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VIRULENCE MECHANISMS OF *CLOSTRIDIUM PERFRINGENS* IN BROILER NECROTIC ENTERITIS

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TABLE OF CONTENTS

List of abbreviations

ADP	adenosine diphosphate
AFLP	amplified fragment length polymorphism
ATP	adenosine triphosphate
BHI	brain heart infusion
BLAST	basic local alignment search tool
BLIS	bacteriocin-like inhibitory substance
CFU	colony forming units
DNA	deoxyribonucleic acid
EF-G	elongation factor G
ELISA	enzyme linked immunosorbent assay
FBA	fructose 1,6-biphosphate aldolase
GAPDA	glyceraldehyde-3-phosphate dehydrogenase
HP	hypothetical protein
LMH	leghorn male hepatoma cell line
LYM	lyophilisation medium
MLST	multilocus sequence typing analysis
MLVA	multiple-locus variable-number tandem repeat analysis
NCBI	national center for biotechnology information
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
PFOR	pyruvate:ferredoxin oxidoreductase
RNA	ribonucleic acid
TMHMM	transmembrane hidden markov model
TSB	tryptic soy broth

Chapter 1

General Introduction

Necrotic enteritis in broilers

Etiology

Clinical signs and gross lesions

Histopathology

Role of *Clostridium perfringens* in the pathogenesis of necrotic enteritis

Basic characteristics

Virulence factors

Genetic diversity

Single strain dominance in necrotic enteritis

Antimicrobial proteins from bacteria

Bacteriocins

Bacteriocins of Gram-positive bacteria

Bacteriocins of *Clostridium perfringens*

CHAPTER 1: GENERAL INTRODUCTION

1. NECROTIC ENTERITIS IN BROILERS

Enteric diseases are an important concern to the poultry industry because of production losses, increased mortality, reduced welfare of birds and increased risk of contamination of poultry products for human consumption. Necrotic enteritis was first described by Parish (1961) and is a common enteric disease, caused by *C. perfringens*. The disease usually occurs in broiler chickens at about 4 weeks after hatching and is found in all poultry-growing areas of the world (Long, 1973; Dahiya et al., 2006).

1.1. ETIOLOGY

Today it is commonly accepted that the bacterium *C. perfringens* plays an important role in the development of necrotic enteritis (Truscott and Al-Sheikhly, 1977; Gholamiandehkordi et al., 2007). However, *C. perfringens* is ubiquitous in the environment and is also a member of the normal gut microbiota of vertebrates (Songer, 1996; Porter, 1998). Moreover, chickens without *C. perfringens* among the normal flora are uncommon (Shane et al., 1984; Miwa et al., 1997a, b; Craven et al., 2001; Van Immerseel et al., 2004). The intestine of birds suffering from necrotic enteritis contains large numbers of *C. perfringens*, up to 10^6-10^8 cfu/g of the intestinal contents, whereas in healthy broilers, counts from 0-10^5 cfu/g of the intestinal contents are normal (Long et al., 1974; Baba et al., 1997; Si et al., 2007). However, the presence of *C. perfringens* in the intestinal tract of broiler chickens, even at high numbers, is not sufficient to produce necrotic enteritis (Long and Truscott, 1976; Cowen et al.; 1987, Kaldhusdal et al., 1999; Craven, 2000; Pedersen et al., 2003; Nauerby et al., 2003). Therefore, it is generally accepted that predisposing factors or risk factors are required for these bacteria to cause disease.

The key risk factor for the development of necrotic enteritis is an intestinal environment that favors growth of the organism. The best-known predisposing factor is mucosal damage caused by coccidial pathogens (Williams, 2005). Coccidiosis is often seen to proceed or occur concurrent with field outbreaks of necrotic enteritis (Long, 1973; Broussard et al., 1986; Gazdzinski and Julian, 1992; Porter, 1998). Moreover, it is shown in experimental infection studies that *C. perfringens* and *Eimeria* act synergistically in inducing necrotic enteritis lesions. Coinfection with *C. perfringens* and *Eimeria* oocysts or commercial coccidiosis vaccines containing attenuated *Eimeria* strains result in more animals with lesions or in higher mortality rates compared with birds receiving only *Eimeria* or only *C. perfringens* (Al-Sheikhly and Al-Saieg, 1980; Shane et al., 1985; Baba et al., 1997; Gholamiandehkordi et al., 2007; Park et al., 2008; Pedersen et al., 2008). *Eimeria* parasites colonize the small intestine and kill epithelial cells as a consequence of the intracellular stages of their life cycle. Through the resulting gaps in the epithelial lining of the intestinal lumen, plasma proteins are leaking into the gut lumen and these can be used as growth-substrate by *C. perfringens* strains (Van Immerseel et al., 2004). Moreover, coccidial infection induces a T-cell mediated inflammatory response that enhances intestinal mucogenesis. This enhanced mucus production provides a growth advantage to *C. perfringens* due to its ability to use mucus as a substrate (Collier et al., 2008). It is pertinent to note that *C. perfringens* is auxotrophic for thirteen amino acids (Shimizu et al., 2002; Myers et al., 2006), an increase in available nutrients would thus allow *C. pefringens* to proliferate extensively. Furthermore, Park et al. (2008) suggested that the exacerbated pathological findings after co-infection with *Eimeria* and *C. perfringens* are caused by an altered cytokine response.

The nature of the diet is an important non-bacterial factor that influences the incidence of necrotic enteritis. Diets with high levels of indigestible, water-soluble non-starch polysaccharides predispose to necrotic enteritis. So, wheat, rye, oat, and barley are risk factors for necrotic enteritis, whereas maize is not (Branton et al., 1987; Hofshagen and Kaldhusdal, 1992; Kaldhusdal and Hofshagen, 1992; Kaldhusdal and Skjerve, 1996; Riddell and Kong, 1992; Craven, 2000; Jia et al., 2009). Some of these effects may be related to differences in digesta viscosity, decreased nutrient digestibility and prolonged intestinal transit time (Choct et al., 1996). High dietary concentrations of animal protein, such as fishmeal, have also been reported to increase the incidence of necrotic enteritis (Truscott and Al-Sheikhly, 1977; Drew

et al., 2004; Gholamiandehkordi et al., 2007). In general, protein-rich diets containing relatively high concentrations of poorly digestible proteins lead to high concentrations of protein in the gastrointestinal tract and thus act as substrates for the bacteria (Williams et al., 2001). The dietary fat source also inflicts on the *C. perfringens* population. Animal fat increases *C. perfringens* counts compared to vegetable oil (Knarreborg et al., 2002). Even the physical form of the feed may influence the incidence of necrotic enteritis. Mashed feeds are associated with higher numbers of *C. perfringens* in the digestive tract compared to pelleted feed (Engberg et al., 2002). Mortality due to necrotic enteritis was higher in groups fed a hammer-mill diet compared to groups fed a roller-mill diet (Branton et al., 1987). The feed particle size in mashed feed and hammer-mill feed varies widely around the geometric mean, it thus contains some large-sized and many small-sized particles. In contrast, feed particles are uniform in size in pelleted feed and roller-mill feed.

Apart from *Eimeria* infections and the feed, any factor that causes stress in broiler chickens could predispose them to necrotic enteritis because it could alter the intestinal environment in such a way that the risk of necrotic enteritis occurring is elevated. Programmed alterations in the feeding regime (moving from starter diets to grower diets), are frequently associated with necrotic enteritis. Furthermore, immunosuppressive agents such as chick anaemia virus, Gumboro disease or Marek's disease, reduces resistance to gut infections and may increase the severity of disease. Also physical rupture of the gastrointestinal tract by rough litter and increases in stocking density predispose to necrotic enteritis (McDevitt et al., 2006).

It is thus generally accepted that any factor that may increase the *C. perfringens* counts in the intestine is a risk factor for the development of necrotic enteritis. Nevertheless, as stated above, presence of *C. perfringens* in high numbers is not sufficient to produce necrotic enteritis. Therefore, additional, unknown factors determine if necrotic enteritis develops.

1.2. CLINICAL SIGNS AND GROSS LESIONS

Many signs of necrotic enteritis are aspecific. Birds are depressed, reluctant to move, and have ruffled feathers and drooping wings and head. They may be somnolent, diarrheic, anorexic, and dehydrated and they can emit a foul smell (Helmboldt and Bryant, 1971; Long 1973; Van Immerseel et al., 2004). The disease can occur in two forms, it may present as acute clinical disease or sub-clinical disease.

1.2.1. CLINICAL NECROTIC ENTERITIS

The acute clinical form of the disease is characterized by a sudden increase in flock mortality, often without premonitory signs, although wet litter is sometimes an early indicator of disease. The course is often peracute, with death in 1-2 h. Mortality may sometimes exceed 1% daily and the duration of the outbreak in a flock is normally one week (Helmboldt and Bryant, 1971).

Gross lesions are usually restricted to the small intestine, but lesions can also occur in other organs, such as caeca, liver and kidney. Upon necropsy, the duodenum, jejunum and ileum are usually thin walled and filled with gas. Confluent mucosal necrosis of large parts of the small intestine, covered by a yellow-brown or bile-stained pseudomembrane, is found (Figure 1A) (Helmbold and Bryant, 1971; Long et al., 1974; Broussard et al., 1986; Olkowski et al., 2006).

