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Clostridium perfringens alpha toxin and NetB toxin derivatives as vaccine antigens for chickens

DORIEN MOT

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List of abbreviations

AGP's	antimicrobial growth promotors
BHI	brain heart infusion
BCA	bicinchoninic acid
BLAST	basic local alignment search tool
BP	base pair
CFU	colony forming units
CN/DAB	chloronaphthol- and diaminobenzidine-based
CP	*Clostridium perfringens*
CPA	alpha toxin
CPE	*Clostridium perfringens* enterotoxin
DNA	deoxyribonucleic acid
EF-G	elongation factor G
EF-Tu	elongation factor Tu
ELISA	enzyme-linked immunosorbent assay
EU	European Union
FBA	Fructose-1,6-biphosphate aldolase
FPV	Recombinant fowl poxvirus
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GMO	genetically modified organisms
GST	glutathione S-transferase
HP	hypothetical protein
HRP	*horseradish peroxidase*

HVT	herpesvirus of turkey
Ig	immunoglobulin
IM	intramuscularly
IPTG	isopropyl-β-D-thiogalactopyranoside
kDa	Kilo Dalton
LMH	leghorn male hepatoma cell line
MALDI TOF	*Matrix assisted laser desorption ionization time-of-flight*
MWCO	*molecular weight cut-off*
Naglu	N-acetylglucosaminidase
NCBI	National center for biotechnology information
NE	necrotic enteritis
NetB	necrotic Enteritis Toxin B-like
netB	gene encoding NetB toxin
Ni-NTA	nitrilotriacetic acid bound Ni^{+2} ions
OD	optical density
ON	overnight
ORF	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PD	prepacked disposable
PES	polyether sulfone
PFO	perfringolysin O
PFT	pore-forming toxin
PFOR	pyruvate:ferredoxin oxidoreductase
Pgm	phosphoglyceromutase

PVDF	polyvinylidene difluoride
rNetB	recombinant NetB
RT	room temperature
SC	subcutaneous
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	standard error of the mean
SN	supernatant
TB	terrific broth
TBS	tris-buffered saline
TCP	total combining power
tHP	truncated hypothetical protein
TMB	tetramethylbenzidine
TpeL	toxin perfringens large
UV-Vis	ultraviolet-visible
v/w	volume/weight

Symbols

>	greater than
™	trade mark
α	alpha
β	beta
ε	epsilon
ι	iota
μ	micro

Chapter 1

General introduction

General introduction

1.1. Necrotic enteritis in broiler chickens

Necrotic enteritis is an important disease of broiler chickens, caused by the bacterium *Clostridium perfringens.* It is one of the gastrointestinal diseases in poultry that has gained worldwide importance during the last decade. Gastrointestinal diseases in broilers, including necrotic enteritis, viral enteritis, coccidiosis and syndromes such as dysbiosis and malabsorption, have become increasingly important worldwide for multiple reasons. First, high-density floor-housing supports easy spread of excreted gut pathogens (Guardia *et al*., 2011). Secondly, due to improvements in genetics, broilers have become incredibly efficient in converting feed into body mass and the gastro-intestinal tract of these animals is highly efficient in absorbing nutrients (Havenstein *et al.,* 2003). Gut micro-organisms play an essential role in degradation of feed components. There is a complex interplay between gut bacteria and the gastro-intestinal mucosa, that can be beneficial or harmful for the host, depending on the microbial composition (Valeria *et al.,* 2011). Nutritionists are also constantly looking for improving the limits of digestibility. As a side effect, gut health problems have arisen, related to shifts in enteric bacterial populations and bacterial overgrowth. Excess feed nutrients in the gut are used by facultative pathogenic micro-organisms, such as certain *Clostridium perfringens* (*C. perfringens*) strains. Shifts in enteric populations also lead to dysbacteriosis, a hitherto poorly defined syndrome causing inflammatory reactions in the gut resulting in poor performance and an increased feed conversion ratio (Teirlynck *et al.,* 2011). It has also been shown that genetics play a role in the development of the intestine, which can affect the microbiota composition (Lumpkins *et al.,* 2010). There is thus an interplay between host genetics and feed utilization that is important in either preventing or causing gastrointestinal problems. Another issue that is important in this context is the public and governmental pressure to reduce the use of antibiotics in broilers. The traditional antimicrobial growth promotors (AGP's), used in the past not only to improve feed conversion ratios and body weight gain, but also to prophylactically control diseases such as necrotic enteritis, have been banned

in the European Union in 2006. Consumers in other countries are also putting pressure on the poultry industry to rear animals without AGP's. Therapeutic antibiotics are nowadays widely used for preventive and curative control of gastro-intestinal pathologies and their preventative use is heavily disputed. All of these factors have led to the emergence of necrotic enteritis in broilers, caused by chicken-adapted NetB toxin-producing *C. perfringens* strains that belong to a certain pathogenic clonal lineage (Lepp et al., 2011). This pathogen clearly benefits from high nutrient diets supporting its fast growth. There is evidence that the use of AGP's in feed protected broilers from disease caused by *C. perfringens* (Johansson *et al.*, 2004; Martel *et al.*, 2004; Lanckriet *et al*., 2010a). Therapeutic antibiotics, such as amoxicillin and tylosin, are often used also to prevent and control necrotic enteritis (Hermans & Morgan, 2007). The use of antibiotics is no longer considered as a valid and viable strategy for keeping gut health problems under control because of issues related to antibiotic resistance. Therefore, better farm management, including biosecurity measures and optimization of feed quality have become more relevant. Additionally, feed additives, including organic acids, essential oils and prebiotics, have been tested in animal models and shown to be, at least partially, efficacious in controlling necrotic enteritis (Lensing *et al.*, 2010a; Timbermont *et al.*, 2010; Jerzsele *et al*., 2012). For a disease caused by a toxin producing bacterium, however, it seems logical, to explore whether vaccines can be developed.

1.2. *Clostridium perfringens*, the causative agent

C. perfringens is the causative bacterium of necrotic enteritis in broiler chickens. It is a Gram-positive, rod-shaped, spore-forming, anaerobic bacterium which can be found in the environment and also in the gastro-intestinal tract of humans and animals (Songer, 1996; Van Immerseel *et al.*, 2004). *C. perfringens* is less strictly anaerobic than other *Clostridia* species, and can survive under extreme conditions due to its switch from vegetative cells to highly resistant dormant spores (Novak *et al.*, 2003). The bacterium grows at an optimal temperature of 43-45°C with generation times often less than 10 minutes. Growth is accompanied by abundant gas production (Cato *et al.*, 1986). *C. perfringens* needs an environment rich in

amino acids because the bacterium lacks the genetic machinery to produce 13 essential amino acids (Myers *et al*., 2006). *C. perfringens* can produce up to 17 different toxins. *C. perfringens* toxin genes are located on the chromosome or on extrachromosomal elements, plasmids. Strains are classified into 5 different toxin types (Table 1), based on the differential production of the four major toxins (Petit *et al*., 1999; Van Immerseel *et al*., 2009).

Table 1: Toxinotypes of *C. perfringens* (Petit *et al.*, 1999)

Toxinotype	Major toxins			
	Alpha toxin	Beta toxin	Epsilon toxin	Iota toxin
A	X			
B	X	X	X	
C	X	X		
D	X		X	
E	X			X
Gene	*plc*	*cpb1*	*etx*	*Iap, ibp*
Location	chromosome	plasmid	plasmid	plasmid

Toxinotype A strains are commonly found in the normal intestinal microbiota of warm blooded animals and in the environment, but are also associated with gas gangrene in humans and enteric diseases in humans and animals. Strains of toxinotype B to E are only sporadic found in the normal intestinal microbiota of the intestines and most commonly associated with enteric diseases. An overview of the main diseases caused by *C. perfringens* in animals and humans is given in Table 2.

Table 2: Diseases produced by toxigenic types of *C. perfringens* (Songer, 1996)

Toxinotype	Major toxin(s)	Diseases
A	α	Myonecrosis, food poisoning, necrotic enteritis in chickens, enterotoxemia in cattle and lambs, necrotizing enterocolitis in piglets; possibly equine colitis, canine hemorrhagic gastroenteritis
B	α, β, ε	Dysentery in newborn lambs, chronic enteritis in older lambs (pine), hemorrhagic enteritis in neonatal calves and foals, hemorrhagic enterotoxemia in adult sheep
C	α, β	Enteritis necroticans (pigbel) in humans; necrotic enteritis in chickens; hemorrhagic or necrotic enterotoxemia in neonatal pigs, lambs, calves, goats, foals; acute enterotoxemia (struck) in adult sheep
D	α, ε	Enterotoxemia in lambs (pulpy kidney) and calves, enterocolitis in neonatal and adult goats, possibly enterotoxemia in adult cattle
E	α, ι	Enterotoxemia likely in calves and lambs, enteritis in rabbits; host range and disease type unclear
A-E	Enterotoxin	Canine and porcine enteritis; possibly bovine and equine enteritis

Various strains can also produce other toxins, which are not part of the toxinotyping scheme. Perfringolysin O (PFO), enterotoxin (CPE), Toxin perfringens Large (TpeL), β2 toxin and Necrotic Enteritis Toxin B-like (NetB) are so-called minor toxins (Uzal *et al.*, 2014).

In poultry, necrotic enteritis is caused mainly by toxinotype A strains, producing the NetB toxin. The alpha toxin was long believed to be the major virulence factor involved in the disease but it has been proven that it is not an essential virulence factor for the development of necrotic enteritis (Keyburn *et al.*, 2006; 2008). Keyburn et al. (2008) showed that a *netB* mutant was unable to induce necrotic lesions in the intestinal tract of experimentally infected broilers, but complementation with an intact *netB* gene restored virulence. NetB is a secreted pore forming toxin with 38% amino acid sequence similarity to the beta-toxin from *C. perfringens* and therefore it was designated Necrotic Enteritis Toxin B-like (NetB).

1.2.1. Alpha toxin

The *C. perfringens* alpha toxin is produced by any *C. perfringens* strain and is the only major toxin that is produced by chickens isolates. It is a zinc-dependent phospholipase C enzyme with lecithinase and sphingomyelinase activities (Saint-Joanis *et al.*, 1989). It is located in one of the most stable regions on the bacterial chromosome, close to the origin of replication. Nucleotide sequencing revealed a single open reading frame with a signal peptide (28 first amino acids) resulting in a mature protein of 370 amino acids. The molecular architecture of the alpha toxin reveals a two-domain protein with an amino-terminal domain composed from nine tightly packed α-helices (N-domain) (residues 1-246) and a carboxy-terminal domain composed of eight-stranded antiparallel β-sheets (C-domain) (residues 256-370) (Figure 1). The domains are joined by a central loop domain (residues 247-255) (Titball, 1999; Oda *et al.*, 2015).

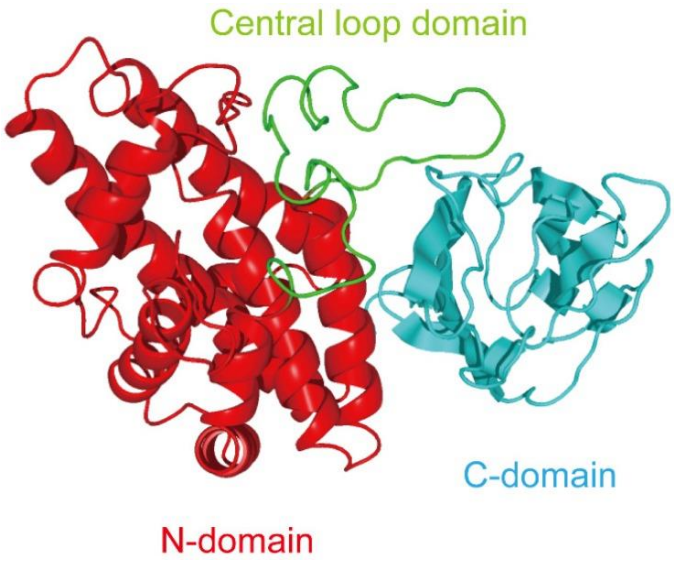


Figure 1: The structure of the *C. perfringens* alpha toxin showing the N- and C-domains linked by a flexible peptide (Oda *et al.*, 2015).

The N-domain of alpha toxin is the active site of the toxin and contains the zinc ions which are essential for the catalytic activity. The C-domain is a membrane binding domain (Eaton *et al*, 2002; Oda *et al.*, 2015). The C-terminal domain shows a strong structural similarity to eukaryotic calcium-binding C2 domains (Naylor et al., 1998). Calcium-ions play a role in the binding to the membrane phospholipids by conferring a positive charge on the polar head groups of the membrane phospholipids. The negatively charged toxin domain interacts with the positive charged calcium-ions and the C-domain can bind into the bilayer membrane. The binding of alpha toxin to membranes appears to result in the opening of the active site allowing hydrolysis of membrane phospholipids (Moreau *et al.*, 1988; Titball *et al*., 1999 and 2000).

Alpha toxin has haemolytic, cytotoxic, myotoxic and lethal activities. It causes membrane damage to erythrocytes and other cultured mammalian cells (Titball *et al.*, 1999). Alpha toxin causes massive degradation of phosphatidylcholine and sphingomyelin, two major components of the eukaryotic cell membranes, followed by membrane disruption and cell lysis (Sakurai *et al.*, 2004). The N-domain acts against phosphatidylcholine but not against sphingomyelin and is not haemolytic or cytotoxic. The

interaction between the N- and C-domain is essential to confer the sphingomyelinase activity (Titball *et al.*, 1999).

1.2.2. NetB

For years, alpha toxin was believed to be the major virulence factor involved in necrotic enteritis, until Keyburn et al. (2008) showed that an alpha toxin deleted mutant was still able to produce necrotic enteritis in an experimental model. A new toxin was identified in avian *C. perfringens* type A strains, a 323 amino acid protein including a 30 amino acid secretion signal sequence. Since it has a similarity to beta-toxin from *C. perfringens* (38% amino acid sequence identity) it was designated as NetB (Necrotic Enteritis Toxin B-like). The molecular architecture of a NetB monomer consists of 16 β-strands and an α-helix, which are arranged into the β-sandwich, latch, rim and prestem domains (Figure 2a). The β-sandwich domain contains a five-stranded and a six-stranded anti-parallel β-sheet. The prestem domain (residues 140 to 186) is formed of three-stranded anti-parallel β-sheets. The rim domain contains a four-stranded antiparallel β-sheet and a well-organized loop formed by residues 205 to 242. The rim domain involves a number of solvent-exposed aromatic groups (Figure 2) (Yan *et al*., 2013). The rim region is involved in membrane recognition and membrane binding and the aromatic residues of the rim region form direct contacts with the outer leaflet of the lipid membrane. Seven NetB monomers form the ring structure. NetB is a heptameric β-pore-forming toxin (PFT) that forms single channels in planar phospholipid bilayers (Rood *et al.,* 2016). The membrane fluidity is not the sole factor affecting NetB pore formation. The activity is also influenced by cholesterol which enhances the oligomerization of NetB and plays an important role in pore formation. NetB has a high hemolytic activity against avian red blood cells (Savva *et al*., 2013; Yan *et al.,* 2013).

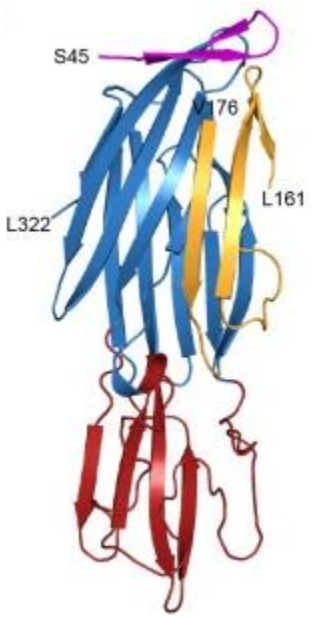
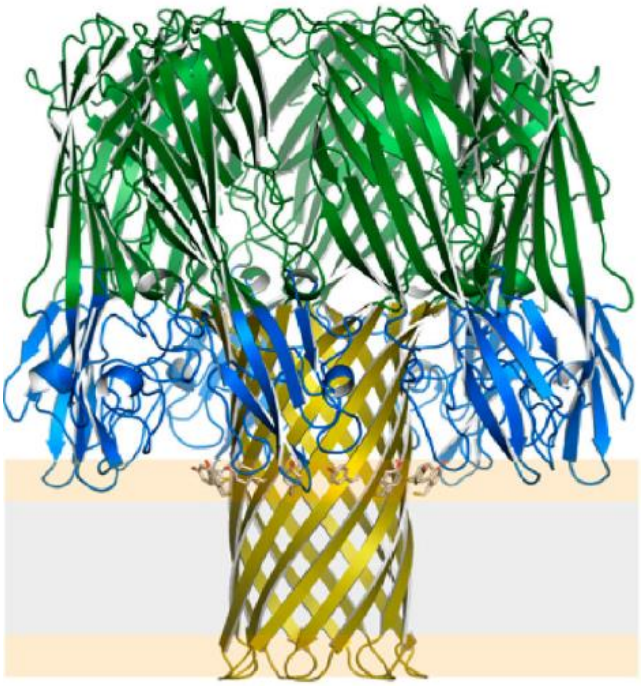


Figure 2: (a, above) The molecular structure of a NetB monomer. The β-sandwich domain is shown in blue. The prestem domain is shown in gold. The rim domain is shown in red and the amino latch domain is shown in magenta (b, below) Structure of the heptameric NetB toxin that forms a pore in the cell membrane (Yan *et al.*, 2013).



1.3. Clinical symptoms and pathogenesis

Necrotic enteritis can present in an acute clinical and in a subclinical form. Broiler chickens are most commonly affected at 2 to 5 weeks of age (Kaldhusdal et al., 2001). The disease develops at an age when maternal antibodies have declined and active production of antibodies is still insufficient (Lovland *et al.*, 2004). Clinical signs of the acute form include depression, ruffled feathers, diarrhea, huddling and anorexia, but more often the disease leads to sudden death, without any premonitory signs. An increased mortality of 1 to 5% in the broiler flock for several consecutive days during the last weeks of the rearing period is common. At necropsy an extensive necrosis of the small intestines is found (Figure 3). Also liver lesions, consisting of multifocal hepatic necrosis, can occur (Ficken & Wages, 1997; Kaldhusdal, *et al*., 2001).

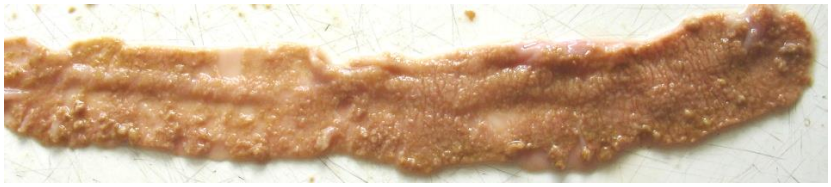


Figure 3: Severe necrotic lesions in the small intestinal tract of a boiler chicken.

The subclinical form causes no mortality but the damage to the intestinal mucosa leads to decreased absorption, reduced weight gain and an increased feed conversion ratio. At necropsy, focal necrotic lesions can be observed (Figure 4). Cholangiohepatitis can occur in the subclinical form (Elwinger *et al.*, 1992; Kaldhusdal *et al.*, 2001). Affected birds recover and areas of necrosis can heal completely so that the infection can go unnoticed. Nevertheless, the subclinical form is economically the most important one. Major causes of profit loss are increased feed conversion ratio, reduced live weight of the chickens and an increased number of condemnations at the slaughter house (Kaldhusdal *et al.*, 2016).

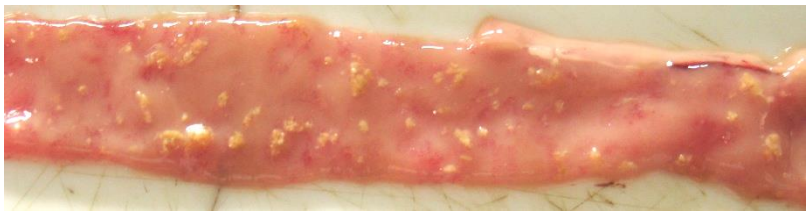


Figure 4: focal necrotic lesions in the intestinal tract of a broiler chicken.

