the antisera to neutralize the *C. perfringens* cytotoxicity to BUVECs was determined using a Neutral Red Uptake assay (NRU)[29]. Briefly, BUVEC cells were seeded in 96-well tissue culture plates at a density of $10^5$ cells per well and cultured for 24 h to obtain cells in the exponential growth phase. The neutralizing ability of the sera was assessed by pre-incubating a twofold dilution series of the sera (100% to 0.4%) prepared in serum-free cell culture medium with an equal volume of undiluted *C. perfringens* supernatant. After 30 min at 37 °C, the cells were treated for 2 h with 100 µL of the supernatant-serum mixture, followed by a standard NRU assay. The inhibitory capacity of the antiserum was expressed as the last dilution associated with 100% cell viability. As a positive control, cells were treated with *C. perfringens*

supernatant pre-incubated with serum-free medium. Untreated cells incubated with serum-free medium served as a negative control. The test was performed in duplicate.

## Neutralization of necrotic lesion development in the intestinal loop model

To study the protection against *C. perfringens*-induced necrosis provided by the antisera obtained from calves vaccinated with the respective vaccines, three intestinal loop experiments were performed using three male Holstein Friesian calves aged three months. In each of the three intestinal loop experiments, the sera for each vaccine were pooled. Intestinal loops were inoculated with a wild-type strain (JIR325) combined with 25% commercial milk replacer suspended in sterile NaCl solution. Before inoculation, serum from calves immunized with the different vaccine preparations was added to the NaCl solution containing milk replacer to a final concentration of 6% serum (v/v). In each calf, five intestinal loops were injected with anti-Quil A, five with anti-native alpha toxin, and five with anti-C-terminal fragment of alpha toxin. Moreover, five control loops per calf were injected with *C. perfringens* without addition of serum (positive control) and five with sterile bacterial growth medium (negative control). This totalled 25 injected loops per calf. Samples were collected and scored as described for the intestinal loop experiments using the alpha toxin-deficient strain.

## Statistical analysis

Differences in the development of necrotic loops between the wild-type and the mutant *C. perfringens* strains were analysed using multivariable logistic regression. The protective effect of the different antisera against development of intestinal necrosis in the loop model was determined by multivariable logistic regression. To account for clustering of loops within a calf, a fixed factor was included describing in which calves the experiments were performed. Significance was set at $p < 0.05$ and analyses were performed in SPSS v. 22.0 (IBM Corporation, New York, USA). Results were reported as means and standard errors of the means (SEM).

# Results

## *C. perfringens* alpha toxin-deficient strain has a decreased ability to cause necrotic lesions in an intestinal loop model

A wild-type strain and an alpha toxin-deficient strain (Δ*plc*) were tested in an intestinal loop model. The wild-type strain caused necrotic lesions in 62.1% (18/29) of the injected loops, whereas the alpha toxin-deficient strain induced necrosis in significantly fewer loops (3.4%; 1/29) ($p < 0.001$). To confirm the role of alpha toxin in lesion development by complementing the deficiency, the Δ*plc* derivatives carrying the empty shuttle vector (JIR4120) or the $plc^+$ plasmid (JIR4121) were used. Necrotic lesions were observed in only one of the ten (10%) loops injected with the alpha toxin-deficient strain carrying the empty shuttle vector. This is significantly fewer than in the loops inoculated with the wild-type strain (62.1%; $p = 0.008$). The *plc*-complemented strain induced necrotic lesions in 50% (5/10) of the loops, which is comparable to the effect of the wild-type strain. No lesions were detected in the control loops treated with sterile bacterial culture medium (Figures 1 and 2).

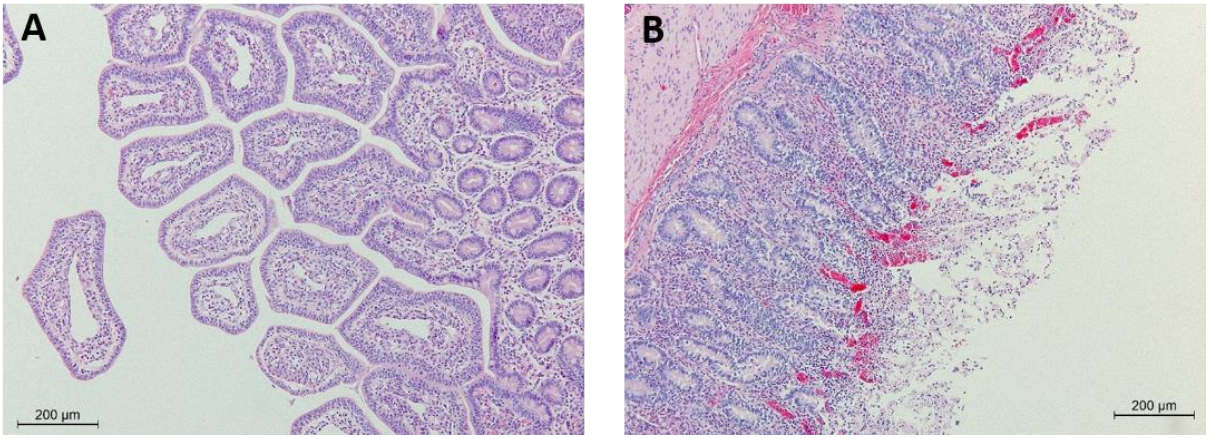


**Figure 1**

***C. perfringens*-induced necrosis in experimentally infected intestinal loops in calves.**

(A) Representative histological section from an intestinal loop injected with sterile bacterial growth medium. There are no lesions in this negative control loop. (B) Representative histological section from an intestinal loop injected with the wild-type *C. perfringens* strain, showing haemorrhages and necrosis of the villi. HE, bars 200 µm.

## Antibody responses against alpha toxin in calves

After vaccination with native alpha toxin, the non-toxic C-terminal domain of alpha toxin or the adjuvant QuilA, serum antibodies produced against native alpha toxin were analysed by ELISA. In all calves vaccinated with the native toxin or with the C-terminal domain, a strong antibody response against alpha toxin was detected six weeks after the first immunization. The calves vaccinated with the native toxin had antibody titres of 69.7 ± 7.8. Calves vaccinated with the non-toxic C-terminal domain of alpha toxin had antibody titres of 91.1 ± 1.6. No anti-alpha toxin response was measured in the calves vaccinated with the adjuvant QuilA.

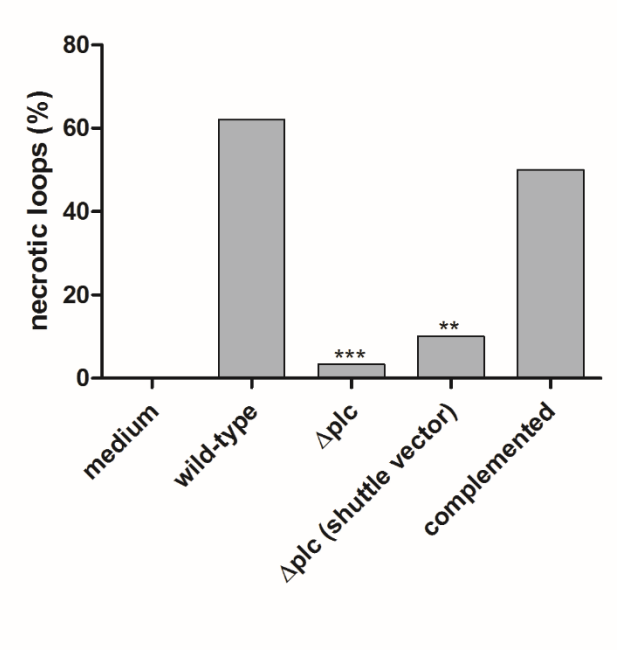


**Figure 2**
**Percentage of necrotic loops after *C. perfringens* inoculation.**

Intestinal loops inoculated with sterile cell culture medium ($n = 29$), the wild-type strain ($n = 29$), the alpha toxin-deficient strain (Δ*plc*) ($n = 29$), the alpha toxin-deficient strain carrying the empty shuttle vector (Δ*plc* (shuttle vector)) ($n = 10$) and the alpha toxin-complemented strain ($n = 10$) were histologically examined for the presence of tissue necrosis. The graph represents the percentage of loops in which necrotic lesions were present after 5 h of incubation with logarithmic stage cultures. ** $0.001 \leq P < 0.01$ and *** $P < 0.001$ indicate a significant difference relative to the loops inoculated with the wild-type strain.

## Neutralization of alpha toxin activity in vitro

Sheep blood agar was used to examine in vitro neutralization of alpha toxin activity of a wild-type *C. perfringens* strain by sera from calves immunized with the native alpha toxin (rCpa) or the non-toxic C-terminal fragment of the alpha toxin ($Cpa_{247-370}$). Plates treated with *C. perfringens* supernatant exhibited both the inner (perfringolysin O) and outer (alpha toxin) zones of haemolysis. Incubation of the supernatant with sera against either rCpa or $Cpa_{247-370}$ did not result in an outer zone of haemolysis, indicating neutralization of alpha toxin activity.

These sera had no effect on perfringolysin O activity. Incubation with sera from the control calves (QuilA) had no effect on *C. perfringens* toxin activities (Figure 3).

To determine whether the antisera against the vaccines can neutralize the lecithinase activity of alpha toxin, serial dilutions of the antisera were incubated with alpha toxin and its activity was measured on egg yolk lipoproteins. The sera of calves immunized with either the native alpha toxin (rCpa) or the C-terminal fragment of alpha toxin ($Cpa_{247-370}$) decreased the activity of alpha toxin, with an inhibitory capacity of respectively $1189.0 \pm 390.4$ for anti-rCpa or $323.8 \pm 133.3$ for the sera raised against $Cpa_{247-370}$. No effect on alpha toxin activity was observed after incubation with sera from calves immunized only with QuilA.