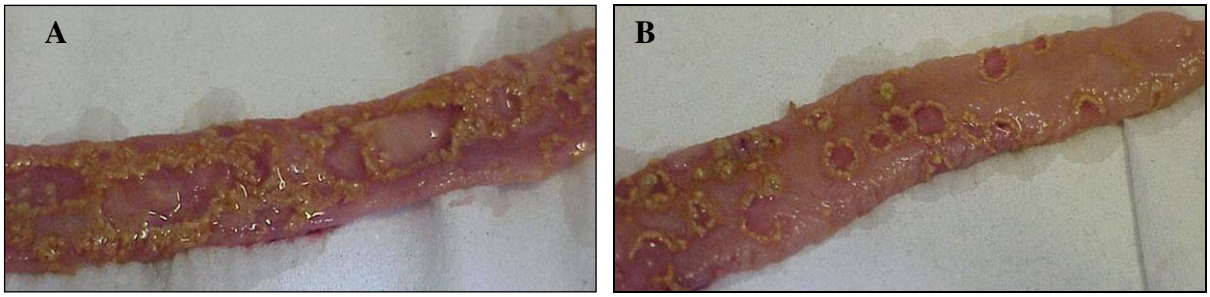

Figure 1: A. Confluent necrosis of the jejunum B. Focal necrosis of the jejunum

1.2.2. SUB-CLINICAL FORM OF *CLOSTRIDIUM PERFRINGENS* INFECTION

In the sub-clinical form, the clinical signs are milder and usually there is no peak mortality. Intestinal damage leads to production losses due to decreased digestion and absorption, reduced growth-rate and increased feed-conversion ratio. Ulcers in the form of a depression in the mucosal surface, with discolored, amorphous material adhering to the mucosal surface are typical for these cases (Figure 1B) (Kaldhusdal and Hofshagen, 1992). Also mucosal gizzard lesions have been associated with *C. perfringens* infection, although it remains unclear whether *C. perfringens* is the cause of the gizzard lesions (Novoa-Garrido et al., 2006). Moreover, it is shown that *C. perfringens* is also able to cause a liver-associated disease, cholangiohepatitis. Gizzard and liver lesions are found during meat inspection at slaughter, often without any sign of clinical disease in the flock. This increases the number of

condemnations at processing (Onderka et al., 1990; Løvland and Kaldhusdal, 1999; Sasaki et al., 2000). There is a general consensus that, although clinical outbreaks of necrotic enteritis may cause high levels of mortality, the sub-clinical form of the disease is more important than the clinical form because it may persist in broiler flocks without overt clinical manifestations. Since the disease is undetected and birds remain untreated, sub-clinical necrosis causes the greatest economic losses in the poultry production industry (Kaldhusdal and Hofshagen, 1992; Dahiya et al., 2006).

1.3. HISTOPATHOLOGY

Microscopic examination of early stages of necrotic enteritis show strong inflammatory reactions to *C. perfringens*. The lamina propria is hyperemic and infiltrated with numerous inflammatory cells. Most significant changes are seen at the interface of the basal domain of enterocytes and lamina propria. These areas are extensively edematous, allowing for the substantial disturbance of the structural integrity between the lamina propria and the enterocytes (Olkowski et al., 2006). Microscopic examination of later stages of necrotic enteritis lesions shows diffuse and severe coagulative necrosis of the mucosa, involving the luminal third to half of the mucosa. Necrosis of enterocytes in these areas is apparent on the villi. A clear line of demarcation between necrotic and viable tissue and an accumulation of heterophilic granulocytes at the junction is seen. If present, the pseudomembrane consists of masses of tissue fragments, necrotic cells, cell debris, and numerous bacterial colonies suspended in mucus. Congestion of blood vessels is seen in the lamina propria and submucosa. Large Gram-positive rods are associated with areas of necrosis but do not invade the epithelium or are never found to be attached to the viable mucosal epithelial cells, despite their massive presence in the lumen tissue debris (Helmboldt and Bryant, 1971; Long et al., 1974; Al-Sheikhly and Al-Saieg, 1980; Broussard et al., 1986; Olkowski et al., 2006).

2. ROLE OF *CLOSTRIDIUM PERFRINGENS* IN THE PATHOGENESIS OF NECROTIC ENTERITIS

C. perfringens was first described as *Bacillus aerogenes capsulatus* in 1892 and has also been commonly known as *C. welchii* (Hatheway, 1990). *C. perfringens* may be the most widely occurring bacterial pathogen in nature (Songer, 1996). The bacterium is commonly found in soil and sewage and is a normal microbiota component of the intestinal tract of warm-blooded animals and men. *C. perfringens* has been shown to be a cause of human diseases, including gas gangrene, necrotic enteritis and food poisoning. It is also the most important cause of clostridial enteric disease in domestic animals (Rood and Cole, 1991; Songer, 1996; Rood, 1998).

2.1. BASIC CHARACTERISTICS

Clostridium perfringens is a Gram-positive, rod-shaped bacterium. The rods are relatively large: 0.6-2.4 x 1.3-19.0 µm (Cato et al., 1986; Hatheway, 1990). *C. perfringens* has no flagella but it is motile by way of type IV pili (Varga et al., 2006). It is classified as an anaerobe, although *C. perfringens* is less strictly anaerobic than other *Clostridia* (Cato et al., 1986; Novak and Juneja, 2002). *C. perfringens* can survive under extreme conditions, due to its differentiation from vegetative cells to highly resistant dormant spores (Novak and Juneja, 2002). Growth has been shown at temperatures as high as 50 °C, while slowly arresting near 6 °C and below. Under optimal conditions (43-45 °C), *C. perfringens* is known as the most rapidly multiplying organism with generation times often less than 10 min and growth is accompanied by abundant gas production. Growth is limited at pH-values lower than 5.0 or higher than 8.0 and optimal growth is between pH 6 and 7 (Cato et al., 1986; Novak and Juneja, 2002). Genome analysis has revealed that *C. perfringens* lacks the genetic machinery to produce 13 essential amino acids (Shimizu et al., 2002; Myers et al., 2006). As a consequence, *C. perfringens* is not able to grow in an environment where amino acids are limiting and it can obtain these via the action of exotoxins, some of which are enzymes.

2.2. VIRULENCE FACTORS

C. perfringens does not invade healthy cells but produces an intimidating arsenal of toxins and enzymes that are responsible for the associated lesions and symptoms (Petit et al., 1999).

2.2.1. TOXINS

The *C. perfringens* toxins are classified in major toxins, an enterotoxin and minor toxins (Hatheway et al., 1990). Individual strains produce only portions of this toxin repertoire and the ability of *C. perfringens* to cause disease is ascribed mainly to the differential production of four major toxins, an enterotoxin, and 9 minor protein toxins (Hatheway, 1990; Songer, 1996; Rood, 1998).

2.2.1.1. MAJOR TOXINS

The major toxins are alpha, beta, epsilon, and iota toxin, all potentially lethal depending on the host. The bacterium is classified into 5 types (A through E) according to different combinations of production of the four major toxins, as shown in Table I (Sterne and Warrack, 1964; Songer, 1996).

Table I: Toxins used for typing *C. perfringens* (Petit et al., 1999).

Type	Toxin(s) produced			
	alpha toxin	beta toxin	epsilon toxin	iota toxin
A	+	-	-	-
B	+	+	+	-
C	+	+	-	-
D	+	-	+	-
E	+	-	-	+
gene	*plc*	*cpb1*	*etx*	*iap, ibp*
location	chromosome	plasmid	plasmid	plasmid

C. perfringens toxin genes are located on the chromosome or on plasmids. The alpha toxin gene is located in a very stable region on the *C. perfringens* chromosome (close to the origin of replication) and this is why all *C. perfringens* strains carry this gene and why it is produced in varying amounts by all isolates (Canard and Cole, 1989). *Clostridium perfringens* phospholipase C (alpha toxin) is a Zn^{2+} metalloenzyme that degrades both lecithin and sphingomyelin. It promotes membrane disorganization resulting in lysis or other forms of cytotoxicity. Alpha toxin displays platelet aggregating, haemolytic, necrotic, and vascular permeabilization activities (Songer, 1997; Rood, 1998; Titball, 1999; Sakurai et al., 2004; Flores-Díaz et al., 2004). It is the main virulence determinant in gas gangrene, which is a life-threatening infection with fever, pain, edema, myonecrosis and gas production. It is shown that mutated strains that are unable to produce alpha toxin failed to cause this disease in mice (Awad et al., 1995; Flores-Díaz and Alape-Girón, 2003). Beta toxin is a protease-sensitive pore-forming toxin. It forms pores by the formation of toxin multimers in the cell membrane, resulting in Ca^{2+}, Na^{+}, and Cl^{-} influx and K^{+} efflux from the cells (Steinthorsdottir et al., 2000; Shatursky et al., 2000; Nagahama et al., 2003). Epsilon toxin acts by forming large membrane pores by oligomerization into a heptamer resulting in potassium and fluid leakage of cells, which leads to the loss of cell viability (Petit et al., 2001; Miyata et al., 2001; Petit et al., 2003). The beta and epsilon toxins seem to have key roles in enterotoxaemia in calves, lambs, piglets and goats, and most of the domesticated livestock in developed countries are immunized against disease with toxoid vaccines. Iota toxin is a binary toxin, it consists of two independent components, the enzymatic component (Ia) and the binding component (Ib). Ia is an ADP-ribosyltransferase that modifies actin. The iota toxin is the only *C. perfringens* toxin that acts intracellularly. All other toxins interact with the cell membrane leading to membrane disruption or pore formation (Rood and Cole, 1991; Songer, 1996; Petit et al., 1999; Gibert et al., 2000; Marvaud et al., 2001).

2.2.1.2. ENTEROTOXIN

Enterotoxin is the cause of human food poisoning. Unlike the other toxins, enterotoxin is not secreted but is produced during sporulation (Adak *et al.,* 2002; Brynestad and Granum, 2002; Lukinmaa *et al.,* 2002). It interacts with epithelial tight junction proteins and induces leakage of water and ions by forming pores or channels in plasma membranes of host cells (McClane, 2001, Smedley et al., 2004).