Since the causative bacterium can be found in the normal intestinal microbiota, the disease must be triggered by a variety of predisposing factors. The most important predisposing factor is coccidial infection (Williams, 2005). *Eimeria* parasites colonize the small intestine and kill epithelial cells. The physical damage to the epithelium yields direct access for *C. perfringens* toxins to the intestinal mucosa and exposes extracellular matrix molecules, such as collagen, to which *C. perfringens* can adhere (Elwinger *et al.*, 1992; Wade *et al.*, 2016). Also plasma proteins are leaking into the gut, acting as a rich nutrient source for *C. perfringens* (Van Immerseel *et al.*, 2004). Eimeria is also resulting in increased mucus production, and mucus can also be used as nutrient source (Collier *et al.*, 2008). A second predisposing factor is the composition of the diet. Diets with high levels of indigestible, water-soluble non-starch polysaccharides, such as wheat and rye, can increase the viscosity and are predisposing (Choct *et al.*, 1996). High levels of animal protein in the diet, particularly fishmeal, are also predisposing for necrotic enteritis because they can act as substrates for the bacteria (Branton *et al.*, 1987; Riddell & Kong, 1992; Branton *et al*., 1997; Gholamiandehkordi *et al*., 2007; Van Immerseel *et al.*, 2009). Any factor that causes stress in broiler chickens can increase the incidence of necrotic enteritis. Infections with viruses such as Marek's disease virus, infectious bursal disease virus and chicken anemia virus can have immunosuppressive effects and increase the severity of necrotic enteritis. Other stress factors that are associated with necrotic enteritis are overcrowding, programmed alterations in the feeding regime and physiological stress (Hoerr, 2010). Some chicken lines have a higher degree of susceptibility so genetics also have an influence (Jang *et al.*, 2013). Mycotoxins can also influence intestinal permeability and affect the microbiota composition and be predisposing (Antonissen *et al*., 2015).

1.4. Antibody responses to *C. perfringens* antigens

The immune response to *C. perfringens* infection, including immune recognition of the pathogen and its secreted proteins and toxins, is still poorly understood. In this section we only describe antibody responses after infection or vaccination. Other host responses in necrotic enteritis are beyond the scope of this thesis. In addition, there are uncertainties about the type of antibodies (IgA, IgY) and the antigen(s) to which the antibodies are directed, when associated with protection. Infection takes place in the small intestine where the pathogen makes contact with the mucosal surface. The enteric immune system of neonatal broilers is poorly developed and matures rapidly during the first 4-6 weeks post hatch (Mast & Goddeeris, 1999). Generally, adaptive immune defense at the mucosal surface is mediated by initiation of lymphocyte activation and local secretion of IgA (Muir *et al.,* 2000; Sharma, 1999). Mucosal IgY may be important in protection against necrotic enteritis, since it is the major transferred maternal antibody and plays an essential role in the protection of young chickens against other pathogens. Maternal antibodies decline by about 3 weeks of age, which may explain why broiler chickens mostly develop necrotic enteritis around this time point (Ulmer-Franco *et al.,* 2012).

It was shown that the level of specific maternal antibodies against alpha toxin is higher in day-old chicks from older hens than in the chicks from younger hens. Broilers with high titers of specific maternal antibodies (IgY) against alpha toxin were shown to have lower mortality (Heier *et al.,* 2001). Levels of antibodies (IgY) against NetB and alpha toxin are significantly higher in apparently healthy chickens compared to chickens with clinical necrotic enteritis. This suggests that these antibodies may play a role in the protection against necrotic enteritis (Lee *et al*., 2012).

In several vaccination studies a mucosal IgA response against alpha toxin, NetB and other immunogenic proteins was reported in chickens that were partially protected against necrotic enteritis (Kulkarni *et al.*, 2007; Kulkarni *et al.,* 2010; Jang *et al*., 2012). However, in intestinal washings from experimentally infected birds only weak reactivity of mucosal IgA against proteins of *C. perfringens* was found. This

might indicate that a serum IgY response plays a more important role in immunity against necrotic enteritis than mucosal IgA. After systemic immunization with recombinant immunogenic proteins, serum IgY still reaches the mucosal surface under inflammatory conditions caused by *C. perfringens* (Williams, 2005; Kulkarni *et al*., 2007; Kulkarni *et al*., 2010).

1.5. The rise of necrotic enteritis and the consequences

The development of a global industry of specialized broiler production coincided with the introduction of anticoccidials and AGP's. Both of these improve the growth performance when added in low doses to the feed of animals, leading to economic advantages for the farmers (Campbell, 1998; Castanon, 2007). It is proven that these in-feed supplementations protected broilers against necrotic enteritis. Concerns about the potential risk of transmission of antimicrobial resistance and antibiotic residues in the food chain pushed the EU to ban the use of all AGP's in poultry feed, except for the ionophore antibiotics salinomycin and monensin (Regulation No. 1831, 2003). This led to a rise of necrotic enteritis with a high economic impact. In some European countries, the proportion of necrotic enteritis affected flocks has risen up to 40% after the ban of AGP's (Kaldhusdal and Lovland, 2000; Hermans and Morgan, 2007). Published studies on the occurrence of necrotic enteritis in chickens are few and characterized by certain limitations. A correct prevalence study has to be based on histological and bacteriological diagnosis whereas diagnosis of necrotic enteritis in the field is done simply by autopsy performed by the veterinarian. Since *C. perfringens* is a member of the normal intestinal microbiota, the bacterium is often isolated out of the post mortem intestinal tract. Empirical data show a very low prevalence of necrotic enteritis nowadays in broiler chickens in Western-Europe. In laying hens 10% of the flock is currently affected. Indirect measuring of the occurrence of necrotic enteritis in broiler chickens could be done by measuring the frequency of *C. perfringens*-associated cholangiohepatitis at the slaughterhouse, where all birds can be evaluated by competent meat inspection personnel (Kaldhusdal *et al.*, 2016). Nowadays, the peak in broiler necrotic enteritis is over, but it should be underlined that anticoccidials of the ionophore type, used

in broiler flocks, have antibacterial effects and act prophylactically against necrotic enteritis (Lanckriet *et al.*, 2010). The European Union allows the use of anticoccidials as feed additives because of the lack of alternatives. Also the use of therapeutic antibiotics is still high (Hermans and Morgan, 2007; Persoons *et al.*, 2012). Since the major economic loss is due to the effects of subclinical disease, the economic impact of necrotic enteritis is difficult to measure but estimated to be at least 2,5 billion dollars on world scale (Wade and Keyburn, 2016). When the ban on AGP's will become worldwide a new emergence can be expected in certain parts of the world.

1.6. Preventive and curative treatments for necrotic enteritis

Nowadays, necrotic enteritis is still controlled by therapeutic antibiotics, such as amoxicillin and tylosin (Hermans & Morgan, 2007). However, due to antibiotic resistance, the use of antibiotics is no longer considered as a viable strategy for keeping gut health problems under control. Therefore, better farm management, including biosecurity measures and optimization of feed quality have become more relevant. Additionally, feed additives in poultry diets improve digestibility and the absorption of dietary nutrients and as a consequence may reduce pathogen colonization. Organic acids, essential oils and prebiotics, all have been tested in *in vivo* animal models and shown to be, at least partially, protective against necrotic enteritis (Lensing *et al.*, 2010a; Timbermont *et al.*, 2010; Jerzsele *et al*., 2012). The concept of competitive exclusion, in which whole gut flora is administered to the animals, is also known to be effective against necrotic enteritis (Elwinger et al., 1992; Kaldhusdal et al., 2001). For a disease caused by a toxin producing bacterium, it seems logical, however, to explore whether vaccines can be developed, which may or may not be based on the causative toxins. Considerable efforts have been made in recent years in this area. Proteins and toxins have been tested as vaccine candidates. In addition, the use of live vectors is under investigation and studies are being carried out on practical strategies for vaccination in the field. A major question is how birds can be protected by vaccination in the limited time span of 3 to 4 weeks before the lesions are most likely to develop. The disease develops at an age when maternal

antibodies have declined, but preventive vaccination of young broilers is hampered by their immature immune system and by practical problems related to vaccination protocols, because mass parental vaccination is possible at day 1 but not beyond this point. Solutions are being developed to solve these issues. Possibilities are vaccination of breeder hens and the use of live bacterial or viral vectors, so antigens can be delivered orally or *in ovo*.

1.7. Vaccination against necrotic enteritis in broiler chickens

1.7.1. An overview of vaccination studies against necrotic enteritis

There are various ways to deliver antigens to chickens for immunization purposes. Candidate bacterial vaccines can be based on live (attenuated) organisms or killed (inactive) organisms. Live (attenuated) vaccine strains may be superior because they often have the ability to induce a stronger and longer lasting immune response and can be administered orally, but there may be some safety concerns (Witter & Hunt, 1994; Plotkin & Plotkin, 2011; Rappuoli *et al*., 2011). For a toxin secreting bacterium, however, it seems logical that culture supernatants or toxin-based formulations are used because the factors that induce lesions are present in these solutions. They should be produced in inactivated form while preserving antigenicity. Formalin inactivation and genetically engineered inactive toxin variants are an option, as is the delivery of immunogenic non-toxin proteins. DNA vaccines that express *Clostridium* toxins, but not *C. perfringens* toxins, have also been tested as vaccine candidates (Saikh *et al*., 1998; Gardiner *et al*., 2009; Li *et al.* 2011; Jin *et al*., 2013). An overview of studies on vaccination against necrotic enteritis is given in Table 3.

1.7.1.1. Live attenuated vaccines

The principle that previous infections with *C. perfringens* strains induce protection against challenge was proven by Thompson *et al*. (2006). These authors orally administered virulent strains to 15 day old broiler chickens during 5 consecutive days, followed by treatment with bacitracin for nine days to clear the

virulent strains. An oral challenge with virulent strain *C. perfringens* CP4 resulted in significantly fewer chickens with lesions (mean lesion score 0.13 instead of 2.09 in the non-immunized group). These data show the potential of vaccination with live strains, but a major issue with live vaccines is the balance between attenuation and protection. Indeed, live strains should be attenuated without losing the ability to protect against disease. When an avirulent strain was used for oral immunization using the same immunization-infection protocol, no protection was conferred. In contrast, an alpha toxin mutant of the challenge strain induced partial protection against infection with an isogenic challenge strain, i.e. a significant decrease in number of birds with necrotic lesions was observed (Thompson *et al.*, 2006). It could very well be that residual virulence (which may include NetB production) is essential for a live vaccine strain to be protective. Indeed, an avirulent strain may not provide protective antigens to the gut associated lymphoid tissues, and as a consequence not confer protection.

1.7.1.2. Protein-based vaccines

Protein-based vaccines are used because they are safer and better characterized when compared to live vaccines, while still providing protection (Unnikrishnan *et al*., 2012). They include toxoids (inactivated bacterial toxins) and subunit vaccines, often based on virulence factors or secreted toxins (Berzofsky *et al.*, 2001). *C. perfringens* is known to produce many different toxins and proteins. Some studies used crude culture supernatants (whether inactivated or not) as vaccines, whilst other vaccination trials were carried out using inactivated toxins and highly antigenic proteins.

1.7.1.2.1. Crude supernatant vaccines

Both non-inactivated supernatant and formaldehyde-inactivated supernatant (crude toxoid) of *C. perfringens* have been studied as potential vaccines for the prevention of clinical and subclinical necrotic enteritis with variable success. In a study by Saleh *et al.* (2011), subcutaneous vaccination of broilers at 7 and 21 days with *C. perfringens* type A, type C and combined type A and C crude toxoids significantly decreased the number of animals developing intestinal lesions. When breeder hens were vaccinated at 14

and 18 weeks of age with type A and type C crude toxoids and their progeny was challenge exposed under both field conditions and in a disease model, type C crude toxoid was shown to provide better protection than type A crude toxoid (Lovland *et al.,* 2004). The safety and efficacy of a *C. perfringens* type A alpha toxoid (Netvax^{TM}) was investigated by vaccinating breeder hens intramuscularly at 11 and 18 weeks of age. In this field trial, the progeny from vaccinated hens had a reduced mortality compared to the progeny from unvaccinated hens (Crouch *et al.,* 2010). It was, however, unclear whether this mortality was related to necrotic enteritis. Lanckriet *et al.* (2010b) compared the non-inactivated supernatant of 8 *C. perfringens* strains, with different alpha toxin and NetB content, using subcutaneous vaccination at 3 and 12 days. They showed important variation in the protective capacity depending on the strain used for supernatant preparation. This suggests that protective immunity is probably determined by an effective combination of different bacterial immunogens or that the expression levels of one or more antigens drives protection conferred by vaccination. The strain used for crude supernatant preparation is thus of crucial importance when designing the vaccine type. It is clear that non-inactivated supernatant always contains a risk because of the presence of active toxins, and thus crude toxoids are preferred for safety reasons. Formaldehyde is generally used for inactivating proteins in vaccines but can reduce the protective capacity of the vaccine.

1.7.1.2.2. Alpha toxin

The alpha toxin is the most investigated *C. perfringens* toxin in terms of vaccine-induced protection, mainly in mouse gangrene models (Stevens *et al*., 2004; Titball, 2009). As mentioned before the alpha toxin is composed of 2 domains, which are associated with phospholipase C activity (N-domain) and membrane recognition (C-domain) (Naylor *et al*., 1998). Monoclonal antibodies against alpha toxin which are capable of neutralizing the phospholipase C activity, are not necessarily effective in protecting against alpha toxin induced hemolysis and mortality in gangrene models (Sato *et al*., 1989, Logan *et al*.,

1991). Nevertheless, the immune response against the C-terminal domain provides protection against challenge with alpha toxin and also against experimental gas gangrene in mice (Stevens *et al*., 2004).

Before the NetB toxin was identified as the major toxin in necrotic enteritis in broilers, alpha toxin was believed to be crucial and thus multiple studies used alpha toxin derivatives as vaccine antigen. It has been shown that broilers with a history of clinical or sub-clinical necrotic enteritis have a natural serum antibody response to alpha toxin (Heier *et al*., 2001; Lovland *et al*., 2003). When broilers are vaccinated subcutaneously with recombinant alpha toxin at 5 and 15 days of age a decrease in the number of animals with necrotic enteritis lesions is found (Cooper *et al.,* 2009). Also Jang *et al*. (2012) vaccinated broilers subcutaneously at day 1 and day 7 with recombinant alpha toxin and could induce protection against challenge. Using double and triple intramuscular vaccination regimens (day 7, 14 and 21), Kulkarni *et al.* (2007) showed that a prior vaccination with alpha toxoid and a boost with active toxin protected against experimental necrotic enteritis. A triple vaccination of either alpha toxoid or active toxin, however, offered no protection. It was suggested that the failure in protection using the active toxin may have resulted from the toxin activity on immune cells and the failure of alpha toxoid may be the consequence of loss of conformation of the protein, resulting in loss of epitopes, as mentioned before. Although alpha toxin has been shown to play no primary role in the induction of necrotic enteritis, the antigen can still induce a certain level of protection. It has been shown by Zekarias *et al*. (2008) that anti-alpha toxin antibodies bind to the cell wall of the bacterium and suppress its growth *in vitro*. Secreted proteins and toxins of Gram-positive bacteria accumulate within the cytoplasm, and most adhere to the bacterial cell membrane (Ton-That *et al.*, 2004; Schneewind and Missiakas, 2012). Binding of antibodies to the membrane-bound preprotein might block protein transport channels and hereby inhibit proliferation of the bacterium. It suggests an unusual effect of vaccines, which directly affects the bacterium rather than neutralizing the toxin. Alpha toxin can thus be used as a protective antigen to vaccinate broilers, even if the toxin does not play a primary role in the pathogenesis of necrotic enteritis. It is possible that other antigens will be identified that have similar mechanisms of action when used for immunizing chickens. A combination of antigens generating antibodies that inhibit bacterial proliferation and other antigens

generating antibodies that inhibit toxin activity could be more efficient than one of the individual approaches.

1.7.1.2.3. NetB toxin

The discovery of the genetically highly conserved NetB toxin as an essential virulence factor opened new perspectives for the development of vaccines for the control of necrotic enteritis (Keyburn *et al.*, 2008; Keyburn *et al.*, 2010a; Keyburn *et al.*, 2010b). After the structure and function of the NetB toxin protein was analyzed, mutants with reduced cytotoxic activity were designed (Savva *et al*., 2013; Yan *et al*., 2013). The mutation of tryptophan to alanine at position 262 (W262A) resulted in a significant reduction in cytotoxicity to LMH cells and hemolytic activity on red blood cells, and thus generated a promising vaccine candidate (Savva *et al*., 2013; Fernandes da Costa *et al.*, 2013). In the present thesis this was further investigated.

1.7.1.2.4. Other proteins

In addition to toxin-derived protein vaccines, highly immunodominant proteins can potentially be used to protect animals against necrotic enteritis by vaccination. Full protection is probably determined by an effective combination of different bacterial immunogens (Lanckriet *et al*., 2010b). Several purified *C. perfringens* proteins have been evaluated as potential vaccine candidates. Studies have identified antigens recognized by post-infection sera from chickens immune to necrotic enteritis. Hypothetical protein (HP), pyruvate:ferredoxin oxidoreductase (PFOR), elongation factor G (EF-G), perfringolysin O, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and a fructose-1,6-biphosphate aldolase (FBA) were identified using post-infection serum from chickens immune to virulent *C. perfringens* challenge in infection-immunization experiments (Kulkarni *et al.*, 2006). Jiang *et al.* (2009) identified the *C. perfringens* large cytotoxin (TpeL), endo-beta-N-acetylglucosaminidase (Naglu) and phosphoglyceromutase (Pgm) as dominant antigens using post-infection serum from chickens immune to necrotic enteritis. Elongation factor Tu (EF-Tu) and PFO were identified by reaction with immune sera

from chickens derived from a clinical outbreak. Kulkarni *et al.* (2007) immunized chickens intramuscularly two (or three) times at an age of 7, 14 (and 21) days with recombinant proteins alpha-toxin/alpha toxoid, GAPDH, HP, FBA, and PFOR. All the proteins were able to decrease the mean intestinal lesion score. The degree of protection depended on the severity of the challenge. Alpha toxin, HP, and PFOR protected significantly against heavy challenge. GAPDH and FBA protected only against mild challenge. More recently, double subcutaneous vaccination regimens using alpha toxin, NetB toxin, PFOR and EF-Tu gave similar protection levels after experimental infection (Jang *et al*., 2012). Immunization with Naglu and Pgm yielded partial protection after challenge with two different strains. Again, the protection level decreased when the challenge severity increased (Jiang *et al*., 2009). All the above described data thus show that multiple proteins, including derivatives from alpha and NetB toxin, have potential as vaccines, and that defined mixtures of these proteins need investigation.