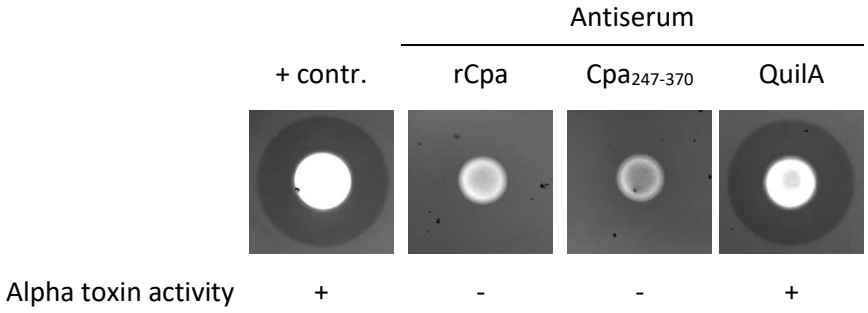


**Figure 3**

***In vitro* neutralization of the haemolytic activity of the alpha toxin of *C. perfringens*.**

Supernatants of *C. perfringens* (+ contr.) were either left untreated or were pre-incubated with serum from calves immunized with native alpha toxin (rCpa), the non-toxic C-terminal fragment of alpha toxin ($Cpa_{247-370}$) or the adjuvant QuilA. They were then spotted on sheep blood agar and incubated overnight at 37 °C. Neutralization of alpha toxin activity results in absence of an outer zone of haemolysis.
+ = no neutralization of toxin activity; - = complete neutralization of toxin activity.
Representative pictures of one out of three independent experiments.

## Neutralization of the cytotoxicity of *C. perfringens* to bovine endothelial cells by anti-alpha toxin antisera

To determine whether the antisera against the vaccines can inhibit the cytotoxicity of *C. perfringens*, serial dilutions of the antisera were incubated with *C. perfringens* supernatant. Exposure of the endothelial cells to untreated supernatant resulted in 100% cell death. Antisera raised against either the native alpha toxin (rCpa) or the C-terminal fragment of alpha toxin ($Cpa_{247-370}$) protected the endothelial cells from *C. perfringens* cytotoxicity. Sera from the control calves did not neutralize the *C. perfringens*-induced cytotoxicity. Pre-incubating the *C. perfringens* supernatant with a 288-fold dilution (±96) of the native alpha toxin antiserum

resulted in 100% neutralization of cytotoxicity, whereas a 32-fold dilution (±0.0) of the antiserum against the C-terminal fragment ($Cpa_{247-370}$) was needed to neutralize the cytotoxicity.

## Protective effect of anti-alpha toxin antisera against *C. perfringens*-induced necrosis in an intestinal loop model

Neutralization of the lesion-inducing potential of *C. perfringens* by sera raised against the respective vaccines was evaluated in the intestinal loop model. Thirteen of the fifteen (86.7%) positive control loops inoculated with *C. perfringens* developed necrosis. Injection of loops with *C. perfringens* combined with sera from control calves (immunized with the adjuvant QuilA) also resulted in a high percentage of necrotic loops (93.3% of the loops, 14/15). Injection of loops with *C. perfringens* combined with antisera raised against native alpha toxin (rCpa) resulted in significantly fewer necrotic loops as compared to the loops containing *C. perfringens* and the QuilA antisera ($p = 0.028$) and borderline significantly fewer necrotic loops as compared to the untreated loops ($p = 0.054$) (53.3% of the loops or 8/15). Antisera raised against the non-toxic C-terminal fragment of alpha toxin ($Cpa_{247-370}$) did not significantly neutralize the lesion-inducing ability of *C. perfringens* (10/15 or 66.7% necrotic loops) (Figure 4).

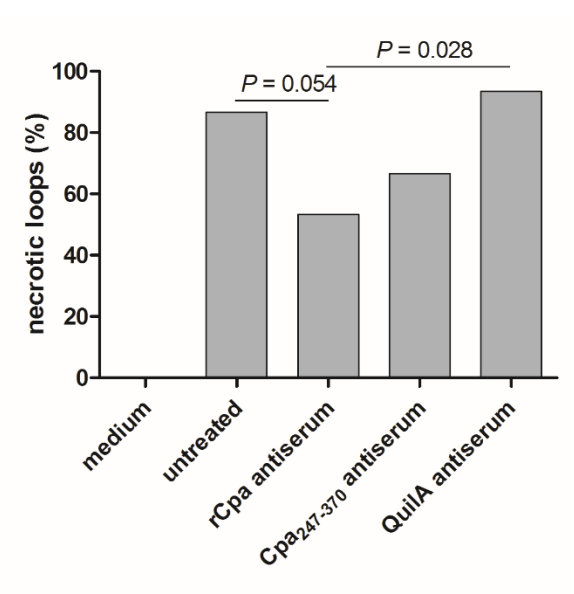


**Figure 4**
**Neutralization of the lesion-inducing potential of *C. perfringens*.**
The graph represents the percentage of intestinal loops in which necrotic lesions were present after 5 h of incubation with five treatments: sterile culture medium, *C. perfringens* alone (untreated) or *C. perfringens* in combination with 6% antiserum to either native alpha toxin (rCpa antiserum), antiserum to the non-toxic C-terminal fragment of alpha toxin (Cpa247-370 antiserum), or antiserum from calves immunized with adjuvant only (QuilA antiserum). The graph represents the data from three intestinal loop experiments (total of 15 loops per condition).

## Discussion

Alpha toxin is involved in the induction of necrotic lesions in a calf intestinal loop model and is thus an important toxin in the pathogenesis of enterotoxaemia. We previously showed that alpha toxin production by *C. perfringens* is required for intestinal virulence by using a double-mutant *C. perfringens* strain devoid of alpha toxin and perfringolysin O, which was complemented for perfringolysin O to generate an alpha toxin-deficient phenotype[39]. In the present study, we supported our earlier conclusions by using an alpha toxin-mutant strain. Mutant strains are frequently used to evaluate the virulence effect of *C. perfringens* genes. A mutant strain was used to show that NetB is crucial for the induction of avian necrotic enteritis[12]. This approach also identified beta toxin as an essential virulence factor of *C. perfringens* type C in infected rabbits[31]. Moreover, Awad *et al.* used mutant strains to demonstrate that both alpha toxin and perfringolysin O are involved in the pathogenesis of gas gangrene[1,2]. In our study, we confirmed that alpha toxin is required for intestinal virulence in a calf intestinal loop model. This conclusion was based on genetic evidence showing that an alpha toxin-deficient strain has a decreased ability to cause necrotic lesions in this model. The alpha toxin-complemented strain regained the ability to cause the disease, unambiguously fulfilling Falkow's molecular Koch's postulates[5].

In the present study, alpha toxin appeared to be a promising vaccine component against bovine necro-haemorrhagic enteritis. Antisera raised against native alpha toxin reduced the lesion-inducing potential of *C. perfringens* in the intestinal loop model. However, alpha toxin is a potent dermonecrotic toxin that is not safe for use in calves. Alpha toxin can be rendered safe by formaldehyde treatment, but a well-known problem of this treatment is that it might reduce protective immunogenicity[9,16,21,36]. Therefore, a recombinant *C. perfringens* alpha toxoid may be preferable to a formaldehyde toxoid. The immunogenicity of the C-terminal fragment of alpha toxin in calves was recently reported for the first time[11]. However, the ability of the antiserum derived after vaccination of calves with $Cpa_{247-370}$ to neutralize the toxicity of *C. perfringens* to bovine cells or bovine intestine has not been evaluated[11]. Here, we report that the non-toxic C-terminal domain of alpha toxin ($Cpa_{247-370}$) may be an effective alternative to the use of native alpha toxin. Indeed, calves immunized with the native alpha toxin or with the C-terminal domain of alpha toxin developed a strong immune response against alpha toxin. Nevertheless, compared to antisera against the native alpha toxin, sera from calves immunized with the C-terminal fragment of alpha toxin showed weaker inhibition of the alpha toxin activity

and weaker neutralization of the *C. perfringens*-induced endothelial cytotoxicity *in vitro*. Additionally, the lesion-inducing potential of *C. perfringens* in the intestinal loop model was significantly reduced only by co-administration of antisera from animals vaccinated with the native alpha toxin.

The diminished protection afforded by antisera against the C-terminal domain may be attributed to the GST tag fused to the C-terminal domain of alpha toxin for protein purification purposes. Distortion of the conformation of the alpha toxin fragment by the GST tag has already been suggested in a previous study reporting that the untagged fragment was more protective against experimental gas gangrene than the C-terminal fragment fused to the GST tag[41]. In contrast to the C-terminal fragment of alpha toxin, the recombinant native alpha toxin used in this study was fused to a HIS tag for purification. This HIS tag is substantially smaller than the GST tag and is less likely to influence the conformation of the alpha toxin. This construct might generate more antibodies against the conformational epitopes that are important for protection. Alternatively, it may be that, in addition to antibodies directed to the C-terminal fragment of alpha toxin, also antibodies against the N-terminal fragment are needed to provide protection. However, a previous study showed that immunization with the N-terminal domain of alpha toxin was not protective against experimental gas gangrene[41]. It is believed that membrane binding induces a conformational change in the N-terminal domain from the closed to open configuration, which could reduce the affinity of antibodies raised against the N-terminal domain and complicates the development of protective antibodies against this N-terminal region[3,28]. Moreover, the combination of both toxin domains as vaccine antigen is not straightforward because combination of both non-toxic fragments restores the biological activity of alpha toxin[24].