2.2.1.3. MINOR TOXINS

All other toxins belong to the group of minor toxins. Theta toxin, also known as theta-hemolysin, perfringolysin O, or the thiol-activated cytolysin is located on the chromosome and produced by all five toxin types of *C. perfringens* (Rood and Cole, 1991). Theta toxin is a member of the cholesterol-binding toxin family and causes complete hemolysis of red blood cells by forming oligomers, which subsequently form pores through the cell membrane (Petit *et al.,* 1999, Awad *et al.,* 2001). A more recently discovered toxin is Beta2 toxin, a pore-forming toxin that is associated with enteritis in neonatal pigs (Gibert *et al.,* 1997; Jost et al., 2005). Other known toxins produced by *C. perfringens* are: delta toxin, a hemolysin; kappa toxin, a collagenase; lambda toxin, a caseinase; mu toxin, a hyaluronidase; nu toxin, a nuclease; neuraminidase or sialidase, a N-acetylneuraminic acid glycohydrolase; and the gamma and eta toxins, whose function is unclear (Hatheway, 1990; Rood and Cole, 1991). The relevance in disease of most of these minor toxins is not fully understood.

The different toxin types of *C. perfringens* are associated with particular human or veterinary diseases (Table II), indicating that variations in toxin production profoundly influence the virulence properties of *C. perfringens* isolates. These isolate-to-isolate differences in toxin production also help explain the pathogenic versatility of *C. perfringens*, which causes both enteric and histotoxic infections and has a disease spectrum ranging from low incidence/high mortality to high incidence/low mortality. Strains of toxin type B-E are always associated with disease processes, indicating that they are frank pathogens. Type A strains are also associated with disease but can equally well be part of the normal flora in the intestinal tract of man and animal (Hatheway, 1990; Songer, 1996). Rather than being a function of a single toxin, the virulence of different *C. perfringens* isolates is considered as a multifactorial trait, with different determinants contributing to adaptation of the organism to its niche and to production of the pathology (Canard et al., 1992; Miyamoto et al., 2006; Sawires and Songer, 2006). The pathogenesis of *C. perfringens* in different diseases has not yet been fully elucidated and probably many potential toxins are yet unidentified.

Table II: Diseases associated with *C. perfringens* toxin types A, B, C, D, and E (Hatheway 1990, Songer 1996, Petit *et al*. 1999, Smedley *et al*. 2004).

Toxin type	Diseases	
	Human	**Animal**
A	gas gangrene	necrotic enteritis (fowl)
		mild necrotizing enteritis (piglets)
		enterotoxaemia (various animals)
		acute gastric dilatation (various animals)
		intestinal clostridiosis (horses)
A (β2, CPE)	food poisoning	diarrhea (various animals)
	non-foodborne gastrointestinal disease	
A (CPE)	non-foodborne gastrointestinal disease	
A (β2)		enteritis (various animals)
B		dysentery (newborn lambs)
		chronic enteritis (older lambs)
		hemorrhagic enteritis (calves, foals)
		enterotoxaemia (sheep, goats, guinea pigs)
C	necrotizing enteritis	enterotoxaemia (pigs, lambs, calves, goats, fowl)
	(i.e. pigbel, darmbrand)	struck (acute enterotoxaemia in sheep)
C (β2)		enteritis (pigs)
D		pulpy kidney disease (lambs and calves)
		enterocolitis (goats, cattle)
E		enteritis (dogs, cattle, pigs)
		enterotoxaemia (calves, guinea pigs, rabbits)
		dysentery (lambs)

CPE: enterotoxin

2.2.1.4. THE ROLE OF ALPHA TOXIN IN NECROTIC ENTERITIS

It is generally accepted that *C. perfringens* type A is the causative agent of both clinical and sub-clinical necrotic enteritis since strains isolated from birds suffering from necrotic enteritis all belong to toxinotype A (Engström et al., 2003; Nauerby et al., 2003; Gholamiandehkordi et al., 2006; Chalmers et al., 2008a). Moreover, clinical and sub-clinical necrotic enteritis were experimentally reproduced using *C. perfringens* type A (Al-Sheikhly and Truscott,

1977a; Al-Sheikhly and Truscott, 1977c; Gholamiandehkordi et al., 2007). Of the major typing toxins, type A strains produce only alpha toxin. Therefore, for a long time it was thought that alpha toxin was the major virulence factor in the pathogenesis of necrotic enteritis in poultry. Several studies presented evidence for this hypothesis. Bacteria-free crude supernatant from *C. perfringens* type A cultures produced necrotic lesions in broilers (Al-Sheikly and Truscott, 1977b) or caused mortality in germ-free chickens (Fukata et al., 1988). After addition of antibodies to *C. perfringens* alpha toxin to the supernatant no mortality was seen (Fukata et al., 1988). Lovland et al. (2004) showed that maternal vaccination with a crude *C. perfringens* type A and C toxoid induces antibodies against alpha toxin in chicks, which are partially protective against necrotic enteritis. However, care must be taken when interpreting these studies. Hence, crude supernatant was used and the assumption that the observed effects were caused by the dominant protein present in the supernatant (i.e. alpha toxin) did not take into account other secreted toxins that the bacteria may have produced. Epidemiological and experimental evidence has supported the proposal that alpha toxin is an important protective antigen. High titers of antibodies to alpha toxin are found in poultry immune to necrotic enteritis (Heier et al., 2001; Kulkarni et al., 2006). Moreover, immunization of broilers with purified alpha toxoid induced protection against experimentally induced necrotic enteritis (Kulkarni et al., 2007). Thompson et al. (2006) showed that spontaneously derived alpha toxin mutants of a virulent strain have an impaired ability to cause NE lesions. However, since it were spontaneously derived mutants, the reduced virulence could be due to the impairment of the production of other toxins than alpha toxin.

Against this background, the role of alpha toxin in the pathogenesis of necrotic enteritis was called somewhat into doubt at the start of this thesis. *C. perfringens* outbreak strains as well as normal broiler microbiota isolates are type A (Nauerby et al., 2003; Gholamiandehkordi et al., 2006). Moreover, no apparent difference in the levels of alpha toxin was found when the alpha toxin production *in vitro* was compared between strains associated with necrotic enteritis and isolates derived from the microbiota of normal broilers (Gholamiandehkordi et al., 2006). Yet another study found that the intestinal level of alpha toxin was not correlated with disease lesion scores (Wilkie et al., 2006). More convincing evidence was produced by Keyburn et al. (2006). They showed that an alpha toxin mutant, constructed from a virulent chicken isolate, was equally able to cause necrotic lesion in broiler chickens compared to the wild-type strain (Keyburn et al., 2006). Another observation that argues strongly against the

role of alpha toxin in necrotic enteritis is the massive heterophil, lymphocyte, and plasma cell infiltration in infected tissues (Al-Sheikhly and Truscott, 1977b; Gazdzinsky and Julian, 1992; Shane et al., 1985). In gas gangrene, a disease proved to be mediated by alpha toxin, marked leukostasis and lack of inflammatory infiltrate is common in tissues infected by *C. perfringens* cells (Flores-Díaz and Alape-Girón, 2003). Alpha toxin-negative mutants of *C. perfringens* are not able to cause gas gangrene in mice but do promote profound inflammatory responses (Awad et al., 1995). Thus, the massive immune-cell influx in necrotic enteritis lesions seems to be inconsistent with the known effects of alpha toxin on the innate immune system.

2.2.2. PROTEOLYTIC ENZYMES

Recent studies of initial stages of the disease process have provided new etiological details on the development of necrotic enteritis in broilers. Olkowski et al. (2006, 2008) showed that damage to the villi initially occurs at the level of the basement membrane and lateral domain of the enterocytes, spreading throughout the lamina propria, while epithelial damage occurs later in the process. The nature of the morphological changes indicates that the initiation of the pathological process leading to necrotic enteritis involves proteolytic factors affecting the extracellular matrix and cellular junctions. Indeed, in broilers undergoing necrotic enteritis, the extracellular matrix is disorganized and can even be completely absent. It was shown that *C. perfringens* strains isolated from field cases of necrotic enteritis secrete several potent collagenolytic enzymes and that broilers challenged with *C. perfringens* show elevated levels of several collagenolytic enzymes in the intestinal tissue in comparison to controls. It was thus suggested that the pathology may be the result of bacterial collagenases, whose action is enhanced when mucosal damage (e.g. induced by coccidia) is present, or of host matrix metalloproteinases that are activated by the host-pathogen interaction (Olkowski *et al.,* 2008). From this point of view, it is interesting to note that one of the recently identified, potentially protective vaccine antigens from *C. perfringens* might be a zinc metallopeptidase (Kulkarni et al, 2007).

2.3. GENETIC DIVERSITY

The genetic diversity among *C. perfringens* isolates originating from humans, calves, pigs, sheep, rabbits, goats, poultry, horses, roe deer and food has been investigated by pulsed-field

gel electrophoresis (PFGE). In all cases a high degree of genetic diversity was found (Canard et al., 1992; Maslanka et al., 1999; Nauerby et al., 2003; Johansson et al., 2006; Gholamiandehordi et al., 2006). Ribotyping of *C. perfringens* isolates obtained from food also confirmed a great diversity (Schalch et al., 1999; Kilic et al., 2002). Using multiple-locus variable-number tandem repeat analysis (MLVA), Sawires and Songer (2006) and Chalmers et al. (2008b) showed also a lack of association between strain phylogeny and host species of disease. Isolates of common host origin did not cluster, while isolates from diverse animal origins were sometimes the same MLVA type. Moreover, some normal flora strains from certain host species are phylogenetically close to genotypes of virulent strains from a different host species.