1.7.1.3. Attenuated live vectors expressing *C. perfringens* proteins

Attenuated or avirulent bacteria can be used as vehicles for the effective delivery of vaccine candidates (Rappuoli *et al*., 2011). Attenuated *Salmonella* strains are often used in poultry for the control of salmonellosis. They can serve as safe and effective oral carrier vaccines to prevent several poultry diseases by expressing heterologous antigens (Hegazy & Hensel, 2012). Because the attenuation is usually induced by a deletion mutation in a gene that is essential for the metabolism of the bacterium, the vaccine carrier strains can not overgrow the immune system of the animal host (Spreng *et al.*, 2006). Zekarias *et al.*(2008) evaluated the efficacy of a live recombinant attenuated *S. enterica* serovar Typhimurium vaccine strain that delivered the C-terminal domain of the alpha toxin. The vaccine strain was twice administered orally at 3 and 17 days of age. Thereafter the birds were challenged by oral inoculation and repeated infection through contaminated feed with a virulent *C. perfringens* strain. A significant reduction in the number of birds with necrotic lesions was observed. Kulkarni *et al*. (2008) showed that the delivery of FBA and HP using an attenuated *S. enterica* serovar Typhimurium vaccine vector by the oral route

induced a significant protective immune response. Broilers immunized with the vaccine strain, expressing PFOR, at day 1 and day 14, however, were not significantly protected against necrotic enteritis. The authors also tested *Salmonella* strains expressing truncated nontoxic alpha toxoid and truncated HP (tHP). The alpha toxoid consisted of a region of 162 amino acid residues that included two sections of immunodominant epitopes as well as regions of weak reactivity. Broiler chickens immunized orally with a *Salmonella* strain expressing nontoxic alpha toxoid, at days 1 and 10 of age, were significantly protected against moderate challenge but not protected against severe challenge, while chickens immunized with tHP were protected against both moderate and severe challenge (Jiang *et al*., 2010; Kulkarni *et al.*, 2010). While *Salmonella* strains are thus potential vaccine carriers for *C. perfringens* proteins, there are other possibilities that, although not yet explored for protection of poultry against necrotic enteritis, can be of value. The expression of the C-terminal domain of alpha toxin on the surface of *Bacillus subtilis* spores was described and shown to be immunogenic in mice (Hoang *et al*., 2008). Lactic acid bacteria can also be used as vaccine carriers for *Clostridium* antigens (Robinson *et al*., 1997; Robinson *et al*., 2004). *B. subtilis* and lactic acid bacteria have the advantage of having a GRAS (generally recognized as safe) status. The use of live vectors to express *C. perfringens* proteins in the gut of broilers is thus a promising approach and deserves further attention, but will be very complex, because the vector needs to present the antigens to the mucosal immune system. The choice of the proteins to be expressed is also an important issue.

Table 3: Summary of studies on vaccination against necrotic enteritis in broilers described in the scientific literature. The table shows the route of administration, vaccine regimen, antigen, dose, vector (if used), adjuvant, the result of the vaccination study and the literature reference.

Route of Administration	Vaccination regime	Vector/Adjuvant	Antigen and dose	Protection	Reference
IM	Double (breeder hens week 14 and 18)	20% Alhydrogel and 0.013% thiomersal	- Type A crude toxoid (0.25ml of 1TCP*) - Type C crude toxoid (0.25ml of 30TCP*)	- Specific antibody response against alpha toxin in breeder hens and their progeny - Less mortality in progeny	(Lovland *et al*., 2004)
Oral	Infection-immunization for 5 consecutive days	Mixed in feed at ratio 2:1 (feed:broth culture)	- Avirulent strain CP5 - Virulent strain CP1 - Virulent strain CP4 - Alpha toxin deficient mutants (Cpa^{-1},Cpa^{-2},Cpa^{-3} and Cpa^{-4})	- Reduction in chickens with lesions that were infection-immunized with CP1, CP4, Cpa^{-2} and Cpa^{-4}	(Thompson *et al*., 2006)
IM	Double or triple (day 7, 14 (and 21)	Quil A	- Alpha toxin - Alpha toxoid - HP* - FBA* - GDP* - tPFOR* 20µg in triple vaccination, 40µg in double vaccination	- Serum and intestinal antibody response against immunogens - Reduction in chickens with lesions depending on the severity of challenge	(Kulkarni *et al*., 2007)
Oral	Double (day 1 and 14)	Attenuated *S. enterica* serovar *Typhimurium X*9241	- FBA* - tPFOR* - tHP* 100µl containing 10^9 CFU	- Serum and intestinal antibody response against immunogens - Reduction in main lesion score and increase in body weight gain (FBA and tHP)	(Kulkarni *et al*., 2008)
Oral SC	Double (day 3 and 13) Double (day 3 and 17) Triple (day 3, 13 and 35)	Attenuated *S. enterica* serovar *Typhimurium X*8914 Complete Freunds adjuvant (SC)	- C-terminal domain of alpha toxin (rPLC) 50µg (SC) 500µl containing 10^9 CFU (oral)	- Low serum antibody response - Reduction in number of chickens with lesions - Reduction in lesion score	(Zekarias *et al*., 2008)

Table 3: Summary of studies on vaccination against necrotic enteritis in broilers described in the scientific literature. The table shows the route of administration, vaccine regimen, antigen, dose, vector (if used), adjuvant, the result of the vaccination study and the literature reference.

SC	Double (day 5 and 15)	Quil A	- Alpha toxin 20µg	- Specific serum antibody response against alpha toxin - Reduction in number of chickens with lesions	(Cooper *et al*., 2009)
IM	Double (breeder hens week 11 and 18-19)	Light mineral oil	- Type A crude toxoid (0.5ml of 3 TCP*)	- Specific antibody response against alpha toxin in breeder hens and their progeny - Lower mortality rate in field trial	(Crouch *et al*., 2010)
Oral IM	Double (day 1 and 10) Triple (day 1, 10 and 17)	Attenuated *S. enterica* serovar *Typhimurium X*9352	- Alpha toxoid (region of 162 amino acid residues) - tHP 100µl containing 10^9 CFU	- Serum and intestinal antibody response against immunogens - Reduction in chickens with lesions depending on the severity of challenge - Increased body weight	(Kulkarni *et al*., 2010)
SC	Double (day 3 and 12)	Quil A	- Supernatant of 8 type A strains (variable NetB and alpha toxin content) 7 and 70µg	- Reduction in number of chickens with necrotic lesions	(Lanckriet *et al*., 2010b)
IM	Double or triple (day 7, 14 (and 21)	Quil A	- Naglu* - Pgm*	- Serum and intestinal antibody response against immunogens - Reduction in chickens with lesions depending on the severity of challenge and challenge strain	(Jiang *et al.,* 2009)
SC	Double (day 7 and 21)	Unknown	- Crude toxoid A - Crude toxoid C - Crude toxoid AC	- Serum antibody response against immunogens - Reduction in number of chickens with necrotic lesions	(Saleh *et al.,* 2011)
SC	Double (day1 and 7)	Montanide ISA 71 VG	- Alpha toxin - NetB - EF-Tu* - PFO* 50µg	- Specific serum antibody response against NetB and PFO - Reduction in lesion score	(Jang *et al.,* 2012)
SC	Triple (day 3, 9 and 15)	Quil A	- NetB toxoid - NetB (W262A) 30µg	- Reduction in number of chickens with necrotic lesions - Reduction in mean lesion score	(Fernandes da Costa *et al*., 2013)

Table 3: Summary of studies on vaccination against necrotic enteritis in broilers described in the scientific literature. The table shows the route of administration, vaccine regimen, antigen, dose, vector (if used), adjuvant, the result of the vaccination study and the literature reference.

SC	Double (day 7 and 17)	60% Montanide 40% Quil A DEAE-dextran	- NetB - Bacterin (50:50 bacterial cells and culture supernatant) - Bacterin + NetB 50µg	- Specific serum antibody response against NetB - Reduction in average lesion score depending on the severity of challenge	(Keyburn *et al.*, 2013b)
SC	Triple (breeder hens week 22, 24 and 26)	60% Montanide 40% Quil A DEAE-dextran	- rNetB(S254L) - Crude toxoid (type A, NetB positive) - Crude toxoid (type A, NetB positive) + rNetB(S254L) 50µg	- Specific antibody response against NetB in breeder hens and progeny - Reduction in number of chickens with necrotic lesions in experimental infection trial in progeny	(Keyburn *et al.*, 2013a)

*TCP (total combining power), (t)HP ((truncated)Hypothetical protein), FBA (fructose-1,6-biphosphate aldolase), GDP (glyceraldehyde-3-phosphate dehydrogenase), (t)PFO(R) ((truncated) pyruvate: ferredoxin oxidoreductase), Naglu (endo-beta-N-acetylglucosaminidase), Pgm (phosphoglyceromutase), EF-Tu (elongation factor Tu), CFU (colony forming units)

SC (subcutaneous), IM (intramuscularly)

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Chapter 2

Scientific aims

Scientific aims

Necrotic enteritis in broilers is caused by *Clostridium perfringens* type A strains that produce the NetB toxin. It is one of the gastrointestinal diseases in poultry that has gained worldwide importance during the last decade. Prevention strategies include avoiding predisposing factors, such as coccidiosis, and in-feed supplementation of a variety of feed additives. All of these measures can reduce the incidence but definitely do not (fully) protect against the disease. Therefore, vaccination seems a logical preventive tool for protection against necrotic enteritis. Considering the importance of bacterial toxin production in the pathogenesis, vaccination with a modified toxin or other secreted immunogenic proteins appears to be the best option. Previous studies in this field have generated promising results, with, however, still some major drawbacks in the practical applicability of the vaccine candidates. Therefore, the general aim of this thesis was to design novel strategies for practical application of vaccination against necrotic enteritis in broilers. Most published studies have used multiple dosage subcutaneous vaccination regimens that are not relevant for practical use in the broiler industry. The use of active toxins or crude supernatant is not possible in the field for safety reasons. It is essential to either use pure immunogenic non-toxic proteins or inactivated proteins. The ***first specific aim of this thesis was to evaluate the efficacy of subcutaneous vaccination with crude formaldehyde toxoids, as compared to crude bacterial supernatant, using different vaccination regimens, and to explore whether single shot vaccination at young age yields protection.*** Several studies have been carried out recently to identify the most important immunogenic and protective proteins that can be used for vaccination and many proteins and toxins have been tested as vaccine candidates in recent years. However, Lanckriet *et al*. (2010) performed, to our knowledge, the only vaccination study which resulted in full protection against necrotic enteritis after challenge with virulent strains. The supernatant of *C. perfringens* strain 23 was clearly shown to be superior to the supernatant of 7 other *C. perfringens* strains. ***The second specific aim of this work was thus to identify unique proteins in the supernatant of C. perfringens strain 23 that contribute to protection of broilers***

against the development of necrotic enteritis. The NetB toxin is known to be the most important virulence factor and acts immunoprotective. Despite the fact that it has been shown that alpha toxin is not essential for the development of necrotic enteritis, it can also be used as a protective antigen to vaccinate broilers. To study the positive effect of vaccination it is necessary to induce the disease. The method used for the experimental reproduction of necrotic enteritis can affect the results. Some antigens are solely protective against mild challenge but not against severe challenge, and there is evidence that a combination of different antigens is needed to obtain an optimal protection. Therefore, the ***third specific aim of this thesis was to investigate whether a combination of a non-toxic NetB W262A variant and a non-toxic fragment of the C-terminal domain of alpha toxin could provide improved protection against disease as compared to vaccination with individual antigens***. Vaccination was evaluated in two experimental infection models (in-feed and oral administration).

Chapter 3

Experimental studies

3.1. Day-of-hatch vaccination is not protective against necrotic enteritis in broiler chickens

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Abstract

Necrotic enteritis (NE), caused by *netB* toxin producing *Clostridium perfringens* type A, is an important disease in broiler chickens worldwide. Attempts to prevent necrotic enteritis by vaccination hitherto have insufficiently taken into account the practical limitations of broiler vaccination. In most published studies on vaccination against necrotic enteritis multiple doses at different ages are administered, which is practically impossible for broilers. The aim of this study was to compare the efficacy of subcutaneous single vaccination at day 1 or day 3 and double vaccination at day 3 and day 12, using crude supernatant containing active toxin or formaldehyde inactivated supernatant (toxoid) of a *netB* positive *C. perfringens* strain in a subclinical necrotic enteritis model. Double vaccination with crude supernatant resulted in a significant decrease in the number of chickens with necrotic enteritis lesions. The efficacy of vaccination using toxoid was lower compared to crude supernatant. Single vaccination with crude supernatant at day 3 resulted in significant protection, while vaccination of one-day old chickens with crude supernatant or toxoid, as envisaged for practical field application, did not induce protection.

Introduction

Necrotic enteritis, caused by *Clostridium perfringens* type A, occurs in broiler chickens and emerged after the ban on the use of antimicrobial growth promoters (AGP's) in the European Union in 2006. *C. perfringens* infections in poultry may present as an acute clinical disease, with high mortality at 2 to 5 weeks of age, or in a subclinical form leading to reduced weight gain (Kaldhusdal *et al*., 2001). The disease develops when several predisposing factors are present, such as a coccidial co-infection and a high protein high non-starch polysaccharide containing diet (Thompson *et al*., 2006; Gholamiandehkordi *et al*., 2007; Van Immerseel *et al*., 2009). Nowadays, necrotic enteritis is typically controlled using antibiotics and anticoccidials (Lanckriet *et al*., 2010). Due to concerns about the spread of antibiotic resistant bacteria and antibiotic residues in the food chain, there is a need for alternative control strategies. The use of feed additives, including organic acids, essential oils and prebiotics, can only marginally decrease the incidence of necrotic enteritis in broilers. No feed additives are as efficient as antibiotics in controlling the disease (Lensing *et al.*, 2010; Thanissery *et al.*, 2010; Timbermont *et al.*, 2010; Jerzsele *et al*., 2012).

Vaccination of broilers may be an interesting option for the prevention of necrotic enteritis. In the literature, vaccination trials are described using crude supernatant, inactivated supernatant and recombinant proteins; the latter either injected or administered using a live bacterial vector. Different vaccination approaches have been used in several necrotic enteritis models in the past few years (Lee *et al*., 2011). Vaccination in broiler chickens can be done using oral, subcutaneous or intramuscular administration. All previous reports on subcutaneous or intramuscular vaccination experiments are based on double or triple vaccination schedules. Both crude supernatant and formaldehyde-inactivated supernatant (toxoid) of *C. perfringens* have been studied as potential vaccines for the prevention of clinical and subclinical necrotic enteritis with variable degrees of success (Kulkarni *et al.*, 2007; Cooper *et al.*, 2009; Jang *et al.*, 2009; Lanckriet *et al.*, 2010; Saleh *et al.*, 2011; Jang *et al.*, 2012).

All studies carried out in the past use vaccination regimens that are not applicable in the field for logistical reasons. Indeed, subcutaneous or intramuscular injection of broilers is only possible at the hatchery at day

of hatch. The use of crude supernatant is not possible for safety reasons and it is thus essential to either use pure immunogenic non-toxic proteins or inactivated proteins. In *Clostridium* vaccines safety is often guaranteed using formaldehyde inactivation to produce a so-called toxoid (Jones *et al.*, 2008).

Therefore, the objective of this study was to compare the efficacy of subcutaneous vaccination with crude supernatant and toxoid, using different vaccination regimens. Broilers were either once vaccinated at day of hatch or at 3 days of age or twice at days 3 and 12.

Materials and Methods

***Clostridium* strains and culture conditions.** *C. perfringens* strain 23 was used for preparing crude supernatant and toxoid vaccines. This strain is a *netB* positive toxin type A strain isolated from a broiler chicken (Gholamiandehkordi *et al*., 2006; Lanckriet *et al*., 2010). The challenge strain used in the *in vivo* trials, *C. perfringens* strain 56, a *netB* positive toxin type A strain, was isolated from a broiler chicken with necrotic lesions and has been shown to be highly virulent in *in vivo* trials (Gholamiandehkordi *et al*., 2007; Timbermont *et al*., 2009; Lanckriet *et al*., 2010). Bacteria were grown at 37°C in Brain Heart Infusion (BHI) broth (Oxoid, Basingstoke, UK) supplemented with 0.375% glucose in an anaerobic (84% N_2, 8% CO_2 and 8% H_2) workstation (Ruskinn Technology, South Wales, UK).

Vaccines. For all *in vivo* trials, supernatant derived from an overnight culture of *C. perfringens* strain 23 was concentrated using Vivaspin containing a 5,000 MWCO PES membrane (Sartorius Stedim Biotech GmbH, Goettingen, Germany). The protein concentration from the supernatant was determined using a commercially available BCA Protein Assay Reagent (Thermo Scientific Pierce, Rockford, USA). The concentrated supernatant was diluted in PBS to final protein concentrations as described in Table 1. The supernatant used for toxoid preparation was inactivated for 16h at 37°C with 1% (trial 1) or for 24h at 37°C with 0.5% (trial 2 and trial 3) formaldehyde solution (Sigma-Aldrich, Bornem, Belgium) to produce

a toxoid. Inactivation of the supernatant was tested by analyzing the inactivation of the alpha toxin and theta toxin by loss of double hemolytic zone when plating droplets on Columbia agar containing 5% sheep blood (Columbia Blood agar base®, Oxoid, Wesel, Germany). Quil A (Brenntag Biosector, Frederikssund, Denmark) was used as adjuvant (10 mg/ml PBS solution) (50 µg/bird/vaccination) in all test groups. The total volume administered to each bird was 0.2 ml. The freshly prepared vaccines were filter-sterilized (0.2 µm).

***In vivo* necrotic enteritis model.** The *in vivo* necrotic enteritis model was based on the subclinical *in vivo* model as described previously (Gholamiandehkordi *et al*., 2007). Groups of a variable number (indicated in Table 1) of one-day-old Ross 308 broiler chickens were fed a wheat/rye-based (43%/7.5%) diet, with soybean meal as protein source. The feed composition was as described elsewhere (Gholamiandehkordi *et al*., 2007). Briefly, the diet contained high levels of (animal) proteins and non-starch polysaccharides which predispose to the development of necrotic enteritis. In all trials, Nobilis Gumboro D 78 vaccine (Schering-Plough Animal Health, Brussels, Belgium) was given in the drinking water on day 16 in all groups. From day 17 onwards, soy bean meal was replaced by fishmeal (30%) as protein source. All groups were orally challenged on day 17, 18, 19 and 20 with approximately 4.10^8 cfu *C. perfringens* strain 56 bacteria per challenge dose (Table 1). On day 18, all groups were orally inoculated with a ten-fold dose of Paracox-5 (Schering-Plough Animal Health, Brussels, Belgium).

In trial 1, 3 control groups were included. Two were left unvaccinated (group 1 and 2). In group 1 all animals were inoculated with *C. perfringens* once a day during 4 consecutive days, while in group 2 three inoculations per day were given from day 17 till day 20. The third control group (group 3) was vaccinated with PBS and Quil A at day 3 and 12 post-hatch, and *C. perfringens* inoculations were done 3 times a day between day 17 and 20. Five test groups were included. They were vaccinated subcutaneously in the neck with a 200 µl dose. Group 4 was vaccinated with crude supernatant containing 7 and 70 µg total protein at day 3 and day 12, respectively. Group 5 was vaccinated with 7 µg crude supernatant at day 3. Group 6

was vaccinated at day 3 and day 12 with 7 and 70 µg toxoid, respectively. Groups 7 and 8 were vaccinated with 7 µg toxoid at day 3 or day 1, respectively (Table 1). On day 22, 23 and 24, each day one-third of the birds were euthanized.

Trial 2 was carried out to clarify whether vaccination at day 3 and 12 with crude supernatant yielded better protection as compared to vaccination at day 3 and 12 using toxoid. One control group was left unvaccinated (group 1) and another control group was vaccinated with PBS and Quil A at day 3 and day 12 post-hatch (group 2). Group 3 was vaccinated at day 3 and day 12 with 7 µg and 70 µg crude supernatant, respectively, while group 4 was vaccinated at day 3 and day 12 with 7 µg and 70 µg toxoid, respectively (Table 1). All birds were euthanized on day 21.

Trial 3 was carried out to compare the efficacy of vaccination at day 1 with crude supernatant or toxoid at different dosages with vaccination at day 3 and 12. One control group was left unvaccinated (group 1) and another control group was vaccinated with PBS and Quil A at day 3 and day 12 post-hatch (group 2). Group 3 and 4 were vaccinated at day 3 and day 12 with 7 µg and 70 µg crude supernatant or toxoid, respectively. The other test groups (group 5 to 12) were vaccinated at day 1 with different doses (35µg, 70µg, 140µg, 210µg) crude supernatant or toxoid (Table 1). On day 21, 22, 23, each time one-third of the birds were euthanized. The bird experiments were carried out according to the recommendations and following approval from the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University.

Table 1: Description of experimental groups used in this study.