Total protection was not obtained even after vaccination with native alpha toxin. It is possible that not all alpha toxin was neutralized by the antisera, leaving residual active alpha toxin to exert cytotoxicity. We also do not know whether in the field serum antibodies leaking into the intestinal lumen after intestinal damage will be sufficient to inhibit alpha toxin and the induction of necrotic lesions. This should be tested in a subsequent study by performing intestinal loop experiments in immunized animals without adding antiserum to the ligated intestinal loops. It is possible that total protection against development of intestinal lesions also requires other neutralizing antibodies, for example, against perfringolysin O and/or other *C. perfringens* proteins. Therefore, other *C. perfringens* proteins in addition to alpha toxin and perfringolysin O might have to be incorporated in a vaccine to obtain complete protection. This is also the case

for avian necrotic enteritis, where NetB is essential to cause disease, but vaccination with NetB provides only partial protection against *C. perfringens* challenge[6,13,14].

Endothelial damage is probably a key event in the pathogenesis of bovine necro-haemorrhagic enteritis[38,39]. Initial epithelial damage could enable alpha toxin to penetrate the epithelial barrier and to act on endothelial cells. In addition to other infectious agents, such as coccidia, enteropathogenic bacteria, coronaviruses and rotaviruses, several *C. perfringens* factors can contribute to initial epithelial damage, such as collagenase (kappa toxin), hyaluronidase (mu toxin) and mucinase[8,15,18,40]. More research is needed to investigate the role of these factors in the pathogenesis of necro-haemorrhagic enteritis and the protective effect of neutralizing antibodies against these proteins.

In this study, we used the calf intestinal loop model to evaluate the vaccine potential of *C. perfringens* alpha toxin. Ideally, vaccinated animals should be challenged with crude toxins or bacterial cultures to obtain conclusive evidence that vaccination against *C. perfringens* alpha toxin protects against bovine necro-haemorrhagic enteritis. However, no challenge model for testing vaccine candidates in calves is yet available[11,26,38]. The intestinal loop model remains currently the best available model.

In conclusion, this study shows that the non-toxic C-terminal domain of alpha toxin is a promising antigen for vaccine development. Although antibodies against *C. perfringens* alpha toxin neutralize alpha toxin activity and *C. perfringens*-induced endothelial cytotoxicity *in vitro*, antibodies against alpha toxin alone are inadequate for complete neutralization of *C. perfringens*-induced necrosis in the intestinal loop model of bovine necro-haemorrhagic enteritis. The development of a multivalent vaccine combining the C-terminal fragment of alpha toxin with other, still unidentified, *C. perfringens* virulence factors might be necessary for complete protection against bovine necro-haemorrhagic enteritis.

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# PART IV
# General discussion

## A plea for a change of name for bovine enterotoxaemia

The term "enterotoxaemia" is widely applied to various diseases caused by *C. perfringens*, however it is appropriate only for diseases in which the major signs are caused by systemic actions of the toxins[36]. Previously, a better descriptive name for "haemorrhagic enterotoxaemia" caused by *C. perfringens* type C has been proposed to be "necrotic enteritis". This suggestion was based on the observation that the disease is not always haemorrhagic and, although the toxin may incidentally reach the circulation, it is produced in the intestine and exerts it major effects locally[36,58,65]. Also for bovine enterotoxaemia, the nomenclature may be confusing. Based on the neurologic signs, without the presence of major intestinal lesions, it should not be questioned whether bovine enterotoxaemia caused by *C. perfringens* type D is a true enterotoxaemia[15]. To the contrary, the situation is less clear for bovine enterotoxaemia caused by *C. perfringens* type A, exhibiting intestinal necrosis and haemorrhage, with systemic effects only sporadically being reported[28,31]. Furthermore, alpha toxin is rapidly metabolized when it enters the circulation, questioning its potential to cause systemic toxicity[42]. It is now known that the effects of TNF-α in the general circulation, rather than alpha toxin itself, are to blame for lethality[44]. In addition, destruction of the intestinal barrier by *C. perfringens* toxins may also allow other intestinal toxins (e.g. lipopolysaccharide of Gram-negative bacteria) to enter the circulation and to cause shock. Therefore, we prefer to describe this syndrome as **bovine necro-haemorrhagic enteritis**, thereby making a clear distinction between the pathologies caused by *C. perfringens* type A or D, and clearly describing the lesions caused by the pathogen.

## Immune development in the calf and its consequences for vaccination against bovine necro-haemorrhagic enteritis

At birth, the calf is immunologically naïve and depends on colostrum intake as an immediate source of antibodies from the mother. The ingestion of colostrum is essential for providing the neonatal calf with immunologic protection during at least the first 2 to 4 weeks of life[7,9]. In addition to maternal antibodies, colostrum also contains, amongst others, cytokines and leukocytes (predominantly macrophages, lymphocytes and neutrophils). The number of circulating B lymphocytes is greatly reduced in neonatal calves (B cells account for less than 5% of the colostral lymphocytes) and gradually increases to normal levels by 6 to 8 weeks of life[24,41]. Furthermore, neonatal calves are immunosuppressed by hormonal influences of

parturition: both absorbed immunosuppressive maternal hormones and production of high levels of cortisol by the calf. The low number of B cells, together with the cumulative effect of these hormones, results in a prolonged lack of endogenous antibody response[7,41]. This neonatal immuno-suppression is needed to prevent excessive inflammation following the sudden transition from a sterile environment in the womb to the external environment[12]. It allows the colonization of the intestinal tract by a diverse commensal population and the establishment of a symbiotic equilibrium, while maternal antibodies protect the neonate from pathogenic bacteria[7,12]. Although essential for the colonization with commensal organisms after parturition, the immunosuppression of the calf, together with the passively derived maternal antibodies, might impair immune responses to vaccination[38]. Upon immunization of young calves, antibodies of maternal origin bind to their specific epitopes at the antigen surface, competing with the calves' B cells and thus limiting B cell activation, proliferation and differentiation[52]. Maternal antibodies gradually decline over time, with different half-life for antibodies directed to different pathogens. Bovine necro-haemorrhagic enteritis is of economic importance mainly near the end of the fattening period[46]. Therefore, vaccination later in life, when maternal antibodies have disappeared, may be considered[68]. In this work, calves were vaccinated at the age of two months, when no maternal antibodies could be measured (**Chapter 5** and **6**). Although this may be an appealing strategy for the prevention of necro-haemorrhagic enteritis and potentially *C. perfringens* type A-associated disorders in adult cattle (e.g. HBS), it is not an option to prevent clostridial abomasitis in neonatal calves. In order to avoid interference with maternal antibodies, the antibody dynamics in calves have to be determined, before effective vaccination schedules can be developed. Alternatively, recent research is focused on immunization routes and adjuvants that break through maternal immunity, when immunizing young animals[37].

In order to develop an efficient vaccine for bovine necro-haemorrhagic enteritis, a better knowledge of the natural development of protective immunity towards *C. perfringens* and its toxins is needed. It is hitherto unknown how active immunity towards *C. perfringens* toxins (which are classified amongst the most potent bacterial toxins) is established without developing disease. In calves, it has been hypothesized that the development of active immunity to *C. perfringens* toxins may be influenced by the diet[68]. In calves raised for beef production, a fluent transition from passive to active immunity is achieved. In contrast, calves raised for veal production do not develop active immunity until the age of at least 26 weeks[68]. The major difference between these production systems is the diet[46,68]. Under conventional circumstances

(as in beef production), new-born calves go through a pre-ruminant phase of 2 to 3 weeks of age where they rely almost entirely on a liquid diet for their nutrients[11]. After this phase, calves begin to consume enough dry feed and the rumen starts to develop. Furthermore, ingestion of dietary fibres results in an increase in colon viscosity and bulking of faecal matter and has an influence on microbiota composition[53]. On the other hand, calves raised for veal production are raised on low-iron all liquid diets to obtain the desired white meat (low iron uptake) and are offered only negligible amounts of dry feed, which counteracts the development of the rumen[46,68]. These dietary differences will inevitably lead to differences in the composition of both the ruminal (altered ruminal microbiota in veal calves) and the intestinal microbiota. It is tempting to speculate that the development of a healthy microbiota, either in the rumen or the large intestine, supports an environment which allows the controlled proliferation of *C. perfringens* and concomitant toxin production. Part of the produced toxins may be absorbed in the blood and processed by the immune system, leading to immune development[28]. This situation might be different in calves raised for veal production. One possible explanation for the lack of active immune development may be *C. perfringens* passing through the intestine without being present for a sufficient period to allow absorption of toxins. Another possibility may be that factors in the diet suppress the production of *C. perfringens* toxins in the large intestine and consequently the immune response to these antigens. Indeed, when grown in the presence of either milk or commercial milk replacer, the production of the main toxins of *C. perfringens* type A is reduced (unpublished results). In fact, this reduction in toxin production is more pronounced with higher concentrations of milk, without affecting the growth of *C. perfringens*. Further research is needed to elucidate the effect of nutrition on the intestinal microbiota composition and the presence of *C. perfringens* in particular. Also the effect of the diet on the *C. perfringens* toxin production *in vivo* remains to be elucidated. In addition to a possible dietary influence, also the genetic background may influence the sensitivity to bovine necro-haemorrhagic enteritis. Differences in disease susceptibility between cattle breeds are frequently reported and may be due to differences in the immune response. Despite the generally higher resistance of Holstein-Friesian cattle to *Psoroptes ovis*-induced psoroptic mange, experimental infection of either Holstein-Friesian or Belgian Blue cattle revealed largely similar immunologic responses[49]. Also the innate immune response of two cattle breeds with different prevalence of mastitis was very similar after intra-mammary infection with either *Staphylococcus aureus* or *Escherichia coli*, two common mastitis-inducing pathogens[3,4]. No reports were found where the immune response of different cattle breeds to *C. perfringens*

toxins was described. However, genetically different chicken lines showed a highly divergent immune responses to *C. perfringens* alpha toxin exposure[61]. This demonstrates that the genetic background can indeed have an influence on the immune response. A possible breed influence on the immune response to *C. perfringens* toxins and concomitantly therewith the increased susceptibility to necro-haemorrhagic enteritis should be further investigated.