The entire genome sequence of three different *C. perfringens* type A strains has been published (Shimizu et al., 2002; Myers et al., 2006). The sequenced strains are a gangrene strain (ATCC 13124) and a food poisoning strain (SM101) isolated from humans and a soil isolate that is able to induce gas gangrene in mice (strain 13). Comparison of the three genomes revealed considerable genomic diversity with discrete islands containing genes likely to confer specific virulence, metabolic, or catabolic capabilities to the host strain. This enables *C. perfringens* to adapt to a variety of environmental conditions and explains the different virulence characteristics of *C. perfringens* (Myers et al., 2006). *C. perfringens* toxin genes are located on the chromosome or on plasmids. The genomic diversity of *C. perfringens* seen during typing could be the result of the presence of toxin genes on extrachromosomal elements. It seems to be the case that, by acquisition of extrachromosomal elements (plasmids, transposons and possibly phages) containing additional toxin genes, different *C. perfringens* types have been derived from the type A strain (Petit et al., 1999). Moreover, Sawires and Songer (2006) suggested that acquisition of the major toxin genes as well as other plasmidborne toxin genes is a recent evolutionary event and that their maintenance is essentially a function of the selective advantage they confer in certain niches under different conditions

2.4. SINGLE STRAIN DOMINANCE IN BROILER NECROTIC ENTERITIS

Genetic characterization by PFGE or amplified fragment length polymorphism (AFLP) has revealed that in healthy flocks, different genotypes of *C. perfringens* type A can be found,

even within individual birds and within the same gut segment. In contrast, outbreak isolates from a flock with necrotic enteritis or cholangiohepatitis are generally clonal, regardless of the animal or the organ of isolation (Engström et al., 2003; Nauerby et al., 2003; Gholamiandehkordi et al., 2006). These results were confirmed by multilocus sequence typing analysis (MLST) (Chalmers et al., 2008a). After natural recovery or treatment, birds again yield multiple genetic types (Nauerby et al., 2003). The reason for the selective presence of a single clone in necrotic enteritis outbreaks is not known. It is possible that during an outbreak, certain *C. perfringens* strains are able to secrete growth-inhibiting molecules which give them a competitive advantage over other *C. perfringens* strains in the broiler gut. Indeed, inhibiting other strains could lead to extensive and selective presence of a strain that contains the genetic make-up to secrete toxins that cause gut lesions.

3. ANTIMICROBIAL PROTEINS FROM BACTERIA

Antimicrobial peptides and proteins are produced by all species of life (prokaryotic and eukaryotic): plants, insects and other invertebrates, fish, amphibians, birds, mammals - including humans, and different microorganisms (Nissen-Meyer and Nes, 1997; Jenssen et al., 2006). In higher organisms these compounds are produced as an innate host defense mechanism to protect against pathogenic attack, whereas microorganisms presumably use these compounds as weapons in the competition for space and nutrients among bacteria living in the same ecological niche. Although they differ greatly in their primary structures, they are nearly all fairly short molecules, cationic and very often amphiphilic (i.e. it possesses both hydrophobic and hydrophilic properties). This is reflected in the fact that many of these peptides kill their target cells by permeabilizing the target cell membrane, resulting in an irreversible leakage of cellular material and consequently cell death (Nissen-Meyer and Nes, 1997). Other modes of action such as the inhibition of nucleic acid synthesis, protein synthesis, enzyme activity and cell wall synthesis have been described (Brogden, 2005). The antimicrobial proteins and peptides from bacteria include toxins, antibiotics, bacteriolytic enzymes, bacteriocins, and bacteriocin-like peptides (Jack et al., 1995).

3.1. BACTERIOCINS

Bacteriocins are defined as a heterogeneous group of ribosomally synthesised, proteinaceous substances (with or without further modifications) produced by bacteria that kill or inhibit the growth of other bacteria. Their mode of activity is primarily bactericidal and directed against closely related strains and species. The bacteriocin family includes a diversity of peptides and proteins in terms of size, amino acid sequence and composition, secretion and processing machinery, post-translational modifications, microbial target, mode of action, and mechanisms of resistance (Klaenhammer, 1993; Jack et al., 1995).

Bacteriocins were first identified in Gram-negative bacteria over 80 years ago when inhibition was observed between two strains of *Escherichia coli* (Gratia, 1925). A heat labile product present in cultures of *Escherichia coli* V was shown to be toxic to *E. coli* S and it was named colicin (Gratia, 1925). Fredericq (1946) demonstrated the protein nature of colicins and their limited range of activity due to the presence or absence of specific receptors on the surface of sensitive cells (Fredericq, 1946). Since then, bacteriocins have been found in all major bacterial lineages and, more recently, some members of the Archaea have also been seen to produce similar antimicrobial proteins (O'Connor and Shand, 2002). According to Klaenhammer (1988), 99% of all bacteria may produce at least one bacteriocin, and the only reason why there have not been isolated more is that few researchers have looked for them. Since bacteriocin production is detected in all surveyed lineages of prokaryotes, bacteriocins must serve some function in microbial communities (Klaenhammer, 1988). They may play a defensive role by inhibiting the invasion of other strains or species into an occupied niche or they may serve as anti-competitors enabling the invasion of a strain into an established microbial community (Riley and Wertz, 2002). Additional roles have been proposed; they may mediate quorum sensing by acting as a signal molecule that induces the transcription of genes involved in its biosynthesis and they may act as communication signals in bacterial consortia e.g. biofilms (Gobbetti et al., 2007; Gillor et al., 2008).

Bacteriocins can be divided into two main groups: those produced by Gram-negative and Gram-positive bacteria (Gillor et al., 2008). Within the scope of the thesis we will focus on bacteriocins of Gram-positive bacteria.

3.2. BACTERIOCINS OF GRAM-POSITIVE BACTERIA

3.2.1. GENERAL CHARACTERISTICS

Bacteriocins of Gram-positive bacteria are abundant and diverse. They resemble many of the antimicrobial peptides produced by eukaryotes; they are generally cationic, amphiphilic, heat stable, membrane-permeabilizing peptides, and smaller than 8 kDa (Maqueda et al., 2008). However, some Gram-positive bacteria have been shown to form relatively high-molecular-weight, heat labile bacteriocin-like substances (Jack et al., 1995). Although by definition all bacteriocins have a protein or peptide component that is essential for their bactericidal function, some have been reported to consist of combinations of different proteins, or are composites of proteins together with lipid or carbohydrate moieties (Jack et al., 1995).

Bacteriocins can specifically target a particular subset of bacterial strains or species. The conventional wisdom about the spectrum of Gram-positive bacteriocins is that they are restricted to killing other Gram-positives (Gillor et al., 2008). However, the range of killing can vary significantly, from relatively narrow to extraordinarily broad. Some bacteriocins are only active against closely related strains, others against a wide range of Gram-positive bacteria and some are even able to inhibit Gram-negative species (Morency et al., 2001). Interpretation of spectra of inhibitory activity in terms of specific bacteriocin activities can sometimes be difficult if the producer strains release more than one bacteriocin-like agent (Higa et al., 1991).

Many bacteriocins appear to elicit their lethal effects by permeabilizing the cell membrane of target organisms, in certain cases by targeting intermediates of cell wall biosynthesis or possibly proteins of sugar phosphotransferase systems (Garneau et al., 2002). Creating pores in the membrane of target cells has deleterious effects such as dissipation of proton motive force, ATP depletion and leakage of nutrients and metabolites. The size, stability, and conductivity of these pores differ considerably from one bacteriocin to another. To form a pore, bacteriocins have to interact with the cytoplasmic membrane of target cells. This process is at least in part governed by electrostatic interactions between the positively charged peptide and the anionic lipids that are abundantly present in the membranes of Gram-positive bacteria (Eijsink et al., 2002). Several factors may contribute to making a cell resistant towards bacteriocins. The composition and structure of both cell wall and cellular membrane(s) may be such that the bacteriocin is physically unable to reach its target. Alternatively, certain

cellular components ('receptors') that are essential for bacteriocin action may be lacking or may be mutated. In some cases, the presence of (aspecific) proteases in and near the target cell may reduce bacteriocin effectiveness. Finally, the ease at which a membrane-bound bacteriocin actually can form pores can be affected by the physiological state of the target cell (Driessen et al., 1995; Eijsink et al., 2002).

Production of bacteriocins in Gram-positive bacteria is generally associated with the shift from logarithmic phase to stationary phase. Gram-positive bacteriocins require several genes and these bacteriocin-associated genes appear to be characteristically arranged in multigene operon-like structures, the first gene typically (but not always) encodes the structural protein (Klaenhammer, 1993). Additional genes encode for proteins that aid in the regulation, proteins that aid in the processing to the active form, proteins that aid in the transport of the bacteriocin across the membrane and proteins that confer immunity to the host producer (Engelke et al., 1992; Klein et al., 1993; Engelke et al., 1994; Klein and Entian, 1994; Diep et al., 1996; Qiao et al., 1996; Diep et al., 2007; Dufour et al., 2007). Due to these additional genes, bacteriocins produced by Gram-positive bacteria are generally not lethal to the producing cell.

3.2.2. CLASSIFICATION

There is a lack of consensus in the classification of bacteriocins from Gram-positive bacteria. Bacteriocins are commonly divided into three or four main categories (Nes et al., 2007; Gillor et al., 2008). This classification is based on bacteriocins produced by lactic acid bacteria since these are the best characterized of this group. Class I is comprised of lantibiotics. These are small, posttranslationally modified peptides that contain unusual amino acids (Guder et al., 2000; Twomey et al., 2002). Class II includes heat stable non-lantibiotics (Eijsink et al., 2002; Héchard and Sahl, 2002; Drider et al., 2006). Larger, heat labile bacteriocins are classified as class III. For this category much less information is available than for the first two. Class IV is comprised of complex bacteriocins that require lipid or carbohydrate moieties for activity. However, presently, no such bacteriocins have been purified and it is suggested that this type of bacteriocin is an artefact due to the cationic and hydrophobic properties of bacteriocins which result in complexing with other macromolecules in the crude extract (Cleveland et al., 2001; Garneau et al., 2002; Gillor et al., 2008). Class I and II are subdivided in subgroups.