Trial	Group	Birds per group	Vaccine	Vaccination dose (µg)	Vaccination day(s)	Challenges per day	Number of animals with lesions/total number	Percentage of animals with necrotic enteritis
1	1	23	-	-	-	1	12/23	52
	2	26	-	-	-	3	14/26	54
	3	26	Quil A + PBS	-	3, 12	3	17/26	65
	4	29	Active SN	7µg, 70µg	3, 12	3	11/29	38[A]
	5	26	Active SN	7µg	3	3	9/26	35[A]
	6	25	Toxoid	7µg, 70µg	3, 12	3	11/25	44
	7	26	Toxoid	7µg	3	3	13/26	50
	8	25	Toxoid	7µg	1	3	11/25	44
2	1	80	-	-	-	1	72/80	90
	2	79	Quil A + PBS	-	3, 12	1	66/79	84
	3	80	Active SN	7µg, 70µg	3, 12	1	59/80	74[B]
	4	78	Toxoid	7µg, 70µg	3, 12	1	64/78	82
3	1	29	-	-	-	1	14/29	48
	2	29	Quil A + PBS	-	3, 12	1	16/29	55
	3	27	Active SN	7µg, 70µg	3, 12	1	6/27	22[A]
	4	25	Toxoid	7µg, 70µg	3, 12	1	13/25	52
	5	26	Active SN	35µg	1	1	16/26	62
	6	26	Active SN	70µg	1	1	17/26	65
	7	26	Active SN	140µg	1	1	17/26	65
	8	25	Active SN	210µg	1	1	16/25	64
	9	26	Toxoid	35µg	1	1	9/26	35
	10	25	Toxoid	70µg	1	1	17/25	68
	11	26	Toxoid	140µg	1	1	14/26	54
	12	26	Toxoid	210µg	1	1	16/26	62

SN, supernatant. [A]Values with uppercase superscripts differ significantly ($P<0.05$ for trial 1 and trial 3) from the Quil A vaccinated control group; [B]Values with uppercase superscripts differ significantly ($P<0.01$ for trial 2) from the unvaccinated control group.

Macroscopical lesion scoring. Lesion scoring in the small intestine (duodenum, jejunum and ileum) was performed as described by Keyburn *et al.* (2006). Chickens with a lesion score of 2 or more were classified as necrotic enteritis positive.

Statistical analysis. A two-tailed non-parametric test (Mann-Whitney U) (GraphPad Prism Software, Inc, USA (Version 5.00)) was used to determine whether there was a significant difference between the percentage positive chickens and the average lesion score in the vaccinated groups and control groups. Statistical significance was determined at a P value of <0.05.

Results

Trial 1. No chickens died during the challenge period. Vaccination with crude supernatant of *C. perfringens* strain 23 at day 3 and 12 resulted in a significant decrease ($P<0.05$) in the number of chickens with necrotic lesions compared to the control group vaccinated with Quil A and PBS. Also single vaccination with crude supernatant at day 3 resulted in significant protection ($P<0.05$) compared to the same control group. Vaccination with inactivated supernatant (toxoid) did not yield significant decreases in necrotic enteritis positive birds (Table 1). When the average lesion score of each group of vaccinated broilers was compared to those of the control groups, no significant decreases were observed between control groups and groups vaccinated with active supernatant or toxoid (Figure 1). To confirm the protective effects of administration of crude supernatant at day 3 and 12, and the loss of protection when using a toxoid, a trial with an increased number of animals per group was performed (trial 2).

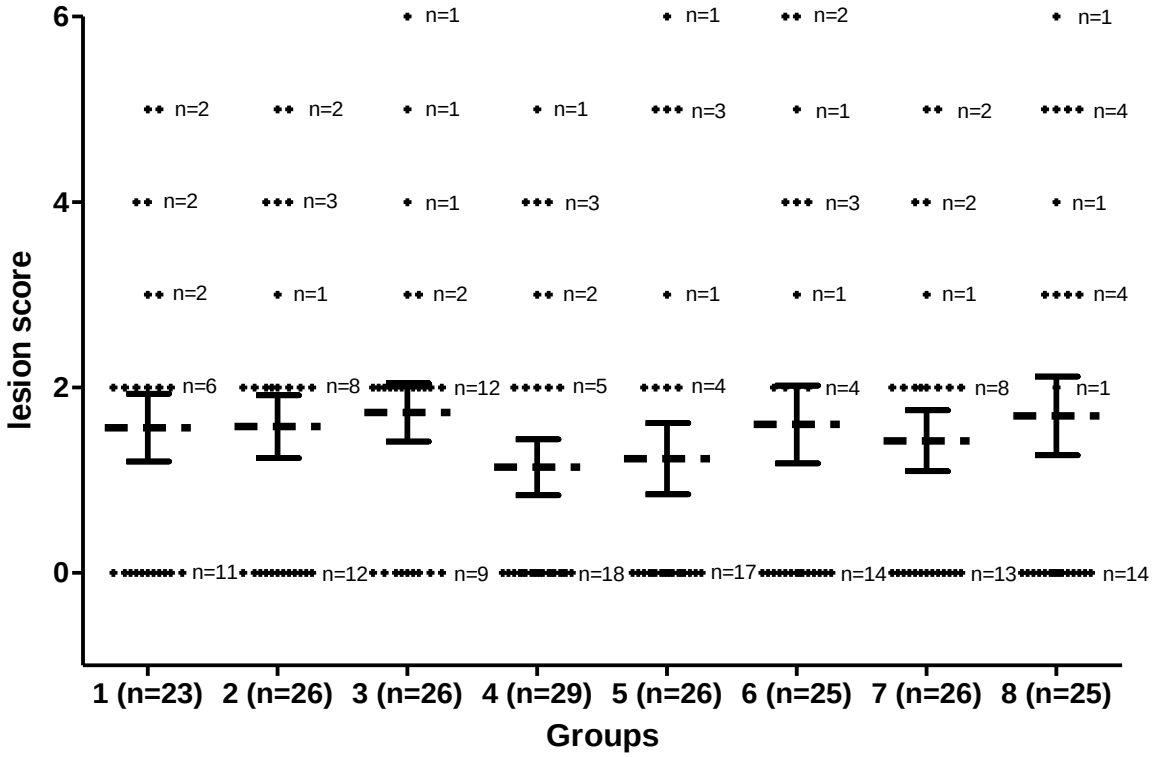

Figure 1. Lesion scores of individual broiler chickens challenged with *C. perfringens* in trial 1 are shown. The striped bars represent the average lesion score in each group. The standard error of the mean (SEM) is represented by the solid bars (GraphPad Prism Software, Inc, USA). A description of the vaccination schedule of group 1-8 is shown in table 1. No significant difference was seen between the groups.

N = number of animals

+ = individual lesion score

Trial 2. Because challenging the chickens once a day during 4 consecutive days with *C. perfringens* resulted in approximately the same average lesion score as challenging three times a day during 4 consecutive days (Table 1, trial1) in the second and third trial the challenge was only done once a day for 4 consecutive days. Double vaccination with crude supernatant of *C. perfringens* strain 23 again resulted in a significant decrease ($P<0.01$) of the number of chickens with necrotic lesions. Twelve chickens, originating from different groups, died during the challenge period and 6 moribund chickens were euthanized. All those birds were necropsied and had the highest possible necrotic enteritis lesion score. The number of chickens with necrotic enteritis lesions was significantly lower in the group vaccinated with active supernatant at day 3 and day 12 compared to the untreated control group. When the average lesion score from vaccinated groups was compared to those from control groups, significant differences were observed between both control groups and the group vaccinated with active supernatant at day 3 and day 12 ($P<0.001$). Also a significant difference was observed between the untreated positive control group and the group vaccinated with toxoid at day 3 and day 12 ($P<0.05$) (Table 1, Figure 2).

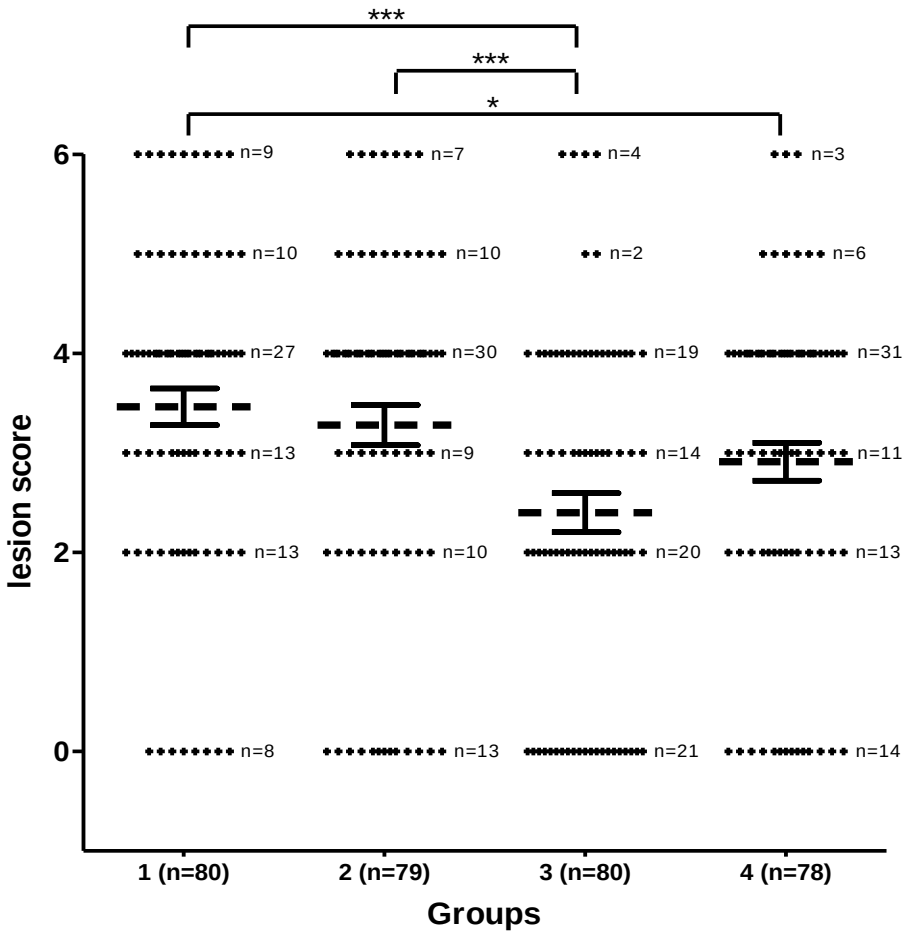

Figure 2. Lesion scores of individual broiler chickens challenged with *C. perfringens* in trial 2 are shown. The striped bars represent the average lesion score in each group. The standard error of the mean (SEM) is represented by the solid bars (GraphPad Prism Software, Inc, USA**)**. A significant decrease was seen between both control groups (groups 1 and 2) and the group vaccinated with active supernatant (group 3) ($P<0.001$)(***). A significant difference was observed between the unvaccinated control group (group 1) and the group vaccinated with toxoid (group 4) ($P<0.05$)(*). A description of the vaccination schedule of groups 1-4 is shown in table 1.

N = number of animals

+ = individual lesion score

Trial 3. Trial 3 was performed to analyze whether a single dose vaccination at day of hatch could induce protection. In addition, multiple toxoid and crude supernatant vaccines containing different protein concentrations were compared. In this trial, repeated dose vaccination with active supernatant of *C. perfringens* strain 23, but not toxoid, protected against necrotic enteritis after challenge ($P<0.05$). No significant difference was seen between both positive control groups (untreated positive control and Quil A treated positive control) and the groups that received a one-dose vaccination at day 1 with either crude supernatant or toxoid, independent of the protein concentration in the vaccines (Table 1). In the group vaccinated with the highest concentration crude supernatant (210µg) 6 chickens died one (4), two (1) and three (1) days after vaccination. Histological examination of the vaccination place showed severe necrosis of the cutis and subcutis. When the average lesion scores from vaccinated groups were compared to those from the control groups, a significant difference was observed between the unvaccinated control group and the group vaccinated with active supernatant at day 3 and day 12 ($P<0.05$). No significant differences were observed between both control groups and the groups vaccinated at day 1 (Figure 3).

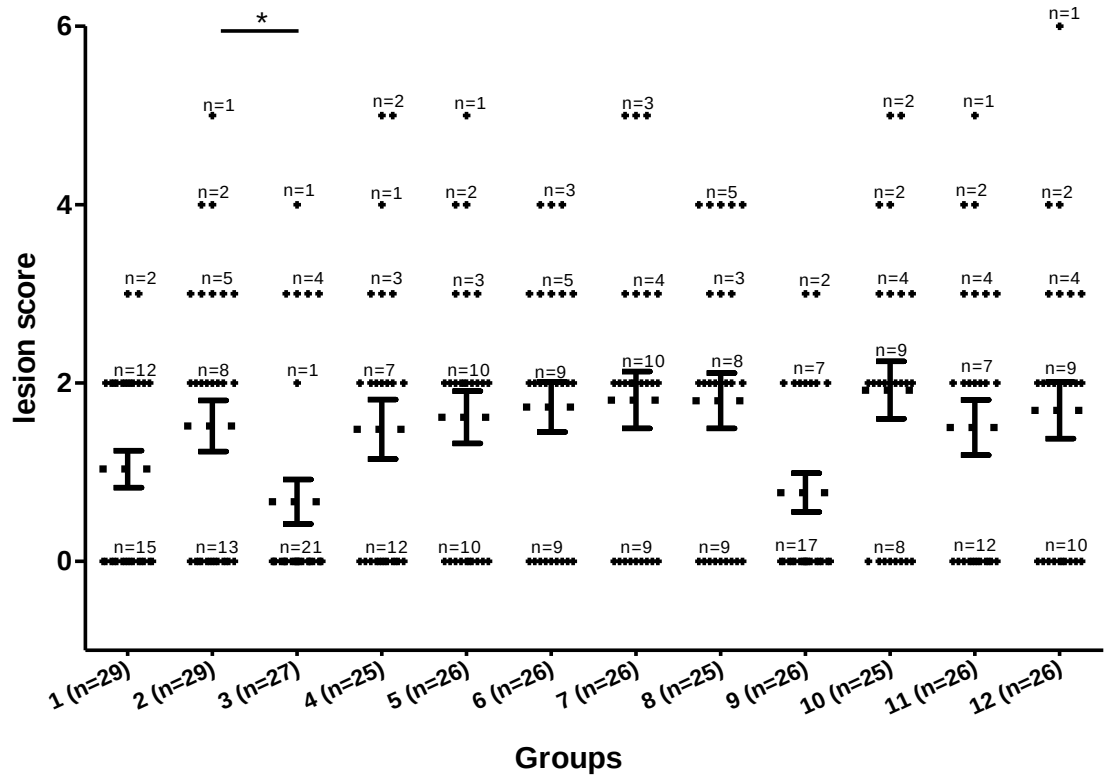

Figure 3. Lesion scores of individual broiler chickens challenged with *C. perfringens* in trial 3 are shown. The dotted bars represent the average lesion score in each group. The standard error of the mean (SEM) is represented by the solid bars (GraphPad Prism Software, Inc, USA). A significant decrease was detected between the unvaccinated control group to which Quil A was administered (group 2), and the group vaccinated with active supernatant at day 3 and day 12 (group 3) ($P<0.05$) (*). No significant differences were detected between the control groups and the groups vaccinated at day 1 with either crude supernatant or toxoid at different dosages (groups 5 to 12). The decrease of the number of animals with lesions in group 9 was not statistically significant different from the control groups. A description of the vaccination schedule of groups 1-12 is shown in table 1.

N = number of animals

+ = individual lesion score

Discussion

Vaccination of broiler chickens at day 1 using a vaccine that is safe and does not affect the performance of the animals would be of high value as a preventive tool for necrotic enteritis. Formaldehyde inactivation has been used to produce *Clostridium* vaccines ensuring safety for vaccinated humans or animals (Thaysen-Andersen *et al*., 2007; Jones *et al*., 2008). Although crude supernatant of *C. perfringens* can induce protection against necrotic enteritis, it contains potent toxins and thus can not be regarded as a vaccine that is safe for both the animals and the user. Indeed, we showed in our third trial that higher dosages induced mortality in the animals. While subcutaneous administration of crude supernatant at day 3 and day 12 resulted in a significant decrease in the number of chickens with necrotic lesions, formaldehyde inactivation affected the efficacy and a toxoid was clearly less protective than the active supernatant. Formaldehyde is widely used in the production of inactivated vaccines. Although bacterial proteins treated with formaldehyde can be highly immunogenic it often occurs that only low levels of neutralizing antibodies are produced. For that reason the protection after vaccination with formaldehyde inactivated proteins can be low (Nencioni *et al.*, 1991; Petre *et al*., 1996; Jones *et al*., 2008). This is believed to be due to the cross-linking capacity of formaldehyde, with major conformational modifications of the cross-linked proteins, resulting in loss of immunogenicity of epitopes (Metz *et al.*, 2004; Thaysen-Andersen *et al*., 2007; Jones *et al.*, 2008). Our current findings are in agreement with this hypothesis. The importance of conformational epitopes in the protection against necrotic enteritis was already suggested by Kulkarni *et al*. (2007), who showed that alpha-toxoid fails to offer protection.

Single vaccination at day of hatch, even with crude supernatant, is not able to protect against necrotic enteritis in the used model, in contrast to repeated vaccination at day 3 and 12 and single vaccination at day 3, yielding partial protection. Other reports show that multiple vaccination regimens can significantly reduce necrotic lesions in challenged animals (Cooper *et al*., 2009; Jang *et al*., 2012; Jiang *et al*., 2009; Kulkarni *et al*., 2007; Lanckriet *et al*., 2010; Saleh *et al*. 2011). The observation in trial 1 that single vaccination at day 3 with active supernatant protected partially against the development of lesions is an

interesting finding, but in practice administration later than day of hatch is logistically not feasible. It has been suggested already that immunization 1 day after hatching does not activate antibody production, most likely due to incomplete structural organization of the secondary lymphoid tissues in neonatal broilers (Mast & Goddeeris, 1999), what could explain the failure of vaccination at day of hatch. Whether the lack of protection of a toxoid vaccine and day-of-hatch vaccine regimes is also valid when using vaccine preparations derived from other *C. perfringens* strains is not clear and should be analyzed in future studies. Indeed, the strain used for toxoid and crude supernatant preparation was isolated from a healthy chicken, and strains isolated from necrotic lesions could have been more appropriate. However, the supernatant of the strain used in the current study was shown to be superior in a comparative study using subcutaneous vaccination with supernatant from 8 different strains at day 3 and 12 (Lanckriet *et al*., 2010). For this reason the use of the vaccine preparations derived from this particular strain seems to be relevant and the data most likely can be extrapolated to the use of vaccine preparations derived from other strains.

Since vaccination at day of hatch is not protective, there are 2 other options that are practically possible in the field. The first one is immunization of parent flocks. Kulkarni *et al*. (2007) suggested the importance of mucosal IgY, the major transferred maternal antibody, in the immunity to necrotic enteritis (Ulmer-Franco *et al*., 2012). Although vaccination can yield maternal antibodies, there is a high chance that these antibodies have disappeared already at the time birds usually develop necrotic enteritis (3 to 4 weeks of age) (Lovland *et al*., 2004). Previous reports (Lovland *et al*., 2004; Crouch *et al*., 2010) described an increase in antibody response in breeder hens and partial passive protection in young chickens. Currently, there is one commercial toxoid vaccine for broiler breeder hens (Netvax®, Intervet/Schering-Plough Animal Health, Summit, New Jersey, USA) containing *C. perfringens* type A toxoid. Another option that can be envisaged, is the use of bacterial or viral vectors expressing recombinant proteins, provided that the strains can be given at day-of-hatch (or in ovo) and persist for a sufficient time to generate a protective

immune response. Orally administered live vaccine strains expressing *C. perfringens* antigens and colonizing the intestinal tract of the broilers have been described (Kulkarni *et al.*, 2008, Zekarias *et al*., 2008; Kulkarni *et al*., 2009). The obtained protection depends on the colonization level and persistence of the vaccine strains.

In conclusion, the current study shows that subcutaneous administration of crude supernatant or toxoid derived from a specific *C. perfringens* strain, at day of hatch, is not effective in controlling necrotic enteritis. Administration of crude supernatant at day 3, or double vaccination with crude supernatant at day 3 and day 12 was able to reduce the number of animals having necrotic enteritis lesions. In addition, subcutaneous toxoid administration was less efficient in double vaccination regimens as compared to crude supernatant.