## Towards a better understanding of current clostridial vaccines as protective measure against bovine necro-haemorrhagic enteritis

Clostridial diseases are common and often rapidly fatal. Since eliciting (dietary) factors are not completely understood and often cannot be avoided in contemporary intensive rearing systems, vaccination is a necessary strategy to control these diseases. Most of the currently marketed vaccines combine (antigens from) several clostridial species, including *C. perfringens*. Despite the fact that these vaccines are licenced for immunization of cattle and the efficacy of these vaccines has been documented in sheep[1], a limited efficacy of these vaccines against bovine necro-haemorrhagic enteritis has been reported (empirical observations from the field in Belgium). No published reports are available describing the efficacy of these vaccines in cattle. In **chapter 5**, it was shown that vaccination with a commercially available multivalent clostridial vaccine resulted in a strong antibody response against both *C. perfringens* alpha toxin and perfringolysin O, the main toxins produced by type A strains. However, antisera from calves immunized with this vaccine were not able to protect against intestinal necrosis when co-injected with *C. perfringens* in bovine intestinal loops, confirming the empirical observations from the field. Several explanations for this lack of protective antibody response in cattle can be suggested.

First, immunological, physical, and/or chemical interactions between the combined components can alter the immune response against specific components[54]. Although there are clear practical benefits of vaccines containing multiple antigens (up to 10 antigens for current clostridial vaccines), there is a risk that the efficacy or safety of the combination is less than that seen with the single antigen vaccines. Indeed, calves vaccinated with the multivalent clostridial vaccine developed more pronounced injection site lesions than calves vaccinated with *C. perfringens* toxins alone (**chapter 5**). This tissue injury and inflammation at the site of injection is suspected to lead to a decreased feed consumption, which may impact animal

growth and productivity[19,60,64,75]. It should be noted that different adjuvants were used in the commercial vaccine formulation (aluminium salt) or in the experimental vaccines (Quil A). It is hitherto unclear whether the differences in development of injection site lesions may be attributed to the number of antigens that are combined in the vaccine or to the choice of the adjuvant that has been used. Nevertheless, all calves developed high antibody titres towards the main *C. perfringens* type A toxins. These results suggest that, although the multivalent clostridial vaccine may cause more adverse reactions (either by combination of multiple clostridial toxoids in one vaccine or by use of an aluminium adjuvant), it does not hamper the ability to develop antibodies towards *C. perfringens*.

Another explanation for the lack of protection of the antisera derived from calves vaccinated with the multivalent clostridial vaccine is the method used to produce the toxoid. All current clostridial vaccines are made from culture supernatants which are inactivated, mostly using formaldehyde. The detoxification process of the bacterial toxins could have effects on antigenicity and immunogenicity[34]. Numerous chemical modifications occur in proteins during the treatment with formaldehyde, thereby altering the conformation of the protein[34,35]. Formaldehyde inactivation is unlikely to have an effect on the antibody titres. However, when antibodies against conformational epitopes are important for protection, it cannot be excluded that the antibodies evoked by the toxoid will be unable to neutralize the activity of the native toxin. Indeed, when compared to antisera from calves vaccinated with native *C. perfringens* toxins, antisera from calves immunized with either the multivalent formaldehyde inactivated clostridial vaccine or with formaldehyde inactivated *C. perfringens* toxins alone, were less able to neutralize the alpha toxin activity or *C. perfringens*-induced cytotoxicity *in vitro* (**chapter 5**). This observation points towards the formaldehyde inactivation process of current clostridial vaccines as the most plausible explanation for their inability to protect against bovine necro-haemorrhagic enteritis.

The discrepancy in protection afforded by current clostridial vaccines against *C. perfringens* diseases in sheep and cattle might be the result of the toxins responsible for the disease. In sheep, *C. perfringens* type D is the main cause of enterotoxaemia[66], whereas bovine necro-haemorrhagic enteritis is caused by *C. perfringens* type A strains[10,31,47]. The involvement of different toxinotypes points to different key virulence factors in these diseases. It seems therefore that the antigens needed to afford protection in sheep are less sensitive to formaldehyde inactivation than those involved in bovine necro-haemorrhagic enteritis.

Taken together, these results indicate that there is need to develop new, effective vaccines against bovine necro-haemorrhagic enteritis and by extension, other *C. perfringens* type A-associated intestinal diseases.

## The quest for a new, effective vaccine against bovine necro-haemorrhagic enteritis

To avoid the above-mentioned obstacles, new vaccines should be more targeted and should contain only the relevant antigens needed to provide protection, and in addition formaldehyde inactivation should be avoided. In order to develop such a vaccine, a thorough knowledge of the pathogenesis of bovine necro-haemorrhagic enteritis and the involvement of different *C. perfringens* virulence factors therein is essential. Hereafter, the main virulence factors to be included in the hypothetical ideal vaccine against bovine necro-haemorrhagic enteritis are discussed in detail.

### Alpha toxin is a key virulence factor in bovine necro-haemorrhagic enteritis

At the time this PhD was started, only little was known about the pathogenesis of bovine necro-haemorrhagic enteritis. *C. perfringens* type A was considered to be the causative agent, but the key virulence factors and toxins involved in the pathogenesis were unknown. Alpha toxin is the major toxin produced by type A strains, but its role in intestinal diseases is controversial and heavily debated. In analogy with the recent discovery of subtypes of type A strains that produce newly identified toxins which are (potentially) involved in other intestinal diseases, it was suspected that a yet undiscovered toxin was essential for the pathogenesis of bovine necro-haemorrhagic enteritis. Indeed, for over 30 years it was believed that alpha toxin was the key virulence factor in necrotic enteritis caused by *C. perfringens* in broiler chickens, until it was shown that a novel toxin, NetB, was crucial for disease[25,26]. In an attempt to elucidate the role of *C. perfringens* type A strains in bovine necro-haemorrhagic enteritis, strains originating from healthy calves as well as from necro-haemorrhagic enteritis cases or isolated from other host species were screened for their lesion-inducing potential in a calf intestinal loop model for bovine necro-haemorrhagic enteritis[67]. Incubation of numerous strains from different origin and toxinotypes induced similar necro-haemorrhagic lesions, suggesting that common virulence factors rather than disease-specific toxins are essential. At the same time, analysis of the

complete genome sequence of a bovine clostridial abomasitis isolate failed to reveal novel toxin genes[43]. Therefore the authors suggested the involvement of known toxins such as alpha toxin and perfringolysin O in clostridial abomasitis, a disease which is closely related to bovine necro-haemorrhagic enteritis[57,69]. Indeed, a possible role of alpha toxin in the pathogenesis of bovine necro-haemorrhagic enteritis was demonstrated in a calf intestinal loop model, by using different approaches. First, an alpha toxin-mutant strain was attenuated in its lesion-inducing potential in the intestinal loop model, whereas complementation of alpha toxin restored its ability to cause necro-haemorrhagic lesions (**chapter 6**). Next, when antisera derived from calves vaccinated with native alpha toxin were co-injected with *C. perfringens* in bovine intestinal loops, the lesion-inducing potential of *C. perfringens* was reduced (**chapter 6**). Furthermore, when pure alpha toxin was injected in bovine intestinal loops, it caused epithelial cell detachment, villus tip blunting, erosion, mild inflammation and haemorrhages of the lamina propria, all events that are seen in natural necro-haemorrhagic enteritis cases[39]. However, no necrosis was observed. These results strongly suggest that alpha toxin is essential in the pathogenesis of bovine necro-haemorrhagic enteritis and is a promising candidate vaccine component. Additional indirect evidence pointing towards alpha toxin as a key virulence factor in bovine necro-haemorrhagic enteritis is the observation that calves in veal production systems do not develop an active immunity towards alpha toxin, when maternal immunity declines. This absence of antibody production after decay of maternal antibodies might explain why calves in veal production systems are at higher risk to develop necro-haemorrhagic enteritis than calves raised for beef production, in which a fluent transition from passive maternal to active immunity is observed[46,68]. Furthermore, it is well known that the protective antigenicity of alpha toxin is easily destroyed by formaldehyde inactivation[21,27,30,62,63], possibly explaining why the current clostridial vaccines are unable to protect against bovine necro-haemorrhagic enteritis.

Nevertheless, even if alpha toxin is indispensable to cause necro-haemorrhagic lesions, the presence of alpha toxin alone seems insufficient to cause the fulminant necrosis seen in natural cases (**chapter 6**, [39]). Furthermore, when comparing antisera from calves immunized with alpha toxin alone versus antisera from calves vaccinated against a mixture of native *C. perfringens* toxins, the latter had a stronger ability to protect against *C. perfringens*-induced necrosis when co-injected with *C. perfringens* in bovine intestinal loops (**chapter 5** and **6**). Therefore other common virulence factors are likely involved in the pathogenesis and might be needed as vaccine components to provide full protection against bovine necro-haemorrhagic enteritis.