Lantibiotics are posttranslationally modified peptides that contain the unusual amino acids lanthionine and β-methyl lanthionine as part of additional intramolecular rings, and often possess other modified residues such as dehydro amino acids (Guder et al., 2000; Twomey et al., 2002; Nagao et al., 2006). They target a broad range of Gram-positive bacteria and are subdivided into three groups on the basis of their structure and mode of action: Type A lantibiotics are small (2 - 5kDa), elongated molecules with a flexible structure in solution, that contain positively charged molecules, which kill via the formation of pores in the bacterial membrane, leading to the dissipation of membrane potential and the efflux of small metabolites from the sensitive cells. Type B lantibiotics tend to have a more rigid and globular structure. They kill by interfering with cellular enzymatic reactions, such as cell wall synthesis (Pag and Sahl, 2002; Garneau et al., 2002). Another subgroup of the lantibiotics is composed of two-component lantibiotics, consisting of two lantibiotic peptides that synergistically display antimicrobial activity (Breukink, 2006; Wiedemann et al., 2006).

Class II bacteriocins are small non-lanthionine containing peptides (Garneau et al., 2002; Drider et al., 2006). The majority of bacteriocins in this group kill by inducing membrane permeabilization and the subsequent leakage of molecules from target bacteria. These bacteriocins are organized into four subgroups. Class IIa is the largest group and its members share activity against *Listeria* and a conserved amino-terminal sequence (YGNGVXaaC) that is thought to facilitate nonspecific binding to the target surface. Class IIa bacteriocins act through the formation of pores in the cytoplasmic membrane. Class IIb bacteriocins are two-peptide bacteriocins and form cation- or anion-specific pores, composed of two different proteins, in the membrane of their target cells (Héchard and Sahl, 2002; Oppegård et al., 2007). A third subgroup (IIc) has been proposed, which consists of leaderless peptide-bacteriocins that are secreted via the general secretory (sec) pathway and not having its own dedicated and specific mechanism (Nes et al., 1996; Garneau et al., 2002). Circular posttranslationally modified bacteriocins are classified in a fourth subgroup (IId) (Nes et al., 2007).

Table III: Classification of bacteriocins

Type	Class	Special features
Lantibiotics	IA	Posttranslationally modified elongated molecules
	IB	Posttranslationally modified globular molecules
	IC	Two-component lantibiotics
Small non-lantibiotics	IIa	Strong antilisterial activity
		Conserved amino-terminal sequence (YGNGVXaaC)
	IIb	Two-peptides
	IIc	No leader
	IId	Circular
Large heat-labile bacteriocins	III	Large, heat labile
Complex bacteriocins	IV	Carry lipid or carbohydrate moieties

3.3. BACTERIOCINS OF *CLOSTRIDIUM PERFRINGENS*

Bacteriocin-like activity in *C. perfringens* was first observed in a study of the bacteriophages of the organism (Smith, 1959). Subsequently, several studies have been carried out to examine the ability of *C. perfringens* strains to produce bacteriocins and to characterize the bacteriocins (Rood and Cole, 1991). It is clear that *C. perfringens* strains produce a large number of different bacteriocins that are distinguishable by examination of activity spectra. Different typing schemes based on patterns of bacteriocin susceptibility of *C. perfringens* strains or on *C. perfringens* bacteriocin activity spectra were developed (Mahony, 1974; Satija and Narayen, 1980a; Satija and Narayen, 1980b; Scott and Mahony, 1982). A bacteriocin typing scheme was used to type human fecal *C. perfringens* isolates and it was shown that in human feces more than one strain of *C. perfringens* could be carried simultaneously (Mahony and Swantee, 1978). Clinical isolates were also typed and it was found that the patterns of strains from the same outbreak clustered together while strains from different outbreaks had different patterns (Watson et al., 1982; Watson, 1985). Despite the effort of developing typing schemes and despite the fact that *C. perfringens* bacteriocins proofed to be useful to type clinical *C. perfringens* isolates, typing of *C. perfringens* strains with bacteriocins or on the basis of bacteriocin production was never generally in widespread or routine use in clinical laboratories (Rood and Cole, 1991). Nowadays, other techniques, such as AFLP, PFGE, and MLVA are used to type *C. perfringens* strains. However, the interest in *C. perfringens*

bacteriocins and the analysis of bacteriocin-encoding plasmids has played a very important role in the development of *C. perfringens* genetics and different *C. perfringens* bacteriocins are described in literature.

3.3.1. BCN5

BCN5 (or N_5) produced by *C. perfringens* CPN50 (or *C. perfringens* BP6k-N_5) is the best characterized bacteriocin of *C. perfringens*. BCN5 was first purified by Wolff and Ionesco (1975). They found a simple protein with an estimated molecular mass of 82 kDa. The purified bacteriocin was inactivated by proteolytic enzymes and was heat labile. Since BCN5 is a large heat labile bacteriocin, it can be classified as class III bacteriocin according to the currently used classification. BCN5 inhibits the initiation of the germination of spores of *C. perfringens* and it inhibits the DNA-, RNA-, and protein synthesis simultaneously in sensitive cells, without DNA degradation (Sebald and Ionesco, 1974; Ionesco and Wolff, 1975). *C. perfringens* CPN50 produces the bacteriocin upon UV induction (Ionesco and Bouanchaud, 1973; Ionesco et al., 1974), which suggest that the production is controlled by an SOS-like system and is induced by DNA-damaging treatment (Walker, 1984). Following activation of the *bcn* gene, copious amounts of a 96 kDa protein accumulate in the cytoplasm, to be released into the medium 90 to 180 minutes after induction by lysis of the bacteria (Ionesco et al., 1974; Garnier and Cole, 1986). From the nucleotide sequence of the *bcn* gene, the primary structure of BCN5 was deduced and revealed a protein with a molecular weight of 96.591 kDa, with high glycine content (11.5%). This is common for bacteriocins and is believed to facilitate their transfer across cell membranes. The primary structure of the protein reveals the presence of an extended lipophilic region near the COOH terminus. This suggests that it may function as an ionophore (Garnier and Cole, 1986). The discrepancy between the estimated molecular weight of 82 kDa by Wolff and Ionesco (1975) could be due to proteolytic processing of the 95 kDa precursor molecule (Garnier and Cole, 1986).

Curing of *C. perfringens* CPN50 has been accomplished after acriflavine treatment (Ionesco and Bouanchaud, 1973). It was shown that these cured derivates produced no longer bacteriocin and that this bacteriocin loss was associated with the loss of plasmid DNA (Ionesco and Bouanchaud, 1973). Electron-microscopic analysis showed more specifically that the loss of both bacteriocin production and immunity to the bacteriocin was associated with the loss of a small (5.7 MDa) plasmid, pIP404. Thus, in addition to encoding BCN5,

pIP404 codes for bacteriocin immunity (Ionesco et al., 1976; Brefort et al., 1977). To locate the *bcn* gene on pIP404, differential dot blot hybridization was performed with RNA samples prepared from cultures before and after induction with UV irradiation. This approach revealed that UV irradiation induces a high rate of transcription of a 4kb segment of pIP404 that includes two contiguous transcription units, *uviAB* and *bcn* (Garnier and Cole, 1986; Garnier and Cole, 1988). The *bcn* gene, encoding BCN5, is transcribed from three promoters (P1, P2 and P3) all of which are dependent on UviA for activation. P1, the promoter closest to the coding sequence, appears to be the strongest of the three (Dupuy et al., 2005). Transcription of *uviAB* is directed by two promoters (P4 and P5). It was hypothesized that the *uviAB* operon might encode proteins needed for BCN5 synthesis or secretion or for immunity to the bacteriocin (Garnier and Cole, 1986; Garnier and Cole, 1988). Dupuy et al. (2005) showed that UviA is an RNA polymerase σ factor. Promotor P4 provides an UviA-independent, basal level of gene expression while the stronger, UviA-dependent promoter (P5) was only utilized after the cell experienced DNA damage. As a result, BCN5 synthesis is induced by treatment with UV light or mitomycin C. The role of UviB, the second product of the uviAB operon, has never been determined. As BCN5-producing cells are immune to the bacteriocin, UviB might be the immunity protein (Dupuy et al., 2005).

3.3.2. BACTERIOCIN 28

Mahony and Butler (1971) screened 33 *C. perfringens* strains for bacteriocin production and four bacteriocin-producing strains were detected. One of the bacteriocins, bacteriocin 28 produced by *C. perfringens* strain 28, was chosen by Mahony and associates to study in more detail. Bacteriocin production by *C. perfringens* strain 28 is associated with a 5.6 MDa plasmid since a cured variant lost its immunity and its ability to produce bacteriocin (Li et al., 1980). High titres of bacteriocin 28 are produced during late logarithmic growth although higher yields are obtainable by induction with mitomycin C but not with UV light (Mahony and Butler, 1971; Mahony, 1977). The bacteriocin was heat labile, only stable at pH between 5 and 7 and sensitive to the action of trypsin and pronase (Mahony and Butler, 1971; Mahony and Li, 1978). Bacteriocin 28 is a glycoprotein with hydrophobic properties and an estimated molecular mass of 100 kDa (Li et al., 1982). Like BCN5, bacteriocin 28 is a large heat labile bacteriocin and can be classified as class III bacteriocin. Investigation of its mode of action indicated that it seemed bacteriostatic and that it does not inhibit the synthesis of DNA, RNA

or proteins but acts on the cell wall of viable indicator cultures, causing conversion of indicator strains to spheroplasts, either by removing the existing wall or inhibiting cell wall synthesis. These spheroplasts are capable of growing as L-form colonies on sucrose containing media. Such colonies were composed of spherical and amorphous structures of considerable size and demonstrated a dense center containing many granular structures (Mahony et al., 1971).