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3.2. Identification of immunogenic *Clostridium perfringens* supernatant proteins of strain 23

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Abstract

Necrotic enteritis is still an important gastro-intestinal disease that affects broiler chickens at an age of 2 to 5 weeks. Preventive vaccination can be a valuable approach against the disease but the ideal combination of antigens that should be used is still under investigation. In our study we aimed to identify proteins from supernatant of *C. perfringens* strain 23 which showed an increased immunogenicity compared to supernatant from other *C. perfringens* strains by Western blotting. Three strong reacting antigens were identified as PFOR (pyruvate:ferredoxin oxidoreductase), Elongation factor G and NetB. *C. perfringens* strain 23 produces the NetB variant A168T and this can be the reason for the strong antigenic reaction of the strain 23-immunized chickens towards NetB. Since chickens immunized with virulent strain 56 (with the consensus NetB) showed not such a high immune response against NetB. Whether the mutation affects immunogenicity is not yet clear and should be investigated in further vaccination studies.

Introduction

Necrotic enteritis in broiler chickens is an important poultry disease worldwide. The disease is caused by NetB toxin producing strains of *Clostridium perfringens* type A, a Gram-positive, anaerobic bacterium. The infection may present as an acute clinical disease, with high mortality, or in a subclinical form leading to reduced weight gain, caused by decreased digestion and malabsorption of feed (Kaldhusdal *et al.,* 2001). Subclinical necrotic enteritis is responsible for a high amount of economic losses as there are no symptoms and it often remains untreated (Dahiya *et al.,* 2006). Therapeutic antibiotics are typically used to control necrotic enteritis. For a disease caused by a toxin-producing-bacterium vaccination may be an interesting option for prevention. Several vaccine studies were already performed with variable degrees of success, yielding partial protection in the used challenge models (Kulkarni *et al.,* 2007; Cooper *et al.,* 2009; Jang *et al.,* 2009; Lanckriet *et al.,* 2010; Saleh *et al.,* 2011; Jang *et al.,* 2012). However, Lanckriet *et al*. (2010) performed a vaccination study which resulted in full protection against necrotic enteritis after challenge with virulent strains. In this study, vaccination was performed with supernatants of different strains and the supernatant of a particular strain, *C. perfringens* strain 23, was clearly shown to be superior in efficacy when parenteral administered, as compared to the supernatant of 7 other *C. perfringens* strains. It is known that strain 23 is less virulent than *C. perfringens* strains 56, 37 and 61 (Lanckriet *et al.,* 2010) Strain 23 was isolated from a healthy broiler flock and its alpha toxin expression level is rather low compared to other strains (Gholamiandehkordi *et al.,* 2006). However, strain 23 possesses the *netB* gene and its supernatant shows a cytotoxic effect towards LMH cells (Lanckriet *et al.,* 2010). The aim of this study was to understand why supernatant of this strain was highly protective by identifying immunoreactive proteins in the supernatant of strain 23 and comparing this to other strains.

Materials and methods

Clostridium strains, culture conditions and vaccine preparation. *C. perfringens* strains 7, 23, 43 and 56 were used for preparing crude supernatant. Strain 7 is a *netB* negative toxin type A strain isolated from a healthy flock. Strain 23 is a *netB* positive toxin type A strain isolated from a healthy flock (Gholamiandehkordi et al., 2006; Lanckriet et al., 2010). Strain 43 is a *netB* negative toxin type A strain isolated from a flock with a necrotic enteritis outbreak. *C. perfringens* strain 56 is a *netB* positive toxin type A strain and was isolated from a necrotic enteritis outbreak and has been shown to be highly virulent in *in vivo* trials (Gholamiandehkordi et al., 2007; Timbermont et al., 2009; Lanckriet et al., 2010). Bacteria were grown overnight at 37°C in Brain Heart Infusion (BHI) broth (Oxoid, Basingstoke, UK) supplemented with 0.375% glucose in an anaerobic (84% N2, 8% CO2 and 8% H2) workstation (Ruskinn Technology, South Wales, UK). Supernatant was concentrated using Vivaspin containing a 5000 molecular weight cut-off polyethersulphone membrane (Sartorius Stedim Biotech GmbH, Goettingen, Germany). The protein concentration from the supernatant was determined using a commercially available BCA protein Assay Reagent (Thermo Scientific Pierce, Rockford, Illinois, USA). The concentrated supernatant was diluted in phosphate-buffered saline (PBS) to the final protein concentration.

***C. perfringens* immune sera.** Five groups of 3 one-day-old Ross 308 broiler chickens were fed a wheat/rye-based (43%/7.5%) diet, with soybean meal (24.6% and 25.3% soybean meal in the starter and grower diet respectively) as a protein source (Gholamiandehkordi et al., 2006). Each group of 3 chickens was immunized with crude supernatant of a *C. perfringens* strain (respectively strain 7, 23, 43 and 56). One group was selected as a negative control group and only vaccinated with the adjuvant Quil A. On days 10 and 20 the chickens were immunized with crude supernatant containing 70µg total protein. On day 30 the chickens were immunized with crude supernatant containing 140µg total protein. Quil A (50 µg; Brenntag Biosector, Frederikssund, Denmark) was used as an adjuvant. The mixture was diluted in PBS to a total volume of 200 µl, mixed well by pipetting up and down, and filter-sterilized (0.2 µm pore size). Birds were vaccinated subcutaneously in the neck with a 200 µl dose. Blood was collected at days

41 and 48 and sera of each group were pooled. This pooled serum was used in the Western Blot experiment.

Characterization of antigenic *C. perfringens* proteins by Western blotting. Supernatant from strain 56 was prepared as described above and heated for 5 min at 95°C. The proteins were separated by one-dimensional SDS-PAGE in an 8% and 15% acrylamide gel. PageRuler Plus Prestained Protein Ladder, 10 to 250 kDa (Thermo Fisher Scientific, Rockford, USA) was used as protein marker. Proteins were electro transferred from unstained acrylamide gels onto 0,45µm Polyvinylidene difluoride (PVDF) membranes (Thermo Fisher Scientific, Rockford, USA) following the manufacturer's instructions (0.8 Ampère during 60 minutes). After blotting, the membranes were blocked with 100% methanol and dried for 15 minutes at room temperature (RT). The membranes were incubated with the primary antibody (*C. perfringens* chicken immune serum derived from chickens immunized with supernatant of respectively *C. perfringens* strain 7, 23, 43 and 56) diluted (1/200) in 5% skimmed milk in phosphate buffered saline (PBS) during 1h at RT and overnight (ON) at 4°C. After the first incubation the membranes were washed (3x5min) in PBS with 0.1% Tween-20, rinsed (1x5min) in PBS and incubated for 1h with purified rabbit anti-chicken immunoglobulin G (IgG), horseradish-peroxidase (HRP)-conjugated (Sigma Aldrich, St-Louis, USA), diluted (1/10000) in 5% skimmed milk in PBS. After the wash steps the specific immunoreactive protein bands were visualized using CN/DAB substrate kit (Thermo Fisher Scientific, Rockford, USA).

In-gel protein digestion and identification of *C. perfringens* proteins by mass spectrometry. Simultaneously with Western blotting, proteins in gels were visualized with Coomassie stain reagent, Brilliant Blue G-Colloidal concentrate (Sigma Aldrich, Missouri, USA). Protein bands, reflecting proteins with identical molecular weight as the ones of interest as detected by Western blotting, were cut from the coomassie stained gels and subjected to in-gel protein digestion with trypsin (Devreese et al., 2002) followed by mass spectrometric characterization. After mixing 1 µl of the digestion mixture with 10 µl a-cyano siniapinic acid (5 mg/ml), one microliter was spotted onto the target plate and analyzed with the 4800 plus MALDI TOF/TOF Analyzer (Applied Biosystems, Foster City, CA). A NCBI BLAST-search to

the corresponding protein sequence was done with the obtained amino acid sequences (http://blast.ncbi.nlm.nih.gov/Blast/).

Sequencing the *netB* gene of *C. perfringens* strain 23 and 56. For sequencing of the *netB* gene, the PCR and sequencing primers were JRP3943 (5'TTTTCTTTTAGACATGTCCATAGGC3'), which binds 268 bp upstream of the netB open reading frame (ORF), and JRP3944 (5'CCATC CCTTATTTCATCAGCATTTA3'), which binds 348 bp downstream of the *netB* ORF. The primers NetB sense (5'TGCTGTTCCATTCCCTTGAG3') and NetB antisense (5'CATGTCCATAGGCGTACCATT3') were used to sequence the amplified PCR products. PCR products were purified using the MSB spin PCRapace (B-Bridge International, CA, USA) before sequencing on an Applied Biosystems sequencer.

Results

Identification of immunoreactive *C. perfringens* proteins. Multiple immunoreactive proteins were identified by Western blot analysis in supernatant of *C. perfringens* strain 23 using sera derived from vaccinated chickens. Three proteins were chosen based on detection by sera from animals vaccinated with strain 23 supernatant, and inferior detection with supernatant of other strains. PVDF membranes incubated with serum derived from chickens immunized with supernatant of *C. perfringens* strain 43 did not react at all. PVDF membranes incubated with serum derived from chickens immunized with supernatant of *C. perfringens* strain 7 reacted very poorly. The protein bands on Western blots were matched with the protein bands that could be seen in the parallel Coomassie blue-stained gel. Several of the Coomassie blue-stained bands were excised from the gel for analysis. Three of these bands were identified by MALDI TOF/TOF Analysis (Fig. 1). Band 1 was identified as pyruvate:ferredoxin oxidoreductase (PFOR) (theoretical molecular mass, 128 kDa) with 62% sequence coverage. Band 2 was identified, based on peptide fragmentation spectra, as elongation factor G (theoretical molecular mass, 76 kDa) with protein

score 84. Band 3 was identified as NetB (theoretical molecular mass, 33 kDa) with 33% sequence coverage.

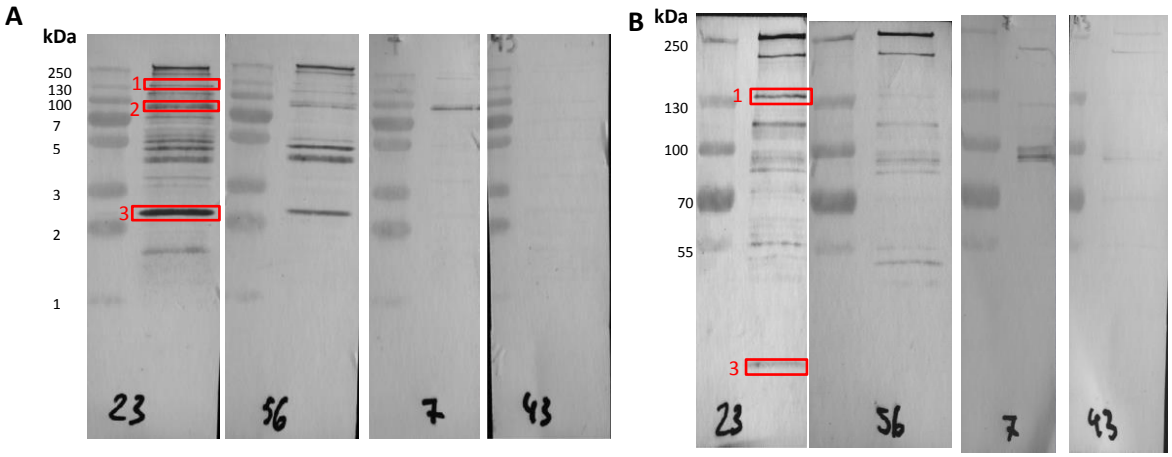

Figure 2. Western Blots of *C. perfringens* proteins. Supernatant of *C. perfringens* strain 56 was loaded on an 8% (A) and a 15% (B) acrylamide gel in quadruplicate and proteins were transferred onto PVDF membranes. Afterwards, the membranes were incubated with chicken serum, derived after immunization of chickens with supernatant of respectively *C. perfringens* strain 7, 23, 43 and 56. Band 1, 2 and 3 indicate the PFOR, elongation factor G and NetB proteins, respectively. The positions of pre-stained protein molecular mass markers are indicated in kilo Daltons (kDa).

Sequencing the *netB* gene of *C. perfringens* strain 23 and 56. A PCR product of the complete *netB* gene was amplified from *C. perfringens* strain 56 and *C. perfringens* strain 23 and sequenced to determine the deduced amino acid sequence of the encoded 322 amino acid full-length NetB proteins. The *netB* sequence of *C. perfringens* strain 56 equals the EHE-NE18 consensus sequence (EU143239). A single nucleotide polymorphism at position 769 altering Guanine to Adenine (G769A) in strain 23 resulted in a NetB amino acid sequence change, altering the alanine residue at position 168 to threonine (A168T). GenBank accession numbers for *netB* sequences are FJ189481-FJ189503.

Discussion

Immunogenicity of proteins derived from *C. perfringens* supernatants is a key issue in the protection conferred by supernatant vaccines. It is logical that ideal *C. perfringens* vaccines for broilers should generate immune responses directed against secreted components of the bacteria, and possibly also to cell wall components. The most evident secreted components against which antibody responses should be targeted at are toxins and enzymes. Remarkably, in the current study, we could not induce antibodies directed against supernatant proteins of strains 7 and 43. In a previous study by Lanckriet *et al*. (2010), it was shown that immunization with supernatant derived from these strains did not induce any kind of protection against challenge with a pathogenic strain. Supernatant derived from strains 56 and 23 did induce partial and full protection, respectively (Lanckriet *et al.,* 2010), and in the current study strong antibody responses against supernatant proteins could be detected using Western blot. There is no clear explanation why differences between antibody responses would exist when supernatants from different strains are used for parenteral vaccination. Strains 7 and 43 are *netB* negative, whereas strains 23 and 56 are *netB* positive. Maybe the other immunoreactive proteins are also not expressed by strain 7 and 43. This should be investigated.

The identification of immunoreactive proteins of *C. perfringens* was already performed by different research groups. We aimed in our study to identify proteins form supernatant of strain 23 with increased immunogenicity compared to supernatant from other strains, and selected 3 possible candidates. PFOR and Elongation factor G were already identified as antigenic proteins of *C. perfringens* (Kulkarni *et al.*, 2006; Lee *et al*., 2011). Together with the two major virulence factors of *C. perfringens*, NetB and alpha toxin, these proteins enhance the protective immunity of chickens against necrotic enteritis, when used as vaccine antigens (Jang *et al.*, 2012; Kulkarni *et al*., 2007 and 2008). The reason for the strong antigenic reaction of the strain 23-immunized chickens towards NetB can probably be explained by the mutation of the *netB* gene in strain 23. Keyburn *et al.* (2010) showed that the *netB* gene is highly conserved in *C.*

perfringens strains that cause necrotic enteritis. In natural occurring strains there are only two variants detected, the consensus wild NetB and the natural occurring A168T variant. Only in a minority of the strains causing necrotic enteritis, the NetB A168T variant could be detected. This mutation however does not lead to decreased cytotoxicity. The A168T substitution occurs in a region that is expected to be within a membrane spanning region (Menestrina *et al.,* 2001). The tertiary structure of the NetB protein was expected to be not significantly affected by the substitution and it was shown that the single amino acid change at residue 168 of NetB did not affected its cytotoxic activity (Keyburn *et al.,* 2010; Lanckriet *et al.*, 2010). Whether the mutation affects immunogenicity is not yet clear. Several vaccination studies with the NetB protein were already performed. A substitution NetB variant (S254L) that is unable to oligomerize and to hemolyse chicken red blood cells was constructed. Vaccination of breeder hens and broilers with formalin treated NetB toxoid and/or NetB variant S254L yielded partial protection (Keyburn *et al*., 2013 a and b). The mutant W262A, the mutation of tryptophan to alanine at position 262, resulted in a significant reduction in cytotoxicity towards LMH cells and hemolytic activity on chicken red blood cells. Vaccination with this W262A variant was also partially protective (Fernandes da Costa *et al*., 2013). The NetB A168T variant does not show decreased cytotoxicity and thus this protein is not a good vaccine candidate.

In conclusion, the supernatant of *C. perfringens* strains elicits antibody responses when parenterally administered to broilers, correlating with protection seen in vaccination-challenge infections. The actual reason why specific supernatants are superior in protecting against disease likely is the cause of antibody responses to a mix of supernatant proteins, and choosing the optimal supernatant vaccine or combination of proteins can be important.

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3.3. Variable protection against experimental broiler necrotic enteritis after immunization with the C-terminal fragment of *Clostridium perfringens* alpha-toxin and a non-toxic NetB variant

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Abstract

Necrotic enteritis toxin B-like (NetB) is a pore-forming toxin produced by *Clostridium perfringens* and has been shown to play a key role in avian necrotic enteritis (NE), a disease causing significant costs to the poultry production industry worldwide. The aim of this work was to determine whether immunization with a non-toxic variant of NetB (NetB W262A) and the C-terminal fragment of *C. perfringens* alpha-toxin ($CPA_{247-370}$) would provide protection against experimental NE. Immunized animals with either antigen or a combination of antigens developed serum antibody levels against NetB and alpha toxin. When $CPA_{247-370}$ and NetB W262A were used in combination as immunogens, an increased protection was observed after oral challenge by individual dosing, but not after in-feed challenge.

Introduction

Necrotic enteritis (NE) is a severe gastro-intestinal disease causing significant costs to the poultry industry worldwide (Parish, 1961; Keyburn *et al*., 2008; Cooper and Songer, 2009). Disease can occur either as an acute clinical or as a mild subclinical form. Acute NE typically leads to high mortality rates during the last weeks of the broiler rearing period. Disease can arise without any previous signs and cause mortality in a couple of hours (Helmboldt and Bryant, 1971; Wijewanta and Senevirtna, 1971). However, most of the NE cases are associated with relatively mild clinical signs (Kaldhusdal and Hofshagen, 1992; Brennan *et al*., 2001a; Brennan *et al*., 2001b). This subclinical form of NE is chronic and induces intestinal damage. Diseased animals show reduced performance parameters such as less feed intake, decreased digestion and absorption of feedstuffs and consequently reduced weight gain over time (Elwinger *et al*., 1992; Kaldhusdal *et al*., 2001). The mild subclinical form is believed to be the most prevalent form of NE and mostly responsible for the associated economic losses as it may go undetected and remain untreated (Dahiya *et al*., 2006).

Clostridium perfringens, a commensal of the intestinal microbiota, has been shown to be the causative agent of NE. A number of predisposing factors have been identified which influence the gut environment of the host organism and favour the growth of NE-inducing *C. perfringens* strains. The nature of the feedstuff is the key predisposing factor for NE. Poor digestible diets, such as non-starch polysaccharides and protein-rich feed, lead to ideal growth conditions for *C. perfringens* in the gut (Branton *et al*., 1987; Riddell and Kong, 1992; Palliyeguru *et al*., 2010). Sudden diet changes, high-density animal housing conditions or extreme environmental temperatures are other important factors that predispose to NE (McDevitt *et al*., 2006; Burkholder *et al*., 2008). Mucosal damage of the gut, caused by organisms such as *Eimeria* species, has often been reported before or during outbreaks of NE in the field (Helmboldt and Bryant, 1971; Broussard *et al*., 1986; Williams, 2005). Co-infection of *C. perfringens* with *Eimeria* species has been shown to synergistically induce NE (Alsheikhly and Alsaieg, 1980; Williams *et al*.,

2003; Gholamiandehkordi *et al*., 2007; Park *et al*., 2008). The molecular makeup of *C. perfringens* strains present in the gut is another essential factor (Shojadoost *et al*., 2012). Most of *C. perfringens* isolates from cases of NE possess the *netB* gene (Chalmers *et al*., 2008; Cooper and Songer, 2009; Martin and Smyth, 2009), encoding the necrotic enteritis toxin B (NetB), a member of the *Staphylococcus aureus* α-hemolysin-like β-pore-forming toxin family (Keyburn *et al*., 2008; Savva *et al*., 2013). This toxin has a proven role in NE development (Keyburn *et al*., 2008).