## Other *C. perfringens* proteins potentially needed in an ideal vaccine formulation

*C. perfringens* type A strains have the ability to produce numerous extracellular toxins and enzymes, of which alpha toxin is the most toxic[48]. Alpha toxin is indispensable to cause disease, but it seems that the activity of alpha toxin alone cannot explain the full package of histopathologic events induced by *C. perfringens* in the intestine[39,40,67]. Furthermore we showed that secreted proteins other than alpha toxin should be included in the ideal vaccine to confer full protection (**chapter 5** and **6**). Up till now the nature of the additional antigens which are needed to provide this protection, is not clear. The most obvious candidate to include in future vaccines is perfringolysin O. Indeed, a synergistic effect between alpha toxin and perfringolysin O has been shown in a mouse model for gas gangrene[2,59] and to bovine endothelial cells[70]. In addition, antibodies towards alpha toxin and perfringolysin O were identified as the most abundant antibodies in the immune sera of calves vaccinated with a mixture of *C. perfringens* toxins (**chapter 5**). It can, however, not be excluded that the better protection afforded by antisera derived from calves vaccinated with a mixture of *C. perfringens* toxins as compared to antisera from calves immunized with alpha toxin alone, is due to other immunogenic proteins. Indeed, when the antisera from calves immunized with *C. perfringens* toxins were less diluted, another, still unidentified, large immunogenic protein was detected via western blot (MW > 100 kDa, unpublished results). Up till now we can only speculate about the nature of this protein. Most logical would be a protein that confers a specific advantage to *C. perfringens* during intestinal colonization and/or infection such as, amongst others, the NanJ sialidase (129 kDa, might be involved in degradation of the protective mucus layer[29]), kappa toxin (≈ 120 kDa, a collagenase which might cause loss of tissue integrity and subsequent necrosis[33]) or mu toxin (≈ 182 kDa, NagH, a hyaluronidase, potentially involved in the degradation of mucins and connective tissue[6,14]). Identification of this protein and further vaccination experiments are needed to elucidate the role of both perfringolysin O and/or the yet unidentified protein as additional vaccine antigen(s), to confer full protection against bovine necro-haemorrhagic enteritis.

## Following steps in vaccine development

### *Optimizing the protective potential of alpha toxoid*

When the essential vaccine components are identified, a lot of further testing is necessary. As mentioned before, the protective antigenicity of alpha toxin is easily destroyed by formaldehyde inactivation[21,27,30,62,63]. The use of active toxins in a vaccine formulation will probably not be allowed, therefore, other approaches for the development of improved toxoids are needed. One strategy that was extensively studied to protect mice against experimental gas gangrene is the use of immunologically active fragments of the toxin. Immunization with either the N-terminal or C-terminal domains of alpha toxin induces high antibody titres against the native toxin[74]. However, only mice immunized with the C-terminal fragment were protected against intraperitoneal challenge with alpha toxin or against experimental gas gangrene[74]. In accordance with these results, we have shown that the C-terminal domain of alpha toxin was highly immunogenic to calves and induced high antibody titres against native alpha toxin (**Chapter 6**). However, when sera derived from calves immunized with this C-terminal fragment were co-injected with *C. perfringens* in bovine intestinal loops, they were less able to prevent the induction of necrotic lesions as compared to sera raised against native alpha toxin. This diminished protection might be attributed to distortion of the conformation or shielding of some parts of the toxin by the GST tag, which was fused to the C-terminal fragment for purification of the protein[74]. Another explanation might be that antibodies against both the N-terminal and C-terminal domain of alpha toxin are needed to confer full protection. Further research is needed to explore both options, either by repeating the vaccination experiment with an untagged C-terminal alpha toxin fragment or by evaluating the use of naturally occurring or genetically engineered variants of alpha toxin with reduced toxicity. One possibility is the use of alpha toxin variant 121A/91-R212H. This protein is a genetically engineered form of a naturally occurring alpha toxin variant devoid of activity, which protects mice against challenge with wild-type alpha toxin[50]. When considering the use of alpha toxin variants as vaccine component, special attention should go to ensure that all toxicity is eliminated and that reversion to full toxicity is unlikely.

## *Exploring the need of systemic and mucosal immunity*

Clostridia are non-invasive bacteria and all clostridial diseases are the result of the activity of their extremely potent toxins. Therefore, it seems only logical that vaccination is focused on the development of humoral immunity. What is less straightforward, is the choice of current vaccines to focus only on generating circulating IgG antibodies for protection against disease. This choice is probably dictated by the confusing nomenclature concerning the term "enterotoxaemia". This term is widely applied to various disease caused by *C. perfringens*, however it is appropriate only for diseases in which the major signs are caused by systemic actions of the toxins[36]. As described above, bovine necro-haemorrhagic enteritis cannot be seen as a true enterotoxaemia.

Since *C. perfringens* is an enteric pathogen and given the local activity of its toxins, we could speculate that mucosal IgA plays a more important role than serum IgG in the protection against bovine necro-haemorrhagic enteritis. In cattle, no reports are found describing the mucosal IgA expression in the intestine during *C. perfringens* infection. Also for other species, the literature concerning this topic is scant. In humans, a correlation between the serum levels of IgA to alpha toxin and the faecal *C. perfringens* counts has been documented, but the relevance of this observation to provide protection against disease is not yet clear[23]. In chickens, it has been shown that systemic antibodies are able to reach the mucosal surface under inflammatory or necrotic conditions[27]. Furthermore, experimental animal work on intestinal *C. difficile* infections has shown that protection can be mediated through simple exudation of serum antitoxin IgG across the inflamed intestinal epithelium[16]. These observations point towards a serum IgG response as major influencer of protective immunity, but more research in cattle is needed to support this hypothesis. The ideal situation probably combines both systemic IgG as well as mucosal IgA immunity. This has been achieved using *Bacillus subtilis* spores as vaccine delivery agent. This organism is able to colonise the gut without causing disease. Oral immunization of mice with *B. subtilis* spores displaying the C-terminal fragment of alpha toxin on the spore surface, resulted in increased serum IgG levels and secretory IgA detected in saliva, faeces or lung wash samples. Moreover, immunised mice were protected against severe challenge with alpha toxin[20]. In addition to the use of *B. subtilis* spores, also the use of other intestinal organisms can be explored, such as, amongst others, the use of *Eimeria*[5] or *Salmonella*[76]. Next, it should be investigated whether immunity against alpha toxin alone is sufficient when both systemic IgG as well as mucosal IgA immunity is obtained.

### *The lack of a real in vivo model hinders vaccine development*

Despite multiple attempts by different research groups to reproduce bovine necro-haemorrhagic enteritis *in vivo*, the intestinal loop model remains the only system closest to an intact animal that is able to reproduce the lesions consistently[40,42,67]. This model allows to study host-pathogen interactions in a way that mimicks normal intestinal physiologic, immunologic, and histopathologic responses[22]. Furthermore, multiple treatments and replicates can be tested in a single animal. Correct ligation of the intestinal loops is, however, a delicate procedure. When done correctly, the vascular and lymphatic functions are not disrupted. In addition to these clear advantages, the intestinal loop model has also some drawbacks, which include the anaesthesia that may affect the general metabolism of the animal, absence of intestinal peristalsis and difficulties to monitor systemic effects. Moreover, the injection of a large amount of *C. perfringens*, which is artificially kept in contact with the same intestinal tissue, makes it an aggressive model. This inevitably complicates the screening of potential vaccine components. Indeed, when antibodies are co-injected with live *C. perfringens* in the intestinal loops, *C. perfringens* is able to keep on producing toxins, while the antibodies injected in the loops are limited. Furthermore, with the experimental setup we used, it is impossible to elucidate whether serum antibodies leaking into the gut will be sufficient or whether mucosal immunity may be needed (**chapter 5** and **6**). To approach this, it should be possible to perform an intestinal loop experiment in vaccinated animals. The difficulty in this approach is the absence of a proper control (unvaccinated loops) within the same animal, and therefore the sample size of this experiment will be much larger, involving more experimental animals. Therefore, this final experiment should only be done with the final vaccine candidate for ethical, practical and financial reasons.

Given the above described limitations of the intestinal loop experiments used during this PhD study, together with the absence of a good *in vivo* model, the next step in determining the efficacy of the vaccine candidates would be to go straight to a field trial. Field studies are often conducted for diseases for which the incidence is high. Because bovine necro-haemorrhagic enteritis is a disease with a low morbidity rate, a statistically significant effect of the vaccine can only be detected when the field study includes a very high number of calves. When calves are immunized with a product that is not licenced for use in Belgium, the animals will not be allowed to go to slaughter for consumption. This implicates a tremendous cost, unless an exemption can be granted by the Belgian Federal Public Service (FPS) Health, Food Chain

Safety and Environment. Such an exception may be realistic for a conventional, parenteral vaccine. More difficulties are expected to get permission of the FPS to evaluate the efficacy of oral immunization with recombinant bacteria, such as *Bacillus* spores. The use of a genetically modified organism that is able to multiply in the intestine and will be shed by the animal indeed generates safety issues.