3.3.3. OTHER *CLOSTRIDIUM PERFRINGENS* BACTERIOCINS

Mahony and Li (1978) compared nine other *C. perfringens* bacteriocins with bacteriocin 28. Two bacteriocins were stable over a wide range of pH values and resisted boiling and three other bacteriocins were resistant to trypsin. Essentially, the bacteriocins could be divided in two major groups, those that inhibit DNA, RNA, and protein synthesis, such as BCN5, and those that interfere with the cell wall of sensitive *C. perfringens* strains, like bacteriocin 28 (Mahony and Li, 1987; Mahony, 1982). Clarke et al. (1975) described perfringocin 11105. This bacteriocin is produced by *C. perfringens* NCIB11105 and is heat stable, trypsin susceptible and stable over a wide range of pH. It has a molecular weight of 76 kDa and the properties of an amphiphilic protein. Perfringocin 11105 is produced at the onset of the stationary phase of the *C. perfringens* culture, and its subsequent production coincides with some lysis of the producer organism. Yield of perfringocin 11105 can be enhanced by mitomycin C treatment (Clarke et al., 1975). Mihelc et al. (1978) reported that a 5.6 MDa plasmid (pCW4) is associated with bacteriocin production and immunity in *C. perfringens* strain CW55. Higa et al. (1991) showed that *C. perfringens* SN-17 produced two types of bacteriocin successively one after the other during late exponential phase, without induction by UV radiation or mitomycin C. The molecular weight of SN-a and SN-b was determined to be about 70 kDa and 100 kDa respectively. Both bacteriocins were heat labile, sensitive to pronase and more stable in alkaline pH than in acidic pH. Both bacteriocins adsorbed to resting cells or even dead cells, indicating that they bind to receptors on the surface of sensitive cells (Higa et al., 1991).

3.3.4. BACTERIOCIN GENES IN GENOME SEQUENCES

Analysis of the genome sequences of the three sequenced *C. perfringens* strains revealed a single bacteriocin gene (*bcn5*) on the chromosome of strain 13 in association with the

prophage remnant (Shimizu et al., 2002). No bacteriocin or accessory genes are found in the ATCC 13124 genome. By contrast, several bacteriocin loci are found in the SM101 genome and plasmid sequences. The two SM101 plasmids each bear a complete UV-inducible bacteriocin operon (*uviA-uviB-bcn5*), similar to the archetypal clostridial bacteriocin bearing plasmid pIP404 of CPN50 (Myer et al., 2006).

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Chapter 2

Scientific Aims

CHAPTER 2: SCIENTIFIC AIMS

Clostridium perfringens type A is a member of the normal intestinal microbiota but can also induce necrotic enteritis in broiler chickens. Necrotic enteritis is characterized clinically by a sudden increase in flock mortality, often without premonitory signs. Also a sub-clinical form of necrotic enteritis is described. At necropsy, necrotic foci are found in the small intestinal mucosa. Despite the clinical and economic importance of necrotic enteritis, the pathogenesis of *C. perfringens* associated disease in poultry is still not fully understood. For that reason, the general aim of this work was to get more insight into the pathogenesis of necrotic enteritis.

C. perfringens is associated with necrotic enteritis but can also be detected in high numbers in the intestinal tract of apparently normal birds. Consequently, the first aim of this works (CHAPTER 3.1) was to compare *C. perfringens* isolates from both clinically healthy broilers and broilers suffering from necrotic enteritis for their ability to induce necrotic enteritis using an experimental necrotic enteritis model in broiler chickens.

Clinically healthy chickens can carry several different *C. perfringens* clones in their intestine. However, in flocks suffering from necrotic enteritis mostly only one single clone is isolated from the gut of all the diseased animals. The reason for the presence of a single clone in necrotic enteritis outbreaks is not known.
Therefore the second aim of this study (CHAPTER 3.2) was to compare virulent and avirulent *C. perfringens* isolates for their ability to inhibit the growth of other *C. perfringens* strains using a lawn-spotting test. The identification of the factor(s) responsible for growth-inhibition was the third aim of the study (CHAPTER 3.3).

Chapter 3

Experimental Studies

Chapter 3.1: Origin of *Clostridium perfringens* isolates determines the ability to induce necrotic enteritis in broilers

Chapter 3.2: Intra-species growth-inhibition by *Clostridium perfringens* is a possible virulence trait in necrotic enteritis in broilers

Chapter 3.3: Purification and partial characterization of a novel antimicrobial peptide from *Clostridium perfringens* strain 56

Chapter 3.1:

Origin of *Clostridium perfringens* isolates determines the ability to induce necrotic enteritis in broilers

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ABSTRACT

Since the ban on growth-promoting antibiotics in animal feed in the European Union, necrotic enteritis has become a major cause of mortality in broiler chickens. Despite the importance of the disease, the pathogenesis is still not completely understood. In the current study, *Clostridium perfringens* strains isolated from healthy flocks and isolates from outbreaks of necrotic enteritis were evaluated for the ability to cause gut necrosis in an intestinal loop model in laying hens and in an experimental infection model in broilers. High, intermediate and low alpha toxin producing strains were chosen from each isolation source. Only the isolates from field outbreaks induced necrotic gut lesions, independent of the amount of alpha toxin produced *in vitro*. It was also shown that alpha toxin producing isolates from calf hemorrhagic enteritis cases were not able to induce necrotic enteritis in poultry. These results suggest the presence of host specific virulence factors in *Clostridium perfringens* strains, isolated from chickens with intestinal necrotic enteritis lesions.

Key words: Clostridium perfringens; Broiler; *In vivo*; Alpha toxin; Host specificity

1. INTRODUCTION

Clostridium perfringens is a Gram-positive spore-forming anaerobic bacterium that is commonly found in soil and sewage and in the gastro-intestinal tract of animals and humans as a member of the normal gut microbiota (Hatheway, 1990; Songer, 1996). *C. perfringens* strains are classified into five types (A-E) on the basis of the production of four major toxins (known as the alpha, beta, epsilon and iota toxins) (McDonel, 1980; Petit et al., 1999). Necrotic enteritis in poultry is an enteric disease which is caused by *C. perfringens* type A (Engström et al., 2003; Nauerby et al., 2003; Gholamiandehkordi et al., 2006). This toxin type produces of the major toxins only alpha toxin. Since the ban on supplementing growth-promoting antibiotics in animal feed in the European Union, *C. perfringens* induced necrotic enteritis and related sub-clinical disease have become economically significant problems in the broiler industry (Grave et al., 2004; Van Immerseel et al., 2004; Williams, 2005). Despite the clinical and economic importance of necrotic enteritis, the pathogenesis of *C. perfringens* associated disease in poultry is still not fully understood. Development of necrotic enteritis depends on the presence of predisposing factors, two of the most important being mucosal damage caused by coccidial pathogens and feed containing high protein levels (Kaldhusdal and Skjerve, 1996; Williams, 2005; Dahiya et al., 2006). These predisposing factors are thought to trigger the proliferation of *C. perfringens* to high numbers in the small intestine, leading to intestinal necrosis due to the production of toxins.

The alpha toxin, the major toxin involved in the pathogenesis of human gas gangrene, has for years been considered as the major virulence factor involved in necrotic enteritis in chickens (Al-sheikhly and Truscott, 1977; Fukata et al., 1988; Hofshagen, and Stenwig, 1992). However, recent studies indicate that alpha toxin is not an essential virulence factor in the induction of necrotic enteritis. An alpha toxin knock-out mutant of *C. perfringens* could cause necrotic enteritis in broilers, and the number and severity of lesions were similar compared to the wild-type strain (Keyburn et al., 2006). Additionally, it was shown that strains isolated from necrotic enteritis outbreaks did not produce more alpha toxin compared to isolates from the gut of clinically healthy broilers (Gholamiandehkordi et al., 2006). To our knowledge, it has not been reported in literature whether any *C. perfringens* strain isolated from any normal, clinically healthy chicken gut can induce necrotic enteritis. If not, this is a specific

characteristic of those strains that are isolated from necrotic enteritis cases. Moreover, it is not described in the literature whether alpha toxin producing isolates from other animal species presenting *C. perfringens* associated enteric disease, such as hemorrhagic enteritis in cattle, are able to cause disease in poultry.

Therefore, in the work described below, *C. perfringens* isolates from both healthy and diseased poultry, and from calf hemorrhagic enteritis cases, producing different concentrations of alpha toxin *in vitro*, were tested for their ability to induce necrotic enteritis in broilers.

2. MATERIALS AND METHODS

2.1. STRAINS AND VACCINES

C. perfringens strains 7, 8 and 17 were isolated from the normal gut microbiota of healthy broiler chickens, while strains 48, 56 and 61 were isolated from the intestine of broiler chickens with severe necrotic gut lesions (Gholamiandehkordi et al., 2006). Strain 56 has been used to optimize the *in vivo* necrotic enteritis model (Gholamiandehkordi et al., 2007), and this strain was used in the present study as a positive control. A chicken *C. perfringens* strain isolated from a field case of necrotic enteritis and its alpha toxin knock-out mutant (EHE-NE18 and EHE-N18-M1) were kindly provided by A. Keyburn, Department of Microbiology, Monash University, Australia (Keyburn et al., 2006). Strains 669 and 670 were isolates from calf hemorrhagic enteritis cases and were kindly provided by Prof. P. Deprez, Department of Internal Medicine and Clinical Biology of Large Animals, Ghent University, Belgium.

Before inoculation of chickens, the bacteria were cultured in Brain Heart Infusion (BHI, Oxoid, Basingstoke, England) broth for 24 h at 37 °C.

The commercial Gumboro vaccine, Nobilis Gumboro D78 (Intervet, Mechelen, Belgium) and the anticoccidial vaccine Paracox-5^{TM} (Schering-Plough Animal Health, Brussels, Belgium), containing live, attenuated oocysts of *Eimeria acervulina* (*E. acervulina*), *E. maxima* (two lines), *E. mitis* and *E. tenella* were used in this study.