Vaccine trials against NE initially focused on the use of *C. perfringens* alpha-toxin as an antigen. Immunization studies with alpha toxin have been shown to partially protect chickens from developing NE (Kulkarni *et al*., 2007; Cooper *et al*., 2009; Kulkarni *et al*., 2010). It has been shown that animals with high alpha toxin titres showed lower mortality rates during the production period than those with low titres (Heier *et al*., 2001). Immunization with either *C. perfringens* crude toxoids or culture supernatants can also provide significant protection against experimental NE (Lanckriet *et al*., 2010; Saleh *et al*., 2011). In addition, a number of immunogenic proteins from *C. perfringens* have been evaluated as sub-unit vaccines providing partial protection against experimental NE. Although a variety of antigens have been tested as vaccine candidates against NE so far, complete protection against disease has not been reported yet. In a previous study, we showed that a non-toxic variant of NetB (NetB W262A) was able to induce partial protection against experimental NE in poultry (Fernandes da Costa *et al*., 2013). In this study we investigated whether a combination of NetB W262A and a fragment of the C-terminal domain of alpha toxin ($CPA_{247-370}$) (Williamson and Titball, 1993) could provide improved protection against disease as compared to vaccination with the individual antigens. Protection was evaluated using an in-feed and oral gavage administration infection model.

Material and methods

Expression and purification of NetB W262A and $CPA_{247-370}$. Expression and purification of NetB W262A or $CPA_{247-370}$ was carried out as described previously (Titball *et al*., 1993; Williamson and Titball, 1993; Fernandes da Costa *et al*., 2013; Savva *et al*., 2013). In short, recombinant *Escherichia coli* (*E. coli)* expressing the toxin variants were grown in terrific broth (TB) supplemented with ampicillin (100 μg/ml) at 37°C and shaken at 300 rpm. For NetB W262A expression, cultures were induced at an optical density (OD_{595nm}) of 0.5 for 6 h by adding arabinose at a final concentration of 0.02% (w/v). Expression of $CPA_{247-370}$ was induced at an OD_{595nm} of 0.5 for 6 h by the addition of IPTG (1mM final concentration). In both cases, bacterial cells were harvested by centrifugation, lysed enzymatically using BugBuster (Invitrogen, Paisley, UK) and NetB or $CPA_{247-370}$ were purified with Ni-NTA or GST GraviTrap chromatography columns (GE Healthcare Life Sciences, Little Chalfont, UK), respectively, according to the manufacturer's instructions. Buffer was exchanged by size-exclusion chromatography using PD-10 desalting columns (GE Healthcare) equilibrated with Tris-buffered saline (TBS; 20 mM Tris pH 7.5, 150 mM NaCl) and protein concentrations were measured with a UV-Vis spectrophotometer (Thermo Scientific, Cramlington, UK).

Animals and housing conditions. Ross 308 broiler chickens were obtained as one-day-old chickens (Vervaeke-Belavi Hatchery, Tielt, Belgium) and the parent flock had not been vaccinated with the commercial Netvax™ or any other *C. perfringens* vaccine. All animals were housed in the same room. The birds were reared in pens at a density of 26-30 animals per 1.5 m^2 on wood shavings. All pens were separated by solid walls to prevent contact between birds from different treatment groups. Before the trials, housing rooms were decontaminated with Metatectyl HQ (Clim'oMedic®, Metatecta, Belgium) and a commercial anticoccidial disinfectant (OOCIDE, DuPont Animal Health Solutions, Wilmington, USA). The chickens received *ad libitum* drinking water and feed. Animal experiments were carried out according

to the recommendations and following approval of the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University, Belgium.

Strains and culture conditions. *C. perfringens* strain 56, a *netB* positive toxin type A strain, was grown during 18 h at 37 °C in Brain Heart Infusion (BHI) broth (Oxoid, Basingstoke, United Kingdom) with 0.375% glucose in an anaerobic (84% N_2, 8% CO_2 and 8% H_2) cabinet (Ruskinn Technology, Bridgend, UK) and used as such.

Vaccine preparation and delivery. On days 3, 9 and 15, chickens were immunized with 30 µg of NetB W262A, $CPA_{247-370}$ or a combination of both. In each case, Quil-A (50 µg; Brenntag Biosector, Frederikssund, Denmark) was used as an adjuvant. The mixture was diluted in PBS to a total volume of 200 µl per bird, mixed by vortexing and filter-sterilised (0.2 µm pore size). Birds were vaccinated subcutaneously in the neck with a 200 µl dose. Controls consisted of an untreated group and a group receiving only the Quil-A adjuvant diluted in PBS.

***In vivo* necrotic enteritis model.** For each trial, five groups of 26 to 30 (indicated in Table 1) one-day-old Ross 308 broiler chickens were fed a wheat/rye-based (43%/7.5%) diet, with soybean meal as protein source. The feed composition was as described elsewhere (Gholamiandehkordi *et al*., 2007). Briefly, the diet contained high levels of (animal) proteins and non-starch polysaccharides which predispose to the development of necrotic enteritis. Nobilis Gumboro D 78 vaccine (Schering-Plough Animal Health, Brussels, Belgium) was given in the drinking water on day 16. From day 17 onwards, soy bean meal was replaced by fishmeal (30%) as a protein source.

Table 1: Description of experimental groups used in this study

Trial	Group	Animals /group	Vaccine	Vaccination dose	Vaccination day(s)	Serum collecting day	Challenge	Number of animals with lesions/Total Number	Percentage of animals with necrotic enteritis
1	1	27	-	-	-	16	Orally on days 17, 18, 19 and 20	10/27	**37**
	2	28	Quil A + PBS	-	3, 9, 15	16		9/28	**32**
	3	28	NetB W262A	30µg	3, 9, 15	16		5/28	**18**
	4	26	CPA (247-370)	30µg	3, 9, 15	16		3/26	**12**^{A}
	5	28	NetB W262A + CPA (247-370)	30µg + 30µg	3, 9, 15	16		0/28	**0**^{A}
2	1	26	-	-	-	17	Culture/feed mixture (3:4) twice a day on days 19, 20, 21, 22	11/26	**42**
	2	27	Quil A + PBS	-	3, 9, 15	17		14/27	**52**
	3	29	NetB W262A	30µg	3, 9, 15	17		8/29	**27**
	4	30	CPA (247-370)	30µg	3, 9, 15	17		6/30	**20**^{B}
	5	30	NetB W262A + CPA (247-370)	30µg + 30µg	3, 9, 15	17		9/30	**30**

^{A} Values with uppercase superscripts differ significantly (P<0.01 for trial 1)

^{B} Values with uppercase superscripts differ significantly (P<0.05 for trial 2)

Trial 1 was carried out to compare the efficacy of vaccination of individual antigens with a combination of antigens using an infection model causing mild disease. The NE model of the first trial was based on a subclinical *in vivo* model described previously (Mot *et al*., 2012). The birds were challenged orally, using a plastic tube inserted in the crop, on days 17, 18, 19 and 20 with a single dose of approximately 4 x 10^8 cfu of *C. perfringens* strain 56. On day 18, all animals were orally inoculated with a 10–fold dose of Paracox-5 (Schering-Plough Animal Health). On days 21, 22, and 23, one-third of the birds in each group were euthanized and necropsied.

Trial 2 was carried out to clarify whether vaccination yielded the same protection when a more severe challenge model was used. In the second trial an in feed-challenge was performed based on the model by Keyburn *et al*. (2006). High level protein feed (30% fishmeal) and BHI broth culture were manually mixed in a ratio of 3:4 (v/w). The mixture was then placed into feed trays. Birds were fed the culture/feed mixture twice a day, on days 19, 20, 21 and 22. The feed trays were cleaned and the remaining feed

discarded prior to each subsequent feeding. On day 20, all animals were orally inoculated with a 10 x dose of Paracox-5 (Schering-Plough Animal Health). The birds were euthanized and necropsied on day 23.

Measurement of antibodies to NetB and alpha toxin using ELISA. Antibody responses to NetB W262A and $CPA_{247-370}$ immunization were determined using an enzyme-linked immunosorbent assay (ELISA). Serum from 10 chickens was collected individually in all groups, on day 16 (first trial) or day 17 (second trial), and pooled. For NetB, ELISA was performed as described previously (Fernandes da Costa *et al*., 2013). First, 96-well microtitre plates (Nunc-Immuno Plates, MaxiSorp, Thermo Scientific, Cramlington, UK) were coated with 0.5 µg/well of recombinant wild-type NetB with N-terminal His tag and incubated overnight at 4°C. Plates were then washed three times with TBS-T (TBS, Tween 0.5% v/v) and blocked with TBS-3% skimmed milk for 1 h at 37°C. Following incubation, plates were rinsed three times with TBS-T and incubated with 100 µl/well of pooled sera (1:20) in TBS-1% skimmed milk for 1 h at 37°C. Wells were then rinsed three times with TBS-T and incubated with a HRP-conjugated goat anti-chicken IgY (H+L) secondary antibody (Abcam, Cambridge, UK) at a dilution of 1:10,000 in TBS-1% skimmed milk. For detection, 100 µl of tetramethylbenzidine (TMB) substrate solution was added to each well and plates were incubated for 30 min at room temperature. The reaction was stopped by the addition of 100 µl of 3 M H_2SO_4 and absorbance was measured at 450 nm using a Model 680 Microplate Reader (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK). For alpha toxin detection, the Bio-X CPA ELISA kit (Bio-X Diagnostics, Jemelle, Belgium) was used according to the manufacturer's instructions. In brief, pooled sera samples (1:2) were added to a recombinant alpha toxin sensitised 96-well microtitre plate and incubated for 2 h at 37°C. Wells were then rinsed three times with washing buffer, HRP-conjugated anti-CPA antibodies added and plates incubated for 30 minutes at 37°C. Antibody detection was performed using TMB as described above. Each ELISA was performed in triplicate.

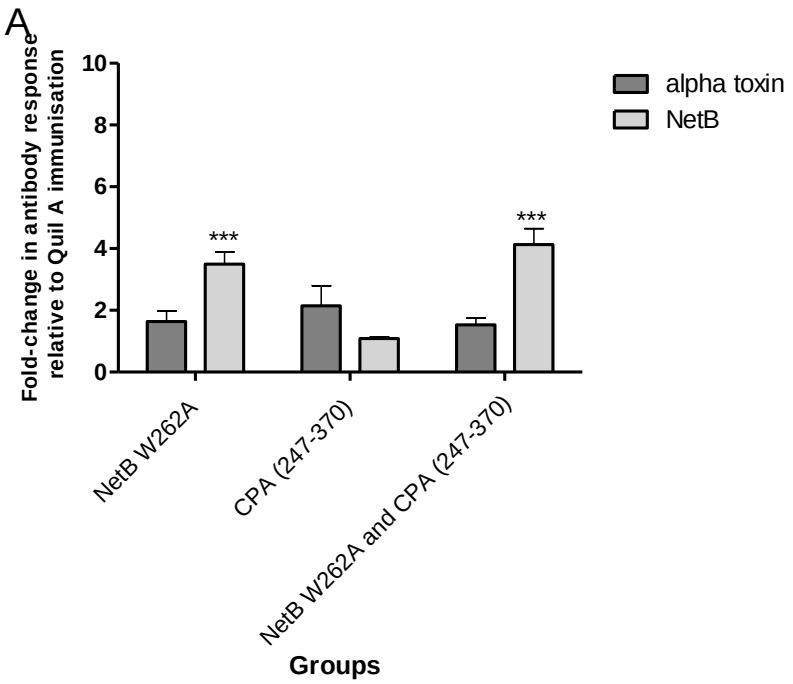
Assessment of protection. NE severity was assessed by scoring lesions within the small intestine of each animal (duodenum to ileum) as described by Keyburn *et al*. (2006) as follows: 0 = no gross lesions; 1 = congested intestinal mucosa; 2 = focal necrosis or ulceration (1-5 foci); 3 = focal necrosis or ulceration (6-15 foci); 4 = focal necrosis or ulceration (≥16 foci); 5 = patches of necrosis 2-3 cm long; 6 = diffuse necrosis typical of field cases. Animals showing lesion scores of 2 or higher were classified as NE positive.

Statistical analyses. To compare the mean values of antibody levels for the ELISA, 1-way ANOVA analysis was carried out followed by Dunnett's multiple comparisons test using GraphPad Prism 5.01 software (GraphPad Software, La Jolla, USA). For the *in vivo* NE model, differences between groups in the occurrence of NE-positive animals were evaluated by binary logistic regression analysis with the SPSS Statistics software 22.0 (SPSS Inc., Chicago, USA). In both analyses, a p-value of less than 0.05 was considered as significant.

Results

Immune response to NetB W262A and $CPA_{247-370}$. An ELISA was used to measure serum antibody responses to NetB or alpha toxin in the immunized birds. Blood samples were taken on day 16 or 17, one day before the first *C. perfringens* challenge. In the first trial chickens immunized with NetB W262A, $CPA_{247-370}$ or a combination of both increased antibody responses to the immunizing antigen relative to the Quil-A immunized control group (Figure 1A). In particular, a statistically significant increase ($p<0.001$) was detected for NetB antibody levels in the NetB W262A immunized group and when a combination of NetB W262A and $CPA_{247-370}$ was used. The second trial confirmed these results (Figure 1B). A significant

increase ($p<0.05$) was detected for NetB antibody levels in the NetB W262A immunized group and when the combination NetB W262A and $CPA_{247-370}$ was used. A significant increase was detected for alpha toxin antibody levels in the $CPA_{247-370}$ immunized group ($p<0.001$) and when the combination NetB W262A and $CPA_{247-370}$ was used ($p<0.05$).



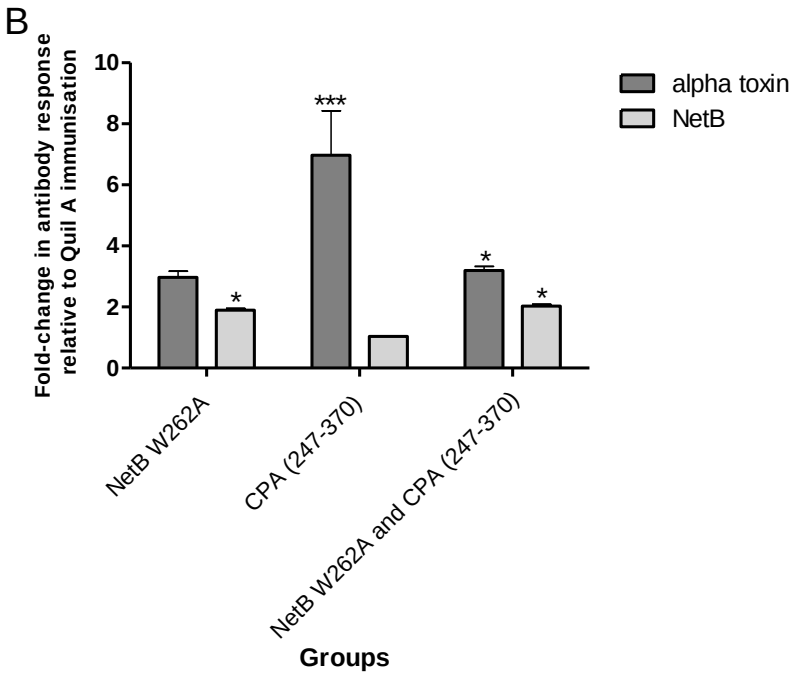
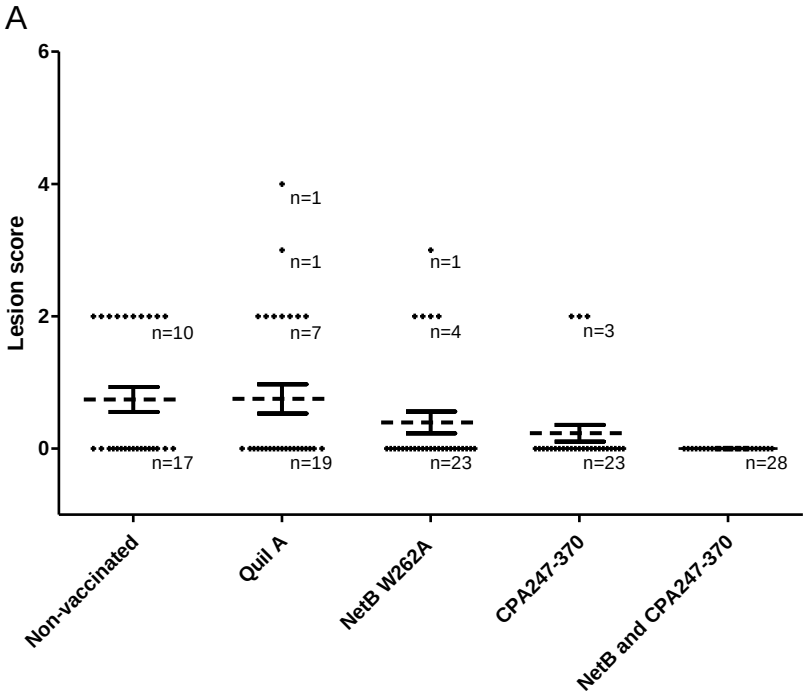


Figure 1: Antibody responses to NetB and alpha toxin using ELISA. Chickens were immunized with NetB W262A, $CPA_{247-370}$ or a combination of both, on days 3, 9 and 15. Sera were taken on day 16 prior to *C. perfringens* challenge. Each bar represents mean ± SEM. Asterisks indicate a statistically significant difference relative to the Quil-A immunized control (*: $p<0.05$, **: $p<0.01$ and ***: $p<0.001$). (A). First trial (B) Second trial

Protection against experimental NE after immunization with genetic toxoids.

Trial 1. The chickens were challenged orally once a day at days 17, 18, 19 and 20. Immunization with NetB W262A, $CPA_{247-370}$ or a combination of both reduced lesion scores and the occurrence of NE-positive animals relative to the control groups. While mean lesion scores were 0.74 and 0.75 in the control groups of untreated chickens and those dosed with adjuvant only, respectively, animals immunized with either NetB W262A or $CPA_{247-370}$ showed reduced mean lesion scores of 0.39 and 0.23, respectively (Figure 2A). No lesions were observed after immunization with a combination of NetB W262A and $CPA_{247-370}$. In the untreated chickens or chickens dosed with adjuvant only, 37% and 32% were NE-

positive, respectively, whereas only 18% of the animals immunized with NetB W262A and 12% of the animals immunized with $CPA_{247-370}$ were NE-positive. This was a significant decrease (Figure 2B). Animals immunized with a combination of NetB W262A and $CPA_{247-370}$ showed no signs of NE.