## The quest for specific virulence factors which are characteristic for bovine necro-haemorrhagic enteritis isolates

As mentioned before, a thorough knowledge of the pathogenesis of bovine necro-haemorrhagic enteritis is essential for the development of efficient control strategies, including vaccine development. *C. perfringens* type A is the causative agent of bovine necro-haemorrhagic enteritis, but is also a commensal of the gastrointestinal tract of healthy animals[28,31,55]. This observation raised the question whether *C. perfringens* type A strains isolated from necro-haemorrhagic enteritis cases are more virulent than other type A strains. Higher activities of the main toxins produced by type A strains (alpha toxin and perfringolysin O) as well as proteolytic and carbohydrate-active factors that degrade the protective mucus layer or extracellular matrix components, may confer a selective advantage to the producing strain. However, compared to strains isolated from healthy cattle or from other animal species, strains originating from bovine necro-haemorrhagic enteritis cases did not have higher alpha toxin, perfringolysin O, mucinolytic or gelatinolytic activity as seen *in vitro* (**chapter 4**). Based on these results we cannot conclude that *C. perfringens* strains isolated from bovine necro-haemorrhagic enteritis cases have characteristics that make them superior to colonize the bovine intestinal tract or even to cause intestinal disease. However, we cannot exclude this possibility either.

First, the production of virulence factors *in vitro* does not necessarily reflect the *in vivo* situation. There is increasing evidence that contact with host tissue alters the *C. perfringens* toxin production[8,72]. This sensing of enterocytes and subsequent toxin production is probably regulated by quorum sensing mechanisms[8,71]. All research concerning this topic was focused mainly on *C. perfringens* type C and to a lesser extent on type D strains. However, these strains are believed to behave differently than type A strains. For example, type C and D strains are shown to adhere to epithelial cells[29]. This is in contrast to the situation of type A strains, for which it is generally believed that they do not adhere to the intestinal epithelium[32,45,55]. So, it

seems only natural that the observations on type C and D strains cannot simply be extrapolated to type A strains and more research is needed in this field.

In addition to this effect of the host on *C. perfringens*, the bacteria can also influence the host. Indeed, after inoculation of bovine intestinal loops with *C. perfringens*, collagen type IV degrading activity can be detected in the bovine tissue. This is in contrast with the control loops, which were not challenged with *C. perfringens*, where no collagen type IV degrading activity could be measured in the host tissue (unpublished observations). More research is needed to see whether necro-haemorrhagic enteritis isolates are more able to exert such effects.

Another point that will need more attention is the fact that it is not because all tested strains of *C. perfringens* were able to cause necro-haemorrhagic lesions in the intestinal loop model that they will do so *in vivo*. For example, strains that cause food poisoning may differ from those that cause gas gangrene only by the presence of an enterotoxin gene in the former, yet food-poisoning strains have never been found to cause gas gangrene[51]. Therefore, it seems not unlikely that necro-haemorrhagic enteritis isolates possess other characteristics that make them more adapted to colonize their host and/or to cause disease in the bovine intestinal tract *in vivo*. One argument pointing in this direction is the difference between alpha toxin produced by *C. perfringens* strains isolated from soft tissue infections (such as gas gangrene) or isolated from the intestine of calves suffering from bovine necro-haemorrhagic enteritis. Although no difference was observed in the enzymatic properties of the toxins, alpha toxin produced by the enteric isolates of *C. perfringens* showed increased resistance to proteolytic inactivation by chymotrypsin, an observation that is consistent with the site of toxin production in the gut[17]. In addition to this variation in sensitivity to proteolytic inactivation, also other factors may render the necro-haemorrhagic enteritis isolates more adapted to their host. These factors are not necessarily toxins, but might equally well be factors involved in more efficient nutrient utilization or host colonization. One property that may lead to enhanced intestinal colonization is the ability to bind to extracellular matrix (ECM) molecules. Affinity to collagen and other ECM molecules is a trait shared by many bacterial pathogens and has been shown to contribute to host colonization[32,73]. Indeed, in necrotic enteritis intestinal lesions in chickens, the villi that are undergoing necrosis are coated with a thick mat of organisms[32]. Furthermore, the ability of *C. perfringens* to adhere to collagen *in vitro* correlates with the ability to cause necrotic enteritis in chickens[73]. It should be noted that *C. perfringens* is never found attached to the epithelium and prior intestinal damage is needed to allow the bacterium to attach to the underlying ECM molecules. Also in bovine intestinal loops, *C. perfringens* is not seen attached to the epithelium

until severe necrosis is present and *C. perfringens* is found in close interaction with the necrotic cells. Furthermore, there seems to be a difference between the ability to adhere to the lamina propria of different strains (unpublished observations). Further research is needed to unravel the ability of isolates from other intestinal disease, such as bovine necro-haemorrhagic enteritis, to adhere to collagen and other ECM molecules. To reveal the full repertoire of characteristics that are unique to *C. perfringens* necro-haemorrhagic enteritis isolates, a collection of necro-haemorrhagic enteritis strains should be sequenced and compared to other *C. perfringens* sequences. This might reveal additional, yet unknown antigens which might be of value for vaccine development.

## Pros and cons of vaccines for bovine nerco-haemorrhagic enteritis

Bovine necro-haemorrhagic enteritis is a disease that only affects a single animal or a very limited number of animals in the same herd[28,31]. Although morbidity is low, mortality is close to 100%. Moreover, there is an increased risk for bovine necro-haemorrhagic enteritis at the end of the rearing period (for calves in veal production systems)[46]. Therefore, the disease has a high economic impact. However, the veal industry, is typically Belgian and compromises only a small target market for the manufacturer. Because of the limited number of cases, the major drawback of a vaccine for necro-haemorrhagic enteritis is the profitability of the vaccine. It may be difficult to pursue a farmer (outside the veal industry) to vaccinate against such a rare disease. Indeed, the vaccine should be provided at low cost to ensure that the vaccination cost is significantly lower than the losses that the farmer suffers through animal loss by bovine necro-haemorrhagic enteritis. It seems clear that commercializing a vaccine targeting only bovine necro-haemorrhagic enteritis is not evident. To circumvent this problem, one can follow the same strategy as current clostridial vaccines, combining antigens against multiple clostridial diseases in one shot. If this vaccine is made using the specific antigenic subunits needed to provide protection against the different diseases, the new vaccine might overcome the inflammatory problems of current vaccines and even provide better protection.

Another appealing strategy is to look beyond the veal calf industry for marketing a vaccine directed against *C. perfringens* type A strains. It is possible that the alpha toxoid will be valuable for the prevention of other diseases in domesticated livestock. There is evidence that alpha toxin plays a key role in the pathogenesis of clostridial abomasitis in calves and

haemorrhagic bowel syndrome in cows, as well as some enteric diseases of sheep[66] (yellow lamb disease), piglets[56,58] (neonatal diarrhoea and type A enteritis), foals[18], broilers (necrotic enteritis), …. Further research is needed to elucidate the exact role of alpha toxin in the pathogenesis of these diseases and the protection afforded by immunization with alpha toxoid. Nevertheless, we strongly believe that, even if other disease-specific toxins (e.g. beta2 toxin, NetF or NetB) might be necessary to cause disease, blocking of the alpha toxin activity will be needed to provide full protection. This hypothesis is supported by the recent finding in broiler chickens, where immunization with either alpha toxoid or NetB toxoid provides equal levels of protection against experimental necrotic enteritis[13].

## Conclusion

The results described in this thesis clearly demonstrate that alpha toxin is essential in the pathogenesis of bovine necro-haemorrhagic enteritis. Furthermore we showed that bovine antisera raised against current clostridial vaccines are unable to block alpha toxin activity *in vitro* or protect against *C. perfringens* challenge in an *in vivo* intestinal loop model for bovine necro-haemorrhagic enteritis. The conformational epitopes of alpha toxin are important to induce a protective immune response and these epitopes are easily destroyed by formaldehyde. Therefore, the inability of current clostridial vaccines to induce protective antibodies against bovine necro-haemorrhagic enteritis is most likely the consequence of the inactivation procedure using formaldehyde. In order to protect animals against *C. perfringens* type A-associated enteric diseases, novel vaccines are needed. Alpha toxin will probably be a key component in these vaccines and the non-toxic C-terminal domain of alpha toxin may be a good candidate for further vaccine development. In addition, the ideal vaccine formulation could also contain other, yet unidentified, factors needed to provide full protection. These factors may be accessory toxins or enzymes involved in lesion induction, or factors that make the strains more adapted to the host environment.

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# PART V
# Appendices

Summary

Samenvatting

Curriculum Vitae

Dankwoord

# Summary

## Summary

Bovine necro-haemorrhagic enteritis caused by *Clostridium perfringens* type A is a sudden death syndrome with necro-haemorrhagic lesions in the small intestine. It mainly affects suckling calves and veal calves in good to excellent body condition. In veal calves, predominantly beef cattle breeds are affected. The syndrome accounts for approximately 20% of the mortalities in these calves, compared to 4% in dairy and mixed breed veal calves. Although morbidity is low, mortality is close to 100%, making it an economical important disease.

At the start of this PhD, only little was known about the pathogenesis of bovine necro-haemorrhagic enteritis. *C. perfringens* type A was considered to be the causative agent, but the key virulence factors and toxins involved in the pathogenesis were unknown. It was suggested that lesions of necro-haemorrhagic enteritis could be induced by all *C. perfringens* isolates, pointing towards the involvement of a common *C. perfringens* virulence factor. The most likely candidates involved in the pathogenesis were thus alpha toxin and perfringolysin O, the two main toxins produced by *C. perfringens* type A strains, as well as the many proteolytic and carbohydrate degrading enzymes produced by this bacterium. In addition to causing disease, type A strains are also normal gut microbiota members. It is hitherto unclear why *C. perfringens* type A strains cause disease in some animals, while acting as commensals in other animals.