2.2. TOXINOTYPING BY PCR

The toxin type of the *C. perfringens* isolates was determined by a multiplex PCR, as described by Yoo et al. (1997), while the presence of the enterotoxin and the beta2 toxin genes were detected with single PCR reactions (Meer and Songer, 1997; Herholz et al., 1999).

2.3. ALPHA TOXIN QUANTIFICATION BY ELISA

For detection of alpha toxin in *C. perfringens* culture supernatant, the Bio-X Alpha Toxin Elisa Kit (Bio-X Diagnostics, Jemelle, Belgium) was used according to the instructions of the manufacturer. A standard curve was constructed based on two-fold serial dilutions of the alpha toxin standard and their corresponding net absorbance values. Based on this standard curve, the amount of alpha toxin produced by the isolates was calculated according to Zhang et al. (2006).

2.4. BIRDS AND HOUSING

Intestinal loop assays were performed in 12 commercial laying hens (Isa Brown Warren) of twenty weeks old.

In vivo necrotic enteritis trials using experimental inoculations were performed with Ross 308 broiler chickens that were obtained as one-day-old chicks from a commercial hatchery. The animals were kept in isolation. All treatment groups were housed in the same room, in cages of 1 m², on litter floor. All cages were separated by solid walls to prevent contact between birds from different treatment groups. Before each trial, the stables were decontaminated with peracetic acid and hydrogen peroxide (Hygiasept® vaporizer climasept, SARL Hygiasept, Sevrey, France) and a commercial anticoccidial desinfectant (OO-CIDE, DuPont Animal Health Solutions, Wilmington, US). For the first trial two hundred and seventy (270) chickens and for the second trial eighty-one (81) chickens were reared, 27 per treatment group. They were given ad libitum drinking water and feed. A 23 h/1 h light/darkness program was applied.

The animal experiments were carried out according to the recommendations and following approval of the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University.

2.5. INTESTINAL LOOP ASSAY

Hens were premedicated with 0.05mg/kg buprenorphine hydrochloride (Temgesic, Schering-Plough, New Jersey, US) and 0.05 mg/kg atropine intramuscularly. The chickens were anaesthetized using mask induction with isoflurane (Schering-Plough) in 100% oxygen. Following intubation with a 3.0 uncuffed tracheal tube (Hudson RCI, Temecula, California, USA), a continuous oxygen flow of 1.5-2.0 l/min was administered carrying 1.5-3% of isoflurane, depending on the depth of the anaesthesia required. The birds were covered with a sterile surgical blanket and the abdominal surface was plucked. After disinfection of the incision area with a polyvidone iodine solution (Braunol, B. Braunol Medical, Prague, Czech Republic), the abdomen was opened through a midline incision avoiding the perforation of the ventral air sacs, and the jejunum was carefully exposed. Jejunal loops of 1.5-2 cm long were ligated using surgical suture (Vicryl 3.0, Johnson-Johnson, New Brunswick, New Jersey, US). Sufficient blood supply was ensured to all separate loops.

In each chicken, 8 loops were created. Six *C. perfringens* strains (7, 8, 17, 48, 56 and 61) isolated from poultry were inoculated in six separate loops and two loops were used as controls. The loops were inoculated with 1 ml of an overnight culture of *C. perfringens* strains in BHI (about 4×10^8 cfu/ml) or with BHI (control loops) using a 27-gauge needle. The loops were repositioned into the abdomen, and the abdominal wall was sutured. After six, ten or 24 h the hens were euthanized by intravenous injection of T61 (Intervet, Mechelen, Belgium). At necropsy, the mucosa of the intestinal loops was macroscopically examined for lesions and samples were taken for histopathology as described below. These experiments were conducted in two chickens per time point and repeated twice to verify results. A total of 12 chickens were used.

2.6. *IN VIVO* NECROTIC ENTERITIS MODEL

These trials were performed as described previously (Gholamiandehkordi et al., 2007). In short: broilers were fed a wheat/rye (43%/7.5%) based diet, with soybean meal as protein source. Gumboro vaccine was given in the drinking water at day 18 in all groups. From day 19 onwards, the same diet was used with the exception that fishmeal (30%) was used as protein source. All groups were challenged orally (three times a day) with approximately 4×10^8 cfu *C. perfringens* bacteria at days 19, 20, 21 and 22. At day 20 all birds, except the

ones in the control group, were orally inoculated with a 10-fold dose of Paracox-5™. At days 23, 24 and 25, nine animals of each group were euthanized by intravenous T61 (Intervet, Mechelen, Belgium) injection. Necrotic enteritis lesion scoring and sample collection was performed, as described below.

In a first trial the three isolates from the gut of healthy broiler chickens (7, 8 and 17) were compared to the *C. perfringens* strains isolated from necrotic enteritis cases (48, 56, 61) for their ability to induce necrotic lesions in the gut. Two control groups were included: a negative control group (not inoculated with *C. perfringens*, not vaccinated with Paracox) and a group only inoculated with a 10-fold dose of Paracox vaccine. A chicken *C. perfringens* strain isolated from a field case of necrotic enteritis and its alpha toxin mutant were also included (EHE-NE18 and EHE-NE18-M1).

In a second trial, the necrotic enteritis inducing ability of *C. perfringens* strains 669 and 670, isolated from bovine enteritis cases, was compared to that of strain 56.

2.7. MACROSCOPICAL LESION SCORING

Intestinal lesions in the small intestine (duodenum to ileum) were scored as previously described (Keyburn et al., 2006). Briefly, the scoring was as follows: 0 = no gross lesions; 1 = congested intestinal mucosa; 2 = small focal necrosis or ulceration (1-5 foci); 3 = focal necrosis or ulceration (6-15 foci); 4 = focal necrosis or ulceration (16 or more foci); 5 = patches of necrosis 2-3 cm long; 6 = diffuse necrosis typical of field cases. Lesion scores of 2 or more were classified as necrotic enteritis positive.

2.8. HISTOPATHOLOGY

Samples from duodenum, jejunum and ileum were fixed in phosphate buffered formalin for at least 24 h, after which they were embedded in paraffin. Sections of 5 µm were cut and stained with haematoxylin and eosin.

3. RESULTS

3.1. TOXINOTYPING BY PCR

All isolates were *C. perfringens* toxin type A, harboring the alpha toxin gene (*cpa*) but not genes encoding beta, iota or epsilon toxin. Isolate 8 was positive for the enterotoxin gene (*cpe*) and isolate 48 was positive for the gene encoding the beta2 toxin (*cpb2*) (Table I).

Table I: Description of the characteristics of the *C. perfringens* isolates used in this study.

Isolate number	Health status of the flock of origin	Toxinotype	Alpha toxin production (µg/ml)
7	healthy	A	0.20 ± 0.07
8	healthy	A (cpe-positive)	14.24 ± 1.40
17	healthy	A	46.91 ± 3.00
48	necrotic enteritis	A (β2-positive)	39.37 ± 4.21
56	necrotic enteritis	A	19.30 ± 2.71
61	necrotic enteritis	A	5.45 ± 1.39
EHE-NE18	necrotic enteritis	A	17.31 ±1.25
EHE-NE18-M1[a]			
669	bovine – hemorrhagic enteritis	A	22.81 ± 2.73
670	bovine – hemorrhagic enteritis	A	46.04 ± 3.84

a: knock-out mutant defective in alpha toxin production derived from strain EHE-NE18, kindly provided by Dr. A. Keyburn.

3.2. ALPHA TOXIN QUANTIFICATION BY ELISA

Quantification of the alpha toxin is shown in Table I. Large differences in the production of alpha toxin were detected when the isolates were compared, independent of their origin. There was a high, moderate and low alpha toxin producer in the group of isolates from healthy chickens (7, 8 and 17) and also in the group of isolates from diseased chickens (48, 56 and 61). The chicken isolate EHE-NE18 produced a moderate amount of alpha toxin and its alpha toxin mutant (EHE-NE18-M1) did not produce any alpha toxin. The bovine strain 670 produced high titers of alpha toxin while the other bovine strain (669) produced a moderate amount of alpha toxin.

3.3. INTESTINAL LOOP ASSAY

Macroscopically, no gross lesions were observed. Microscopically, in one chicken necrosis was induced by clinical isolates 48, 56 and 61 after 10 h of incubation but not by strains isolated from healthy chickens. The lesions were villus atrophy and blunting, necrosis of villus tips, heterophil infiltration and demarcation line formation (Figure 1). The histological sections of the clinical isolates (48, 56 and 61) in the other experiments showed detached epithelial cells and eosinophylic amorphic debris in the intestinal lumen but no necrosis of villus tips.

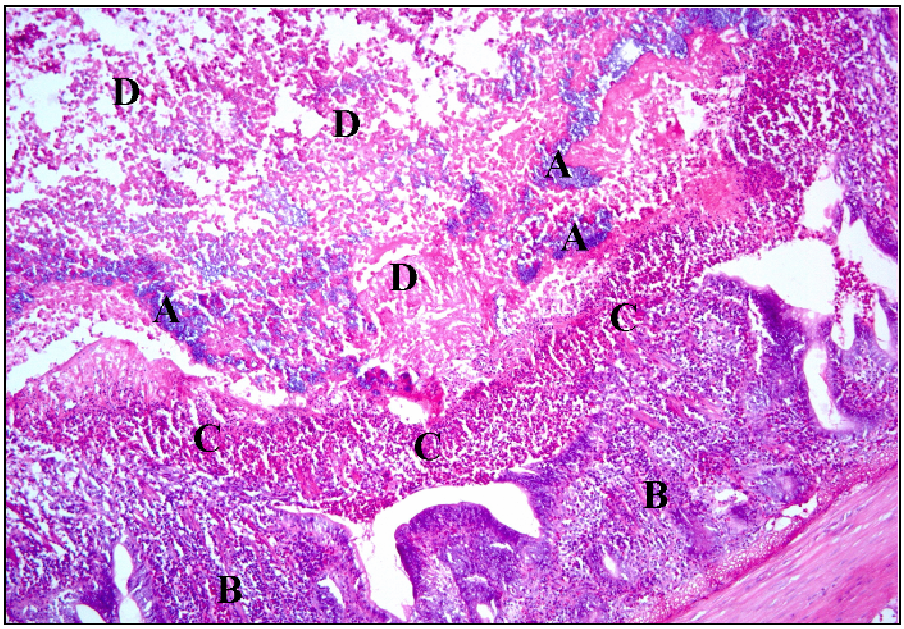

Figure 1: Necrotic lesions after inoculation of intestinal loops with strain 61 for 10 h, typically visible as necrosis of villus tips and tissue with an abundance of dead cells and fibrin-like material in the lumen in which large clusters of bacteria are present. A: *C. perfringens*–like bacteria; B: Villi; C: demarcation line, D: cellular debris and fibrin-like material.