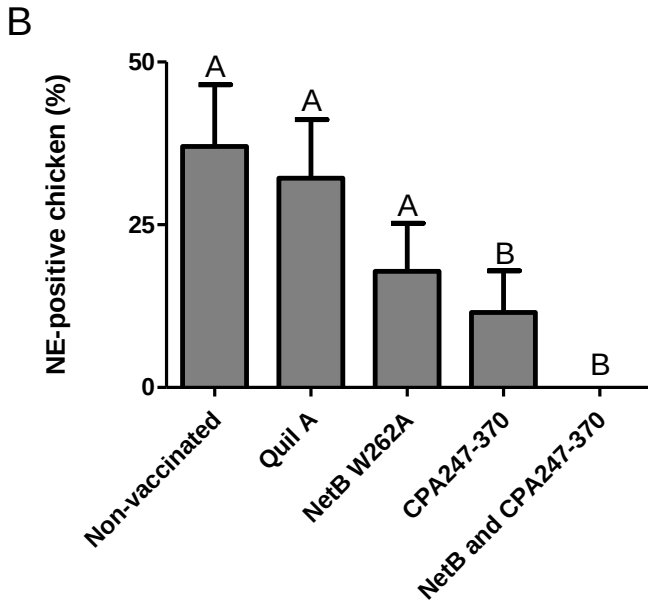
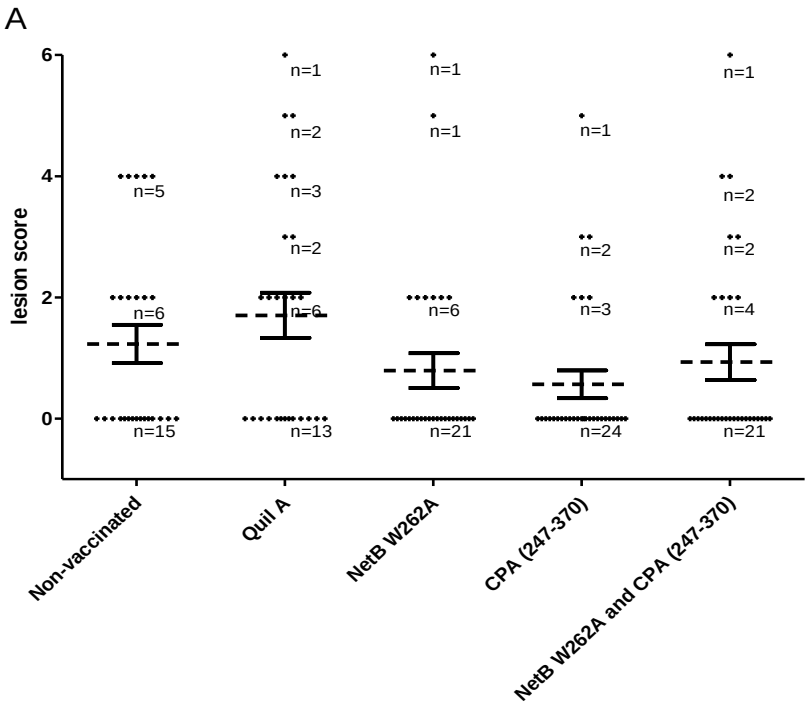


Figure 2: Data from the *in vivo* NE model of the first trial. Chickens were challenged orally once a day at days 17, 18, 19 and 20. (A) Lesion scores of individual chickens. According to severity, lesions in the small intestine were scored from 0 (no gross lesions) to 6 (diffuse necrosis). The striped bars represent the average lesion score in each group. The standard error of the mean (SEM) is represented by the solid bars. Individual chickens are marked as (+). n = number of animals. (B) Percentage NE-positive chickens. Animals with lesion scores of 2 or higher were classified as NE-positive. Black bars represent SEM. Groups not sharing the indicated letters are significantly different ($p<0.01$).

Trial 2. Chickens were challenged in-feed at days 19, 20, 21 and 22. Again, immunization with NetB W262A, $CPA_{247-370}$ or a combination of both reduced lesion scores and the occurrence of NE-positive animals relative to the control groups. Mean lesion scores were 1.23 and 1.70 in the control groups of untreated chickens and those dosed with adjuvant only, respectively. Chickens immunized with NetB W262A showed a mean lesion score of 0.79. The group immunized with $CPA_{247-370}$ showed a mean lesion score of 0.56. After immunization with the combination of NetB W262A and $CPA_{247-370}$ a mean lesion score of 0.93 was observed (Figure 3A). In the untreated chickens or chickens dosed with adjuvant only, 42% and 52% were NE-positive, respectively, whereas only 27% of the animals immunized with NetB

W262A and 20% of the animals immunized with $CPA_{247-370}$ were NE-positive (Figure 3B). In the group immunized with the combination of NetB W262A and $CPA_{247-370}$ 30% of the chickens were NE-positive.



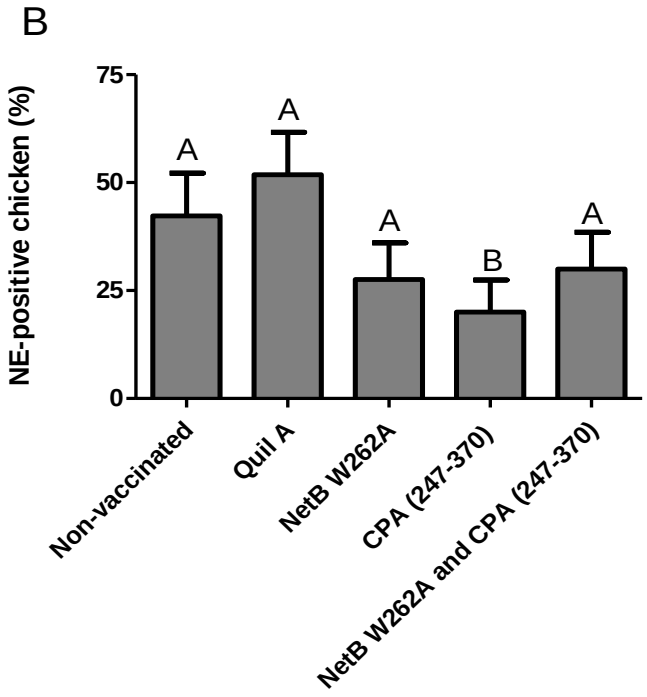


Figure 3: *In vivo* NE model of the second trial. Chickens were challenged in-feed at days 19, 20, 21 and 22. (A) Lesion scores of individual chickens. According to severity, lesions in the small intestine were scored from 0 (no gross lesions) to 6 (diffuse necrosis). The striped bars represent the average lesion score in each group. The standard error of the mean (SEM) is represented by the solid bars. Individual chickens are marked as (+). n = number of animals. (B) Percentage NE-positive chickens. Animals with lesion scores of 2 or higher were classified as NE-positive. Black bars represent SEM. Groups not sharing the indicated letters are significantly different ($p<0.05$).

Discussion

In recent years, a number of studies have been carried out on the development of a potential vaccine against NE. Significant protection has been shown by immunization with crude or inactivated *C. perfringens* supernatant (Lanckriet *et al*., 2010). However, the antigens responsible

for the induction of protective immunity have not been identified. A range of recombinant proteins from *C. perfringens* has been evaluated as vaccines, including glyceraldehyde-3-phosphate dehydrogenase, pyruvate-ferredoxin oxidoreductase, fructose-1,6-biphosphate-aldolase and a hypothetical protein (Kulkarni *et al*., 2007). Immunization with any of these proteins provided partial protection against experimental NE. Oral immunization with an attenuated *Salmonella enterica* serovar Typhimurium vaccine vector expressing fructose-1,6-biphosphate-aldolase, the carboxy-terminal domain of alpha toxin or a hypothetical protein induced protective responses against NE in chickens (Kulkarni *et al*., 2008; Zekarias *et al*., 2008). Partial protection against NE has also been reported after immunization with *C. perfringens* large cytotoxin TpeL, endo-beta-N-acetylglucosaminidase or phosphoglyceromutase (Jiang *et al*., 2009). A more recent study in which alpha toxin, NetB, pyruvate-ferredoxin oxidoreductase and elongation factor-Tu were compared as protective antigens concluded that NetB and pyruvate-ferredoxin oxidoreductase given with ISA71 adjuvant provided the best protective immunity (Jang *et al*., 2012). In an attempt to improve the level of protection afforded by these vaccine antigens, some of these have been expressed in attenuated mutants of *Salmonella enterica* (Zekarias *et al*., 2008; Kulkarni *et al*., 2008; Kulkarni *et al*., 2010). Whilst these recombinant *Salmonella* are well suited for oral delivery, these vaccines also failed to confer complete protection against disease.

In a previous study, we have shown that immunization of poultry with a formaldehyde NetB toxoid or NetB W262A resulted in the induction of antibody responses against NetB and provided partial protection against experimental NE (Fernandes da Costa *et al*., 2013). The current study was conducted to test if a combination of NetB W262A with $CPA_{247\text{-}370}$, which individually have been shown to provide partial protection against disease, provided enhanced protection relative to single protein immunization. Immunization led in both trials to increased antibody responses to NetB and alpha toxin and to protection against experimental NE. However,

the enhanced protection by immunization with a combination of NetB W262A and $CPA_{247-370}$ depended on the severity of challenge in the *in vivo* trials. In the first trial, in which an oral challenge was performed resulting in mild subclinical disease, the protection in the group vaccinated with the combination of NetB W262A and $CPA_{247-370}$ was complete. In the second trial, in which a more severe in-feed model was used, the protection was partial.

The importance of the challenge method used in an *in vivo* NE-model was already mentioned by Shojadoost *et al*. (2012). The severity of the disease, and also the protection against the disease, depends strongly on the challenge method used.

Our data show that vaccination with the combination of both antigens enhances the protection against a mild challenge but is not sufficient enough against a severe challenge. Also, the vaccination scheme used would not be practical in the field since the vaccine was parenteral administered three times. An alternative route may lie in breeder hen vaccination, but the antibody decline in the progeny may decrease efficacy (Keyburn *et al*., 2013). Expression of NetB W262A and $CPA_{247-370}$ in a bacterial vector could allow this vaccine to be given by an oral route, such as in drinking water or feed. Suitable vectors would include attenuated mutants of bacteria, such as attenuated strains of *S. enterica*. Alternatively, it may be possible to express NetB W262A and $CPA_{247-370}$ in a bacterium that is normally a member of the poultry gut microbiota, such as *Bacillus* species. The use of a live bacterial vector for expressing antigens, however, would mean the vaccines would be classified as genetically modified organisms (GMO). In contrast to other vaccines they require special attention concerning their impact on the environment. The regulatory restrictions of GMO products are significantly larger in many countries than those for the release of conventional live vaccines (Frey, 2007).

In conclusion, the present study shows the potential of $CPA_{247-370}$ and NetB W262A to be used as a combination vaccine to provide protection against mild NE. Further studies are required to

determine a suitable delivery route for practical immunization in the poultry industry, and to enhance protection.

Disclosure statement

University of Ghent P13/069 PATENT APPLICATION NUMBER 1322463.9

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Chapter 4

General discussion

General discussion

Necrotic enteritis is one of the gastrointestinal diseases in poultry that have become important during the last decade. The acute clinical form causes sudden death in the broiler flock with an increased mortality rate up to 5% (Ficken & Wages, 1997; Kaldhusdal, *et al*., 2001). The sublicinal form of necrotic enteritis is economically the most important one because broilers are incredibly efficient in converting food into body mass. It causes reduced weight gain and an increased feed conversion ratio (Kaldhusdal, *et al*., 2001). The use of AGP's in broiler feed protected against the disease in the past. Antibiotics such as amoxicillin and tylosin are still used to prevent and controle necrotic enteritis but this should be considered as a temporary solution while other preventive strategies are being developed.

Since the occurrence of the disease depends on predisposing factors an efficient poultry farm management can help to reduce the occurrence of the disease. Diet-related strategies and nutriceutical alternatives (pro- and prebiotics, herbs, organic acids and essential oils) have become important in the poultry industry (Lensing *et al.*, 2010a; Timbermont *et al.*, 2010; Jerzsele *et al*., 2012), and should be applied in combination with good biosecurity measures. Until now, although vaccines exist to control *C. perfringens* related disease in other animal species (e.g. sheep), no good vaccine against necrotic enteritis in birds is used in practice.

4.1. Potential vaccine candidates

As already suggested in the introduction, multiple proteins, with derivates from alpha toxin and NetB toxin as the important ones, have potential as vaccine candidates. There is still need to investigate the value of other immunogenic proteins and develop perfect mixture of the proteins. In this thesis we aimed to identify proteins from supernatant of strain 23 with increased immunogenicity compared to supernatant from other strains (Chapter 3.2). PFOR and Elongation factor G were already identified as antigenic proteins of *C. perfringens* (Kulkarni *et al.*, 2006; Lee *et al*., 2012). Together with the two major virulence factors of *C. perfringens*, NetB and alpha toxin, these proteins induce protective immunity of chickens

against necrotic enteritis, when used as vaccine antigens (Jang *et al.*, 2012; Kulkarni *et al*., 2007 and 2008).

The *netB* gene is highly conserved in *C. perfringens* strains that cause necrotic enteritis (Keyburn *et al.*, 2010). Only in a minority of the strains causing necrotic enteritis, the NetB A168T variant, present in strain 23 supernatant and part of a highly protective supernatant vaccine, could be detected. This mutation however does not lead to decreased cytotoxicity. The A168T substitution occurs in a region that is expected to be within a membrane spanning region (Menestrina *et al.*, 2001). The tertiary structure of the NetB protein was expected to be not significantly affected by the substitution and it was shown that the single amino acid change at residue 168 of NetB did not affected its cytotoxic activity (Keyburn *et al.*, 2010 and Lanckriet *et al.*, 2010). Whether the specific mutation affects immunogenicity is not yet clear. This should be tested in an *in vivo* necrotic enteritis trial.

NetB has been shown to have considerable potential for the development of vaccines against necrotic enteritis. Several vaccination studies with recombinant native NetB, NetB variant S254L and NetB variant W262A were already performed (Fernandes da Costa et al., 2013 and 2016; Jang et al., 2012; Keyburn et al., 2013 a and b). The best protection was observed when birds were vaccinated with the crude toxoid or bacterin supplemented with rNetB (Keyburn *et al.*, 2013b). The study of Keyburn *et al.* (2013b) confirmed that NetB alone is not yielding full protection and that supplementation with other antigens increases the protective response. Keyburn *et al.* (2013a) also used a non-toxic NetB variant (S254L) for vaccinating breeder hens. Neither single NetB nor alpha toxin were capable of inducing full protection against the development of lesions after experimental infection. Our study (Chapter 3.3) showed that the combination of both antigens enhances the protection against a mild challenge but is not sufficient enough against a severe challenge (Fernandes da Costa *et al.*, 2016).

Vaccines using recombinant alpha toxin in toxoid and active form and live delivery of a non-toxic C-terminal domain of alpha toxin have been tested in different models (Kulkarni *et al.*, 2007, Zekarias *et al.*, 2008, Cooper *et al.*, 2009). It was suggested that alpha toxin can be used as an protective antigen. Binding of antibodies to the membrane-bound preprotein might block protein transport channels and as a

consequence, inhibit proliferation of the bacterium (Zekarias *et al.,* 2008). The use of the C-terminal domain of alpha toxin ($CPA_{247-370}$) as an antigen in this thesis can confirm this previously findings. It is likely that a combined alpha and NetB-toxin based vaccine can induce protection in the field, where challenge conditions are possibly less severe than in experimental models.

4.2. Reproduction of necrotic enteritis in experimental models

The evalaution of potential vaccine candidates depends strongly on challenge method used in an *in vivo* NE-model (Shojadoost *et al*., 2012). The severity of the disease, and also the protection against the disease, depends strongly on the challenge method used. As mentioned in the general introduction, reproducing necrotic enteritis is not straightforward. Since necrotic enteritis is a complex and multifactorial disease researchers need to decide which infectious agents, which route of administration for *C. perfringens* and which dietary manipulations they will use as predisposing triggers. It was already mentioned that the challenge strain absolutely needs to possess the *netB* gene to reproduce necrotic enteritis. Strains that also possess the *tPeL* gene appear to cause more severe disease (Coursodon *et al*., 2012). Challenge strains cultured in several different culture media and incubation times were used in experimental models and these factors have an influence. Challenge can occur through supplementation of large volumes of culture broth in-feed (severe challenge) or by oral crop gavage (mild challenge) (Shojadoost *et al*., 2012).Of the other predisposing factors affecting the reproduction of necrotic enteritis that can be manipulated, coccidiosis co-infection is the most important one. Different types of attenuated *Eimeria* vaccines are used in different models and also the time of administration and the dose plays an important role. Necrotic lesions produced by *C. perfringens* combined with coccidia are usually more severe (Williams *et al*., 2003). Some researchers use methods to induce immunosuppression with antiviral vaccines, such as IBD and Gumboro vaccines, but also the mycotoxin fumonisin can be used (Antonissen *et al*., 2015). A dietary factor that is often used as predisposing factor is the addition of fish meal (Shojadoost *et al.,* 2012). It is described that breed, sex and age of the chickens can also play a role (Jang

et al., 2013). The reason for reproducing the disease will have an impact on the design of studies. Production of severe experimental disease will mask some beneficial effects of preventive strategies such as vaccination. Indeed, in chapter 3.3 we confirmed this idea and the vaccination protocol was more effective when a mild as compared to a severe challenge model was applied. For all these reasons it is not possible to compare the positive effects of studied vaccines performed by different researchers and/or in different models. Moreover, it is impossible to compare studies that use different scoring systems. Scales in systems for scoring necrotic enteritis lesions can vary from 0-3 (Gholamiandehkordi *et al.*, 2007; Lovland *et al*., 2004), 0-4 (Cooper *et al*., 2009) to 0-6 (Keyburn *et al.*, 2013a and b; Lanckriet *et al.*, 2010b). Shojadoost *et al.* (2012) recommends that an ideal scoring system should cover the severity of the disease with a wide range for purpose of statistical analysis. The six-point scoring system designed by Keyburn *et al.* (2006) approximates best to this criterium and we used this scoring system in our studies. To measure performance parameters like weight gain, feed intake and feed conversion ratio, replicated test groups with a sufficiently large number of animals are essential for statistical purposes (Shojadoost *et al.*, 2012).Most of the studies performed to test vaccine candidates against necrotic enteritis do not use replicates in groups. We did not do that either (except for trial 2 in chapter 3.1) because these studies are orientating and for animal welfare reasons. Promising results need to be replicated however and even compared to each other in the same experiment.

4.3. Practical application of vaccine candidates

As mentioned in the introduction there are various ways to deliver antigens to chickens for immunization purposes. Live (attenuated) vaccines can be administered orally and induce a stronger immune response (Witter & Hunt, 1994; Plotkin & Plotkin, 2011; Rappuoli *et al*., 2011). Toxin-based formulations must be produced in inactivated form while still preserving antigenicity. Formalin inactivation and genetically engineered inactive toxin variants are an option, as is the delivery of immunogenic non-toxin proteins.

Formalin inactivation showed to be not successful in the use of *C. perfringens*-based vaccines in our study done in Chapter 3.1 (Mot *et al.,* 2014). This is believed to be due to the cross-linking capacity of formaldehyde, with major conformational modifications of the cross-linked proteins, resulting in loss of immunogenicity of epitopes (Metz *et al.,* 2004; Thaysen-Andersen *et al*., 2007; Jones *et al.,* 2008). The importance of conformational epitopes in the protection against necrotic enteritis was also suggested by Kulkarni *et al*. (2007), who showed that alpha-toxoid fails to offer protection. The impact of other inactivation methods such as heat-inactivation and alkalization was investigated (unpublished data) but showed no significant differences.

Our study in Chapter 3.1 showed that one parenteral single vaccination, at day of hatch, which is the only practical feasible way in the field, offers no protection. Whether the lack of protection of a formalin-based toxoid vaccine and day-of-hatch vaccine regimes is also valid when using vaccine preparations derived from other *C. perfringens* strains (and not the one used in our study) is not clear and should be analyzed in future studies. Because it was shown that formalin-based toxoid has detrimental effects on the ability of the toxins to protect against necrotic enteritis when used as vaccine antigens, non-toxic protein fragments may be the antigens of choice.

Since vaccination at day of hatch is not protective, there are only a few options left. These are breeder hen vaccination and the use of live bacterial or viral vectors that can deliver antigens *in ovo* or during rearing (eg. as feed or drinking water additive, thus oral vaccination), therefore presenting the antigens for a longer period as compared to parenteral administration of antigens at day-of-hatch.

4.4. Vaccine delivery and immunization methods for necrotic enteritis

Ease of administration of a vaccine is important for making vaccines acceptable for the poultry industry. Because large populations of animals must be vaccinated, the most widely accepted vaccines are those that can be delivered simultaneously to large numbers of birds with minimum amount of labor (Sharma, 1999). For practical reasons, vaccines are mostly given in the hatchery. Parenteral vaccination

(intramuscularly or subcutaneously) of broiler chickens is theoretically possible at day-of-hatch, but vaccination using live vaccines by spray methods or drinking water application is easier to apply. Parenteral booster vaccinations are practically impossible for broilers in the field. Other options are breeder hen vaccination and the use of live bacterial or viral vectors that can deliver antigens *in ovo* or during rearing (eg. as feed or drinking water additive, thus oral vaccination), thereby presenting the antigens for a longer period as compared to parenteral administration of antigens at day-of-hatch.