The first aim of this thesis was therefore to obtain more insight in the virulence properties of *C. perfringens* strains isolated from bovine necro-haemorrhagic enteritis cases. In **chapter 4** the production of virulence factors that are potentially involved in bovine necro-haemorrhagic enteritis were analysed. To approach this, a collection of strains isolated from necro-haemorrhagic enteritis cases was compared to bovine strains originating from healthy animals and to strains isolated from other animal species. These strains were screened for the *in vitro* production of alpha toxin, the most toxic factor produced by type A strains, and perfringolysin O, which was shown to act in synergy with alpha toxin to cause bovine endothelial cytotoxicity *in vitro*. In addition to these toxins, the activity of proteolytic factors that degrade the protective mucus layer or extracellular matrix components was measured *in vitro.* No differences in the production of these potential virulence factors was found between isolates form the normal microbiota and isolates derived from necro-haemorrhagic enteritis cases. This could indicate that yet another, hitherto unknown, *C. perfringens* virulence factor might be involved in the pathogenesis of bovine necro-haemorrhagic enteritis. In the light of recent findings by other

research groups, it seems most likely that this pathogenesis trait will comprise factors that make *C. perfringens* necro-haemorrhagic enteritis isolates superior in colonizing the bovine intestinal tract, such as, amongst others, factors involved in the host-pathogen cross talk or more proteolytic resistant toxins. However, when taking into account that *C. perfringens* strains from various origin can induce the typical lesions in an intestinal loop model, it seems more plausible that the primary trigger in bovine necro-haemorrhagic enteritis is not *C. perfringens* and that *C. perfringens* is merely responsible for propagating and exacerbating the intestinal damage to the point that is becomes haemorrhagic and necrotic.

Because alpha toxin is the major virulence factor of *C. perfringens* type A strains, the role of this toxin in the pathogenesis of bovine enterotoxaemia was evaluated. Indeed, alpha toxin seems to be essential for the induction of necrotic lesions. This was demonstrated in **chapter 6** by inoculation of mutant strains in bovine intestinal loops. An alpha toxin-mutant strain was attenuated in its lesion-inducing potential, whereas complementation of alpha toxin restored its ability to cause necro-haemorrhagic lesions in the bovine intestine. These results show that alpha toxin is required for intestinal virulence in a bovine intestinal loop model.

Bovine necro-haemorrhagic enteritis is rapidly fatal and no effective control measures are available. This makes vaccination a necessary strategy. Most of the currently marketed clostridial vaccines combine several clostridial species, including *C. perfringens*. These vaccines are made from culture supernatants which are inactivated, mostly using formaldehyde. Current clostridial vaccines were developed for immunization of sheep, but are also licenced for use in cattle. Despite the efficacy of these vaccines in sheep, a limited efficacy of these vaccines against bovine necro-haemorrhagic enteritis has been reported (empirical observations from the field in Belgium). Furthermore, no scientific literature is available describing the efficacy of these vaccines in cattle. In **chapter 5** the protective potential of the antibodies raised against *C. perfringens*, developed after immunization of calves with a commercial multivalent clostridial vaccine, was evaluated. Additionally, the protective potential of antibodies derived from calves immunized with a mixture of *C. perfringens* toxins, either as native toxins or as formaldehyde inactivated toxins, was studied. The most immunogenic proteins in the vaccine preparations were identified as alpha toxin and perfringolysin O. All vaccines evoked a high antibody response against the major *C. perfringens* type A toxins, alpha toxin and perfringolysin O, as detected by ELISA. However, the antibodies raised against the native toxins had a higher capacity to inhibit alpha toxin and perfringolysin O activity *in vitro* and were more inhibitory to the *C. perfringens*-induced cytotoxicity to bovine endothelial cells.

Moreover only antibodies raised against native toxins protected against *C. perfringens* challenge in an intestinal loop model for bovine necro-haemorrhagic enteritis. These results strongly suggest that detoxification using formaldehyde has an influence on the capacity of *C. perfringens* toxins to induce protective antibodies. Moreover, it clearly indicates the need to develop new, effective vaccines against bovine necro-haemorrhagic enteritis.

Given the importance of alpha toxin for the induction of intestinal lesions, the potential of alpha toxin as a vaccine antigen was assessed in **chapter 6**. Since native toxins are not safe, the protective potential of native alpha toxin as well as the enzymatically inactive C-terminal domain of alpha toxin was evaluated. Immunization of calves with either of the vaccine preparations induced a strong antibody response against native alpha toxin. The resulting antisera were able to neutralize the alpha toxin activity and the *C. perfringens*-induced endothelial cytotoxicity *in vitro.* However, antisera raised against the native toxin had a stronger neutralizing activity than those against the C-terminal fragment. Additionally, the lesion-inducing potential of *C. perfringens* in the intestinal loop model was significantly reduced only by co-administration of antisera from animals vaccinated with the native alpha toxin and not with the C-terminal fragment. This diminished protective potential afforded by antisera against the C-terminal domain might be attributed to distortion of the conformation or shielding of some parts of the C-terminal fragment by the GST tag, which was used for purification of the protein. By contrast, the native toxin was fused to a HIS tag for purification, which is substantially smaller and less likely to influence the conformation of the alpha toxin. Another explanation might be that antibodies against both the N-terminal and C-terminal domain of alpha toxin are needed to evoke protective antibodies. It should be noted that, when co-injected with *C. perfringens* in bovine intestinal loops, antibodies raised against native alpha toxin reduced the lesion-inducing potential of the bacterium. However, they were not sufficient to completely neutralize the *C. perfringens*-induced necrosis. Therefore other common virulence factors might be needed as vaccine components to provide full protection against bovine enterotoxaemia.

In conclusion, the results described in this thesis clearly demonstrate that alpha toxin is essential in the pathogenesis of bovine necro-haemorrhagic enteritis. Furthermore, we showed that vaccines based on formaldehyde inactivated toxins (e.g. current clostridial vaccines) are unable to protect against *C. perfringens* challenge in an intestinal loop model for bovine necro-

haemorrhagic enteritis, and novel vaccines are thus needed. Alpha toxin might be a valuable component of a vaccine for bovine necro-haemorrhagic enteritis and the C-terminal domain of alpha toxin may be a good candidate for further vaccine development. In addition, the ideal vaccine formulation will also contain other, yet unidentified, factors needed to provide full protection.

# Samenvatting

# Samenvatting

Necro-hemorragische enteritis bij kalveren veroorzaakt door *Clostridium perfringens* type A wordt gekarakteriseerd door plotse sterfte zonder voorafgaande symptomen. Bij autopsie wordt een hemorragisch necrotiserende enteritis van de dunne darm aangetroffen. De ziekte treft voornamelijk zogende kalveren en kalveren bestemd voor kalfsvleesproductie in goede tot uitstekende voedingstoestand. Binnen de kalveren in kalfsvlees-productiesystemen worden overwegend vleesveerassen getroffen. Hoewel de morbiditeit laag is, sterven zo goed als alle getroffen dieren. Vanuit economisch standpunt wordt necro-hemorragische enteritis dus beschouwd als een belangrijke ziekte.

Bij de aanvang van dit doctoraatsonderzoek was er slechts weinig kennis beschikbaar over de pathogenese van necro-hemorragische enteritis bij kalveren. *C. perfringens* type A werd als oorzakelijk agens beschouwd, maar de belangrijkste virulentiefactoren en toxines die betrokken zijn in de pathogenese waren onbekend. Voorgaand onderzoek heeft aangetoond dat letsels van necro-hemorragische enteritis veroorzaakt kunnen worden door alle *C. perfringens* isolaten, wat de betrokkenheid van een algemene virulentiefactor suggereert. Onder deze factoren die mogelijks betrokken zijn in de pathogenese van necro-hemorragische enteritis zijn alfa toxine en perfringolysin O (de twee belangrijkste toxines geproduceerd door *C. perfringens* type A stammen), evenals de vele proteolytische en koolhydraat-afbrekende enzymen die door deze bacterie worden geproduceerd. Type A stammen zijn niet enkel pathogeen, maar behoren eveneens tot de normale darmflora. Het is tot op heden onduidelijk waarom *C. perfringens* type A stammen bij sommige dieren ziektes veroorzaken, terwijl ze zich in ander dieren gedragen als leden van de normale darmflora.

Het eerste doel van dit proefschrift was dan ook om meer inzicht te krijgen in de virulentiefactoren die aanwezig zijn in *C. perfringens* isolaten uit runderen gestorven aan necro-hemorragische enteritis. In **hoofdstuk 4** werd de productie van virulentiefactoren die mogelijk betrokken zijn bij boviene necro-hemorragische enteritis geanalyseerd. Hiervoor werd een collectie necro-hemorrhagische enteritis-stammen vergeleken met runder-stammen afkomstig van gezonde dieren en met stammen geïsoleerd van andere diersoorten. Deze stammen werden gescreend voor de *in vitro* productie van alfa-toxine (de meest toxische factor die door type A stammen wordt geproduceerd) en perfringolysin O (waarvan een synergie met alfa toxine was aangetoond bij het veroorzaken van endotheliale cytotoxiciteit *in vitro*). Ook de activiteit van

proteolytische factoren die in staat zijn de beschermende mucuslaag in de darm of extracellulaire matrixcomponenten af te breken werd gemeten. Er kon geen verschil waargenomen worden in de productie van deze potentiële virulentiefactoren tussen normale microbiota isolaten en stammen afkomstig van necro-hemorragische enteritis gevallen. Dit kan betekenen dat een andere virulentiefactor van *C. perfringens* betrokken is in de pathogenese van boviene necro-hemorragische enteritis. Wanneer echter rekening wordt gehouden met het feit dat *C. perfringens* stammen van verschillende oorsprong in staat zijn de typische letsels op te wekken in een darmlusexperiment, lijkt het aannemelijk dat *C. perfringens* niet de primaire trigger is in het ontstaan van boviene necro-hemorragische enteritis, maar dat *C. perfringens* alleen verantwoordelijk is voor het verderzetten en het verergeren van de intestinale schade tot op het punt dat de darm hemorragisch en necrotisch wordt.

Aangezien alfa toxine de belangrijkste virulentiefactor is van type A stammen, werd de rol van dit toxine in de pathogenese van boviene necro-hemorragische enteritis geëvalueerd. Alfa toxine blijkt essentieel te zijn voor de inductie van necrotische letsels in de runderdarm. Dit werd aangetoond in **hoofdstuk 6** door gebruik van mutante stammen in een bovien darmlusmodel. Een alfa toxine-mutant was geattenueerd in zijn letsel-inducerend vermogen, terwijl complementering van alfa-toxine dit vermogen om necro-hemorragische letsels te veroorzaken herstelde. Deze resultaten tonen aan dat alfa toxine essentieel is voor intestinale virulentie in het darmlusmodel.

Necro-hemorragische enteritis in kalveren veroorzaakt acute sterfte. Bovendien zijn er geen effectieve preventiestrategieën beschikbaar, wat van vaccinatie een noodzakelijke strategie maakt. De meeste clostridium vaccins die momenteel op de markt zijn bestaan uit een combinatie van verschillende *Clostridium* species, waaronder *C. perfringens*. Deze vaccins worden gemaakt van geïnactiveerd supernatans van een bacteriële cultuur. De detoxificatie van de bacteriële culturen gebeurt voornamelijk door formaldehyde. De huidige vaccins zijn ontwikkeld voor immunisatie van schapen, maar zijn ook geregistreerd voor gebruik bij rundvee. Ondanks het feit dat deze vaccins doeltreffend zijn voor de bescherming van schapen, wordt slechts een beperkte werkzaamheid van deze vaccins tegen boviene necro-hemorragische enteritis gemeld (empirische waarnemingen van het veld in België). Bovendien is er geen wetenschappelijke literatuur beschikbaar over de werkzaamheid van deze vaccins in rundvee. In **hoofdstuk 5** werd het beschermende vermogen geëvalueerd van de antilichamen die opgewekt worden tegen *C. perfringens*, na immunisatie van kalveren met een commercieel clostridium multivalent vaccin. Daarnaast werd ook het beschermende potentieel onderzocht

van antilichamen afkomstig van kalveren geïmmuniseerd met een mengsel van *C. perfringens* toxinen, hetzij als natieve toxines of als formaldehyde geïnactiveerde toxines. De meest immunogene eiwitten in de vaccinpreparaten werden geïdentificeerd als alfa toxine en perfringolysine O. Alle vaccin formulaties induceerden een hoge antilichaamrespons tegen zowel alfa toxine als perfringolysine O, zoals gemeten via ELISA. Deze antilichaamtiters komen echter niet overeen met de neutraliserende eigenschappen van de antilichamen. Antilichamen opgewekt tegen de natieve *C. perfringens* toxines waren beter in staat om de alfa toxine- en perfingolysin O-activiteit te remmen *in vitro* en konden de endotheliale cytotoxiciteit veroorzaakt door *C. perfringens* beter inhiberen dan de antilichamen opgewekt na vaccinatie met het multivalent clostridium vaccin of na vaccinatie met de formaldehyde geïnactiveerde *C. perfringens* toxines. Bovendien konden alleen antilichamen opgewekt tegen de natieve toxines het ontstaan van necrotische letsels na injectie van boviene darmlussen met *C. perfringens* tegengaan. Deze resultaten suggereren dat het gebruik van formaldehyde voor detoxificatie de structuur van de *C. perfringens* toxines zo verandert dat het onmogelijk is om beschermende antilichamen op te wekken. Bovendien tonen deze resultaten duidelijk aan dat er nood is aan de ontwikkeling van nieuwe, effectieve vaccins tegen boviene necro-hemorragische enteritis.

Gezien het belang van alfa toxine in de inductie van intestinale letsels, werd in **hoofdstuk 6** het potentieel van alfa-toxine als een vaccin antigeen nagegaan. Het gebruik van natieve toxines kan niet als veilig worden beschouwd. Daarom werd naast natief alfa toxine ook het gebruik van het enzymatisch inactieve C- terminale domein van alfa toxine als vaccin antigeen geëvalueerd. Immunisatie van kalveren met deze vaccinpreparaten induceerde een sterke antilichaamrespons tegen natief alfa toxine. De verkregen antisera waren in staat zowel de alfa toxine activiteit als de cytotoxiciteit van *C. perfringens* op runder-endotheel te neutraliseren *in vitro*. Antisera opgewekt tegen het natieve toxine hadden echter een sterkere neutraliserende activiteit dan antisera opgewekt tegen het C-terminale fragment. Daarnaast kon enkel de gelijktijdige injectie van antisera gericht tegen natief alfa toxine in het darmlusmodel het letsels-inducerende vermogen van *C. perfringens* significant verlagen. De verminderde bescherming van antisera tegen het C-terminale domein kan worden toegeschreven aan verstoring van de conformatie of afscherming van sommige delen van het C-terminale fragment door de GST-tag, die werd gebruikt voor zuivering van het eiwit. Daarentegen werd het natieve alfa toxine gefuseerd aan een His-tag voor zuivering. Deze His-tag is aanzienlijk kleiner en zal de conformatie van alfa toxine waarschijnlijk minder beïnvloeden. Een andere verklaring kan zijn

dat antilichamen tegen zowel het N-terminale als C-terminale domein van alfa toxine nodig zijn om beschermende antilichamen op te wekken. Er moet opgemerkt worden dat, ondanks het feit dat antilichamen tegen natief alfa toxine het letsel-inducerende vermogen van de bacterie verminderen, de toediening van deze antilichamen toch niet voldoende bleek om de necrose die wordt veroorzaakt door *C. perfringens* volledig te neutraliseren. Het zou dus kunnen dat andere virulentiefactoren, die geproduceerd worden door alle *C. perfringens* stammen, moeten worden toegevoegd aan een vaccin om volledige bescherming tegen boviene necro-hemorrhagische enteritis te bieden.

De resultaten beschreven in deze doctoraatsthesis tonen duidelijk aan dat alfa toxine van essentieel belang is in de pathogenese van necro-hemorragische enteritis in kalveren. Bovendien werd aangetoond dat vaccins op basis van formaldehyde geïnactiveerde toxines (zoals de huidige clostridium vaccins) niet in staat zijn antilichamen op te wekken die bescherming bieden tegen de letsels veroorzaakt door *C. perfringens*, zoals gezien in de darmlus-experimenten. De ontwikkeling van nieuwe vaccins tegen boviene necro-hemorrhagische enteritis is dus noodzakelijk. Alfa toxine zou een waardevolle component kunnen zijn van zo'n nieuw vaccin tegen boviene necro-hemorragische enteritis en het C-terminale domein van alfa toxine vormt een goede optie voor verdere vaccinontwikkeling. Daarnaast zal de ideale vaccinformulering ook andere, nog ongeïdentificeerde factoren moeten bevatten om volledige bescherming te kunnen bieden tegen boviene necro-hemorrhagische enteritis.

# Curriculum Vitae

# Curriculum Vitae

Evy Goossens was born on October 22, 1987 in Ghent, Belgium. In 2010, she obtained her Master's degree in Biochemistry and Biotechnology, Major Biomedical Biotechnology with distinction at Ghent University, Belgium.

As from September 2010, Evy Goossens started her PhD research at the Department of Pathology, Bacteriology and Avian Diseases, Faculty of Veterinary Medicine, Ghent University on an IWT Agriculture project. This research project focussed on the pathogenesis and control of enterotoxaemia in veal calves. Since September 2014 she works in the same department on a project funded by Evonik Industries AG entitled 'Identification of biomarkers for subclinical necrotic enteritis'. In 2016 she obtained the certificate of the Doctoral Training Programme of Life Sciences and Medicine.

Evy Goossens is (co-)author of several papers in international peer-reviewed journals. She has given multiple oral/poster presentations at international conferences.

# Curriculum Vitae

Evy Goossens werd geboren op 22 oktober 1987 te Gent. Na het behalen van het diploma secundair onderwijs aan 'EDUGO De Toren' te Oostakker, richting Wetenschappen-Wiskunde, startte ze in 2005 met de studies Biochemie en Biotechnologie aan de Universiteit Gent. In 2010 behaalde ze met onderscheiding het diploma van Master in de Biochemie en Biotechnologie (optie biomedische biotechnologie).

Onmiddellijk na het afstuderen, startte ze aan diezelfde universiteit een doctoraatsonderzoek aan de vakgroep Pathologie, Bacteriologie en Pluimveeziekten, Faculteit Diergeneeskunde. In het kader van een IWT landbouwproject verrichtte ze voornamelijk onderzoek naar de pathogenese en bestrijding van enterotoxemie bij kalveren. Ze beëindigde ook met succes de doctoraatsopleiding.

Sinds september 2014 is ze aan dezelfde vakgroep tewerkgesteld op een project getiteld 'Identification of biomarkers for subclinical necrotic enteritis', gefinancierd door Evonik Industries AG.

Evy Goossens is auteur en co-auteur van meerdere wetenschappelijke publicaties in internationale tijdschriften en gaf meermaals presentaties op internationale congressen.

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# Dankwoord

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*Evy*