4.4.1. Breeder hen vaccination

Vaccination of breeder hens is often preferred in the poultry industry. Due to the generation of large numbers of protected progeny per vaccinated hen, the vaccine cost per chicken is lower as compared to post-hatch vaccination (Schijns *et al*., 2008). Passive protection by maternal antibodies in broiler chickens by breeder hen vaccination could have some limitations with regard to necrotic enteritis. Indeed, outbreaks of necrotic enteritis mostly occur at the age of 3-4 weeks. The immune system of broiler chickens is still developing at that age and maternal antibodies have declined already (Lovland *et al*., 2004). Until now, three studies have reported data on maternal vaccination against necrotic enteritis, two of them using crude supernatant toxoids and one using rNetB (S254L) either in combination or not with crude toxoid (Lovland *et al*., 2004, Crouch *et al.,* 2010, Keyburn *et al.,* 2013a). When breeder hens were vaccinated intramuscularly at 14 and 18 weeks of age with *C. perfringens* type A or type C crude toxoid, an increase in antibody response to alpha toxin in serum samples of parent hens was shown. In a field trial under predisposing conditions a partial protection against necrotic enteritis in their progeny was shown (Lovland *et al*., 2004). The safety and efficacy of a commercial *C. perfringens* type A alpha toxoid (Netvax^{TM}) was analyzed by immunizing breeder hens intramuscularly at 11 and 18/19 weeks of age. An increase in specific alpha toxin IgY antibody response was shown in serum from hens, in the egg yolk from eggs collected from these hens and in serum from 7-day-old chicks hatched from these eggs (Crouch *et al*., 2010). In a field trial, the progeny (from eggs collected at 27 and 32 weeks) from a group of NetVax^{TM}-vaccinated hens had a reduced overall mortality as compared to the progeny from an unvaccinated group,

especially at those time points at which necrotic lesions were observed in the progeny from the unvaccinated group (Crouch *et al.*, 2010). Recently, a recombinant non-toxic NetB variant (S254L) was tested in breeder hens, singly or combined with crude toxoid (Keyburn *et al.*, 2013a). Hens were vaccinated subcutaneously at 22, 24 and 26 weeks of age. A significant IgY antibody response against NetB was detected in serum samples from hens, in the egg yolk of their eggs and in serum from hatched chicks from vaccinated hens. When the progeny (from eggs collected at 30 weeks) of vaccinated hens was infected with in-feed *C. perfringens* at 26 and 27 days of age, only chickens derived from hens vaccinated with rNetB (S254L) combined with crude toxoid had a significantly lower lesion score. When the *C. perfringens* infection was performed at 14 days of age, chickens derived from hens vaccinated with single rNetB (S254L) or single crude toxoid were also protected partially (Keyburn *et al*., 2013a). The authors hypothesized that a higher level of specific antibodies at the time of challenge is responsible for the protection against challenge at the earlier age.

4.4.2. *In ovo* vaccination and oral immunization using viral or bacterial vector vaccines

Chickens can be vaccinated using vector vaccines *in ovo* or during rearing. Benefits of *in ovo* vaccination compared to post-hatch vaccination include earlier immunity, reduction in bird stress, precise and uniform injection and reduced labor costs (Ricks *et al*., 1999; Schijns *et al*., 2008). The vaccine is injected in eggs during the late embryonation stage, usually at 17-18 days of incubation (Muir *et al*., 2000). Recombinant fowl poxvirus (FPV) and herpesvirus of turkey (HVT) replicating viruses are examples of vector vaccines for *in ovo* application (Schijns *et al*., 2008). When a non-replicative vector for *C. perfringens* antigens is injected *in ovo*, it is possible that protective antibodies would already be in decline at the time the disease occurs. Furthermore the choice of the adjuvant is important as some adjuvants are known for inducing embryotoxic side effects (Asif *et al.*, 2004). To the best of our knowledge there are no studies reporting efficacy of *in ovo* vaccine against necrotic enteritis.

Oral immunization of broilers can be done through the feed or drinking water or by spraying the vaccine onto the chickens (Sharma, 1999). These delivery systems are labor- and time-saving and practically feasible for the broiler industry. Chickens do not always drink regularly in the first days after hatching. In contrast, (coarse) spray application may increase the vaccine uptake and lead to a more consistent level of protection against the pathogen (Atterbury *et al*., 2010). Orally administered live vaccine strains expressing *C. perfringens* antigens and colonizing the intestinal tract of the broilers have been described (Kulkarni *et al.*, 2008, Zekarias *et al*., 2008; Kulkarni *et al.*, 2010). The protection obtained depends on the colonization level and persistence of the vaccine strains. Kulkarni *et al.* (2008, 2009) immunized broilers orally at day of hatch and at day 14 with a recombinant *S. enterica* serovar Typhimurium strain expressing truncated proteins of the alpha toxin, FBA, PFOR or HP. They induced a significant protective immune response but the degree of protection was lower than observed when these proteins were administered intramuscularly in multiple dosages (Kulkarni *et al*., 2006). Zekarias *et al.* (2008) inoculated chickens orally with a *S. enterica* serovar Typhimurium strain expressing a nontoxic fragment of alpha toxin at day 3 and 13. The antibody response was low, but the immunized chickens had a reduced number of necrotic lesions after challenge. The above mentioned studies, however, used oral gavage of the vaccine strains. Practical delivery methods, such as in-feed, drinking water or spray application have not been tested yet. Recombinant *B. subtilis* endospores that express the C-terminal domain of alpha toxin have been used to vaccinate mice against *C. perfringens* infection (Hoang *et al.*, 2008). The endospores appear to provide an adjuvant effect, boosting the immune response to the antigens. The use of these heat-stable endospores as vaccine delivery agents is a promising idea because they could be incorporated into feed. This type of bacterial vector has not been evaluated for necrotic enteritis in broilers yet.

4.5. Concluding remarks and future perspectives

Before the identification of the toxin NetB and important immunogenic proteins, formalin-inactivated crude supernatants were tested as experimental vaccines. During the last few years vaccination studies

have been carried out with purified proteins or combinations of proteins, mostly by parenteral immunization. The impact of these studies has been important to identify proteins as vaccine candidates (such as the NetB toxin), and it has become clear that combinations of immunogenic proteins are yielding better protection compared to single protein immunization (Keyburn *et al.*, 2013a). To determine relevant vaccine candidates it is necessary to understand completely the pathogenesis and know exactly which proteins are expressed by *C. perfringens* at a necrotic lesion in the small intestine. It is known that the NetB toxin is crucial for the development of necrotic enteritis but knowing which factors are produced at the lesions and contribute to lesion severity can help to understand the pathogenesis and will enhance the vaccine development (Prescott *et al*., 2016). Our studies were focused on practical application and the search of an ideal combination of antigens. Most researchers used multiple dosage parenteral immunization regimes which suffer from lack of practical value for broilers. Single dosing at day-of-hatch, a possible method that can be used in the field, results in total loss of protection compared to multiple dosage vaccination. Breeder hen vaccination is an option and several studies have shown promising results, but the antibody decline in the progeny will decrease the efficacy at later ages, which may be important for necrotic enteritis which typically occurs at 3 to 4 weeks of age. *In ovo* vaccination could be a valuable method, but no data have been reported so far on this strategy. Since immunogenic proteins need to be presented to the immune system for a more prolonged period of time using a single dosage, live attenuated bacterial (or viral or parasitic) vectors are a potential strategy for the future. The protection depends on the colonization level and persistence of the live vaccine strains and the combination and levels of the expressed antigens. It is observed that the severity of the challenge, feed and housing conditions of the animals, the strain used for infection, and many more factors affect the protection conferred by a vaccine under experimental conditions. In this regard, a universal infection model that can be used to test the efficacy of vaccines could be of value, as suggested by Shojadoost *et al*. (2012), but this will be difficult to establish. The ideal vaccine strain would be one that, apart from inducing immunity and protection, can be added to the feed or drinking water, or sprayed on the day-old chicks in the hatchery. As is the case for many other bacterial diseases in livestock, active immunization

will only be effective when it is part of an approach that takes into account appropriate management and sanitation measures, feed quality optimization and preventive measures that focus on limiting the presence of predisposing factors. Since subclinical necrotic enteritis has an impact on broiler performance, with high economical loss as a consequence vaccination, against *C. perfringens* induced necrotic enteritis, is still a promising preventive control method.

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Summary

Summary

Necrotic enteritis is an important gastrointestinal disease broiler chickens. It is caused by *Clostridium perfringens* type A strains that produce the NetB toxin. It is an anaerobic bacterium which can be found in the environment and also in the normal gastro-intestinal microbiota of humans and animals. The disease, necrotic enteritis, can occur as an acute clinical disease, with high mortality at 2 to 5 weeks of age, or in a subclinical form leading to reduced weight gain and an increased feed conversion ratio. The disease can go unnoticed but increased feed conversion ratio makes the subclinical form economically the most important one. Necrotic enteritis develops when several predisposing factors are present, such as coccidial co-infection leading to damage of the intestinal mucosa and high protein high non-starch polysaccharide containing diet. After the ban of antimicrobial growth promotors, which acted prophylactic against necrotic enteritis, the proportion of necrotic enteritis affected flocks has risen. Nowadays, the disease is prophylactically controlled by the use of anticoccidials of the ionophore type, which have antibacterial effects, and therapeutically by the use of antibiotics. But their use is no longer considered as viable due to resistance problems. There is a focus on prevention strategies like avoiding predisposing factors and in-feed supplementation of a variety of feed additives. However, vaccination of broilers may be an interesting option for the prevention of necrotic enteritis since it seems a logical tool for protection against a toxin-producing bacterium. In recent years, several studies have been done in this area. Vaccination in broiler chickens can be done using oral, subcutaneous or intramuscular administration. Live attenuated *C. perfringens* vaccine strains were tested but protein-based vaccines should be safer and better characterized. Several proteins and toxins have been tested as vaccine candidates. The use of attenuated or avirulent live vectors is a promising approach and deserves further investigation. The choice of the proteins that are expressed by the vector strains is an important issue.

During in vivo trials, we tested whether subcutaneous vaccination is practical applicable in the field **(Chapter 3.1).** Since crude supernatant of *C. perfringens* contains potent toxins it is not safe for the

animals. Formaldehyde inactivation was used ensuring safety but this affected the efficacy. A formaldehyde toxoid was clearly less protective against necrotic enteritis than the active supernatant. The reason for the loss of protection can be found in the cross-linking capacity of formaldehyde, with major conformational modifications of the cross-linked proteins, resulting in loss of immunogenicity of epitopes. The results showed also that single vaccination at day of hatch, which is practical feasible in the field, even with crude supernatant, failed to offer protection against experimental necrotic enteritis, in contrast to double vaccination at day 3 and 12 and single vaccination at day 3 with crude supernatant.

The ideal combination of antigens in the protection against necrotic enteritis is still under investigation. In our second study **(Chapter 3.2),** we aimed to identify proteins from supernatant of *C. perfringens* strain 23 with increased immunogenicity compared to supernatant from other strains by Western blotting. Three strong reacting antigens were identified as PFOR (pyruvate:ferredoxin oxidoreductase), Elongation factor G and NetB. The NetB variant A168T was detected in *C. perfringens* strain 23 and this could be the reason for the strong antigenic reaction of the strain 23-immunized chickens towards. Since chickens immunized with virulent strain 56 (with the consensus NetB) showed not such a high immune response. The mutation A168T in the NetB toxin does not lead to decreased cytotoxicity and the tertiary structure of the NetB protein was expected to be not significantly affected by the substitution. Whether the mutation affects immunogenicity is not yet clear and should be investigated in further vaccination studies.

The combination of protective antigens is a crucial factor in the development of a vaccine against necrotic enteritis. Despite the fact that is has been shown that alpha toxin is not an essential virulence factor in the disease it can be used as a protective antigen against necrotic enteritis. NetB, identified as the responsible toxin in the development of the disease, can be considered as an important antigen against necrotic enteritis. Since it was shown that formaldehyde inactivation of toxins has negative effects on the ability of toxins to protect animals, the non-toxic protein fragments $CPA_{247\text{-}370}$ and NetB W262A were used **(Chapter 3.3)**. The combination of these antigens was tested in different experimental models to reproduce the disease since it is known the severity of challenge can affect the results. Our data show that

that the combination of $CPA_{247-370}$ and NetB W262A resulted in complete protection in a mild subclinical disease, however in a severe in-feed model the protection was only partial. Since the vaccination scheme, three times subcutaneous administration, is not practical in the field there is a need for alternative routes of delivery.

In conclusion, this thesis offers new insights in the development of an optimal vaccine against necrotic enteritis in broiler chickens. It was shown that the ideal vaccine strain, consisting of an optimal combination of proteins, should be tested in an universal infection model in a way that is practical feasible in the field. This means adding to the feed or drinking water, or sprayed on the day-old chicks in the hatchery.

Samenvatting

Samenvatting

Necrotische enteritis is wereldwijd een belangrijke gastro-intestinale aandoening bij vleeskuikens. De ziekte wordt veroorzaakt door de grampositieve bacterie *Clostridium perfringens* en meer bepaald de toxinotype A stammen die het NetB toxine produceren. Deze anaerobe bacterie komt, dankzij zijn vermogen om resistente sporen te vormen, wijdverspreid voor in de omgeving. De kiem kan ook deel uitmaken van de normale gastro-intestinale flora van mens en dier. Onder bepaalde predisponerende factoren kan *C. perfringens* echter exponentieel gaan vermeerderen en ziekte veroorzaken. Deze ziekte, necrotische enteritis, kan voorkomen in een acute klinische vorm die gekenmerkt wordt door een verhoogde mortaliteit, op een leeftijd van 2 tot 5 weken, in de pluimveetoom, of in een mildere subklinische vorm die leidt tot een verminderde gewichtstoename en dus een toegenomen voederconversie. Een kippentoom kan onopgemerkt de subklinische vorm doormaken maar de gestegen voederconversie veroorzaakt grote financiële verliezen in de pluimvee industrie. Door het ontstaan van intensieve pluimveehouderijen heeft de ziekte in belang gewonnen. Na het verbod op antimicrobiële groeipromotoren door de Europese Unie kende het voorkomen van necrotische enteritis een enorme piek. Momenteel wordt de ziekte in de praktijk onder controle gehouden door het preventief gebruik van coccidiostatica en het curatieve gebruik van antibiotica maar omwille van de gekende resistentieproblematiek is de nood aan alternatieven groot. Een optimaal management van de pluimveetoom en bepaalde voedingssupplementen zijn een gedeeltelijk succes, maar vaccinatie zou de ideale preventiestrategie tegen necrotische enteritis zijn. Vaccins kunnen oraal, intramusculair of subcutaan toegediend worden maar omwille van praktische redenen kunnen de twee laatste enkel toegediend worden in de broeierij. Het gebruik van een oraal vaccin met verzwakte of niet-virulente vectoren lijkt de beste optie waarbij de combinatie van de *C. perfringens* proteïnen die tot expressie gebracht worden een cruciale rol spelen.

In het eerste hoofdstuk van deze thesis werd nagegaan of subcutane vaccinatie tegen necrotische enteritis zou kunnen toegepast worden in de praktijk **(Chapter 3.1)**. Omdat natief supernatans van *C. perfringens* potentieel toxisch kan zijn voor het gevaccineerde dier werd formaldehyde inactivatie toegepast. Er werd aangetoond dat dit de efficaciteit benadeelt, met andere woorden, de bescherming van het vaccin tegen het ontstaan van necrotische enteritis ging verloren. Deze resultaten suggereren dat het gebruik van formaldehyde voor detoxificatie grote structurele veranderingen aan de *C. perfringens* proteïnes veroorzaakt zodanig dat het onmogelijk is om beschermende antilichamen op te wekken. De resultaten tonen ook aan dat een eenmalige toediening van het vaccin, op de dag van uitkippen, de manier waarop in de praktijk gevaccineerd wordt, geen bescherming biedt. Dit in tegenstelling tot de dubbele vaccinatie met natief supernatans op dag 3 en 12 na uitkippen. Ook een eenmalige vaccinatie op dag 3 was gedeeltelijk protectief.

De combinatie van antigenen die gebruikt moeten worden speelt een cruciale rol in de ontwikkeling van een vaccin tegen necrotische enteritis en onderzoek hiernaar is nog steeds lopende, onder meer ook omdat de pathogenese nog steeds niet volledig gekend is. In onze tweede studie **(Chapter 3.2)** gingen we op zoek naar proteïnen die een verhoogde immunogeniciteit vertoonden in supernatans van *C. perfringens* stam 23 aangezien uit voorgaande studies reeds bleek dat vaccinatie met het supernatans van deze stam volledige bescherming bood. Door gebruik te maken van Western Blots konden drie sterk reagerende antigenen geïdentificeerd worden, PFOR (pyruvate:ferredoxin oxidoreductase), Elongation factor G en NetB. *C. perfringens* stam 23 is drager van de NetB variant A168T wat de oorzaak kan zijn van de sterk antigene reactie want serum van kippen die geïmmuniseerd werden met de virulente stam 56 (drager van het consensus NetB toxine) vertoonden een minder sterke antigene reactie. De A168T mutatie in het NetB toxine tast de cytotoxiciteit en de tertiaire structuur van het toxine niet aan. Of deze mutatie gevolgen heeft voor de immunogeniciteit zou moeten onderwerp uitmaken van verder onderzoek. Ondanks het feit dat aangetoond werd dat het *C. perfringens* alpha toxine geen essentiële virulentiefactor is in de pathogenese, werd in het verleden reeds aangetoond dat het gebruik van het toxine in een vaccin wel

bescherming induceert. Het NetB toxine, verantwoordelijk voor het ontstaan van de ziekte, is vanzelfsprekend een belangrijk antigen. Omdat in het eerste hoofdstuk aangetoond werd dat formaldehyde inactivatie nadelige gevolgen had voor de efficaciteit werd in het derde hoofdstuk gebruik gemaakt van de niet-toxische proteïne fragmenten $CPA_{247-370}$ en NetB W262A **(Chapter 3.3)**. Een combinatievaccin van deze twee antigenen werd getest in twee experimentele proeven met vleeskuikens met een verschillende infectiegraad. Er werd aangetoond dat het combinatievaccin met $CPA_{247-370}$ en NetB W262A volledige bescherming bood tegen een milde subklinische infectie maar slechts gedeeltelijke bescherming bood in een zwaar infectiemodel.

De resultaten van deze thesis hebben geleid tot nieuwe inzichten in de ontwikkeling van een doeltreffend vaccin tegen necrotische enteritis bij vleeskuikens. Er werd aangetoond dat het ideale vaccin, bestaande uit een optimale combinatie van proteïnen, getest dient te worden in een universeel infectiemodel op een manier die toe te passen is in de praktijk.

Curriculum Vitae

Curriculum Vitae

Dorien Mot werd geboren op 27 april 1985 te Halle. Na het beëindigen van haar studies algemeen secundair onderwijs, richting Latijn-Wetenschappen, aan het Onze-Lieve-Vrouwecollege te Halle, begon ze in 2003 met de studies Diergeneeskunde aan de Universiteit Gent. In 2010 behaalde ze het diploma Master in de Diergeneeskunde (optie gezelschapsdieren).

In juli 2010 trad ze in dienst bij de Vakgroep Pathologie, Bacteriologie en Pluimveeziekten van de Faculteit Diergeneeskunde en was werkzaam op een FOD-project over *Salmonella Gallinarum*. In oktober van datzelfde jaar startte ze haar doctoraatsonderzoek over vaccinatie strategieën tegen necrotische enteritis bij pluimvee, gefinancierd door Pfizer. Verder begeleidde zij verschillende studenten in het behalen van hun masterproef en vervolledigde ze in 2016 het programma van de Doctoral Schools of Life Sciences and Medicine aan de Universiteit Gent.

Sinds juli 2014 is zij werkzaam als inspecteur-dierenarts bij het Federaal Agentschap voor de Veiligheid van de Voedselketen.

Dorien Mot is auteur of medeauteur van verschillende wetenschappelijke publicaties in internationale tijdschriften en nam deel aan internationale congressen waar ze de resultaten van haar onderzoek presenteerde.

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Dankwoord

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