3.4. MACROSCOPICAL OBSERVATIONS IN THE *IN VIVO* TRIALS

Table II summarizes the number of birds having macroscopic necrotic lesions in all trials. These lesions presented as multiple foci, mostly in the jejunum. In trial 1, only the animals inoculated with strains originally isolated from necrotic enteritis outbreaks (48, 56, 61, and EHE-NE18) developed lesions. The alpha toxin mutant EHE-NE18-M1 induced necrotic

enteritis lesions in about the same number of birds as compared with the wild-type strain. No animals with lesions were found in the groups inoculated with *C. perfringens* strains from clinically healthy birds (7, 8 and 17). No necrotic enteritis lesions were observed in the control groups, i.e. the negative control group and the group only inoculated with a 10-fold dose of Paracox. The mean of the lesion scores is also shown in Table II. The mean of the lesion scores was lower in the groups infected with strains derived from clinically healthy flocks compared to the groups infected with necrotic enteritis outbreak strains.

In trial 2, no gross lesions were observed in the groups that were inoculated with the bovine strains (669 and 670) isolated from calf hemorrhagic enteritis cases, while in the group inoculated with the poultry derived strain 56, 55.56% of the animals showed lesions (Table II).

Table II: Number of birds with macroscopic necrotic enteritis lesions on the three sampling days in all trials.

Trial	Group	Day 23	Day 24	Day 25	Total	Total (%)	Mean score
1	negative control	0/9	0/9	0/9	0/27	0	0
	only coccidia	0/9	0/9	0/9	0/27	0	0,30
	7[h]	0/9	0/9	0/9	0/27	0	0,41
	8[h]	0/9	0/9	0/9	0/27	0	0,56
	17[h]	0/9	0/9	0/9	0/27	0	0,52
	48[ne]	2/9	0/9	1/9	3/27	11.11	0,81
	56[ne]	1/9	5/9	7/9	13/27	48.15	1,56
	61[ne]	4/9	5/9	6/9	15/27	55.56	2,07
	EHE-NE18[ne]	2/8	2/8	6/8	10/24	41.67	1,5
	EHE-NE18-M1[a]	1/8	3/8	7/8	11/24	45.83	2,21
2	56[ne]	4/9	5/9	6/9	15/27	55.56	1,48
	669[b]	0/8	0/8	0/8	0/24	0	0,40
	670[b]	0/8	0/8	0/8	0/24	0	0,58

Birds with macroscopic necrotic enteritis lesions are the birds with a lesion score of 2 or above. The mean of the scores is also shown.

[h] *C. perfringens* isolate from clinically healthy broilers

[ne] *C. perfringens* isolate from broilers with necrotic enteritis

[b] *C. perfringens* isolate from calf hemorrhagic enteritis, kindly provided by Prof. Dr. P. Deprez

[a] knock-out mutant defective in alpha toxin production derived from strain EHE-NE18, kindly provided by Dr. A. Keyburn.

4. DISCUSSION

C. perfringens strains derived from clinically healthy broilers did not produce necrotic enteritis in broilers, even in an experimental model using predisposing factors. In contrast, strains isolated from outbreaks of necrotic enteritis did produce necrotic gut lesions in both trials performed in this study. To our knowledge, this is the first report describing the inability of *C. perfringens* normal gut microbiota isolates to produce necrotic enteritis in a model where the disease is consistently reproduced by virulent strains. These results suggest that high numbers of *C. perfringens* in the gut is not sufficient to induce necrotic enteritis but that strains from cases of necrotic enteritis do possess virulence factors required to induce disease while *C. perfringens* strains from healthy chickens lack these virulence factors. Thus, comparisons of expressed proteins or genome sequences from necrotic enteritis strains versus normal gut microbiota strains should allow identification of virulence factors required for induction of necrotic enteritis in broilers.

The ability to induce necrotic lesions was independent of the ability of *C. perfringens* to produce alpha toxin. Indeed, the four strains that were able to induce lesions (48, 56, 61, and EHE-NE18), produce different amounts of alpha toxin (Table I), and similar levels of alpha toxin were produced *in vitro* by the normal gut microbiota isolates. The alpha toxin, the major toxin involved in the pathogenesis of human gas gangrene, has for many years been proposed as being the major virulence factor in causing necrotic enteritis in chickens (Al-Sheikhly and Truscott, 1977; Fukata et al., 1988; Hofshagen and Stenwig, 1992). However, recent studies indicate that alpha toxin is not an essential virulence factor of necrotic enteritis. It was shown that strains isolated from necrotic enteritis outbreaks do not produce more alpha toxin *in vitro* compared to normal broiler gut microbiota isolates (Gholamiandehkordi et al., 2006). Additionally, using a gene knock-out mutant, Keyburn et al. (2006) showed that alpha toxin is not essential for pathogenesis. Their *C. perfringens* wild-type strain and its alpha toxin mutant were also included in the present experiment and their data were confirmed.

A multiple-locus variable-number tandem repeat analysis to investigate the evolution of virulence and population structure of *C. perfringens* has shown that strains isolated from different animal species can be closely related (Sawires and Songer, 2006), but this does not give information on the phenotypical characteristics of the isolates. In our study, isolates from calf hemorrhagic enteritis were not able to induce disease in broilers. Keyburn et al. (2006) showed that a human gas gangrene isolate (JIR325) was also not able to induce necrotic

enteritis in broilers. It is therefore likely that certain *C. perfringens* strains engage in host-specific interactions with the chicken host. Especially cytotoxic effects and induction of lesions, but maybe not colonization of the gut itself, could be host-specific. Although more studies are needed to definitely prove this hypothesis, our data suggest that *C. perfringens* type A isolates from hemorrhagic enteritis in calves do not cause disease in broilers. It is unclear whether these strains could be part of the normal gut microbiota of broilers.

In conclusion, data were produced in agreement with the hypothesis that isolates from broilers with necrotic enteritis are virulent while isolates from healthy chickens are not. In addition it was demonstrated that induction of necrotic lesions in the broiler gut is not associated with the ability to produce alpha toxin *in vitro*. Moreover, the results also suggest that the virulence of *C. perfringens* strains is to some extent host specific since two *C. perfringens* strains isolated from calf hemorrhagic enteritis were not able to produce necrotic lesions in chickens.

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Chapter 3.2:

Intra-species growth-inhibition by *Clostridium perfringens* is a possible virulence trait in necrotic enteritis in broilers

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ABSTRACT

Necrotic enteritis in broiler chickens is associated with *Clostridium perfringens* type A, carrying the NetB toxin. *Clostridium perfringens* type A is also a member of the normal intestinal microbiota of broilers. Clinically healthy chickens carry several different *Clostridium perfringens* clones in their intestine. In flocks suffering from necrotic enteritis, however, mostly only one single clone is isolated from the gut of all the diseased animals. Selective proliferation of these clinical outbreak strains in the gut and spread within the flock seems likely, but an explanation has not yet been given. The hypothesis that necrotic enteritis associated *Clostridium perfringens* strains might suppress the growth of normal microbiota *Clostridium perfringens* strains, was therefore tested. Twenty-six *Clostridium perfringens* strains isolated from healthy broilers and 24 clinical outbreak isolates were evaluated for their ability to induce intra-species growth-inhibition in an *in vitro* setup. A significantly higher proportion of the *Clostridium perfringens* clinical outbreak strains inhibited the growth of other *Clostridium perfringens* strains compared to *Clostridium perfringens* strains isolated from the gut of healthy chickens. It is proposed that, in addition to toxin production, intra-species growth-inhibition may be a virulence trait that contributes to the ability of certain *Clostridium perfringens* strains to cause necrotic enteritis in broilers.

Keywords: *Clostridium perfringens*; Broiler; Necrotic enteritis; Growth-inhibition

1. INTRODUCTION

Since the ban on growth-promoting antibiotics in animal feed in the European Union, *Clostridium perfringens* associated necrotic enteritis is re-emerging in broilers (Grave *et al*., 2004; Van Immerseel *et al*., 2004; Williams, 2005). Necrotic enteritis in poultry is associated with *C. perfringens* type A, carrying the NetB toxin (Keyburn et al., 2008; Van Immerseel et al., 2009). *C. perfringens* type A is also a member of the normal intestinal microbiota of broilers. Strains isolated from healthy broilers, however, do not induce necrotic enteritis in an experimental model using predisposing factors, in contrast to strains isolated from outbreaks of necrotic enteritis (Timbermont et al., 2009).

In *C. perfringens* isolates from healthy birds, a high degree of genetic diversity is found, even between isolates from the same animal. In contrast, different isolates from a flock suffering from a clinical outbreak are generally of the same pulsed-field gel electrophoresis (PFGE) type, regardless of the animal or the part of the intestine from which the strain was isolated (Nauerby et al., 2003; Gholamiandehkordi et al., 2006). The reason for the presence of a single clone in necrotic enteritis outbreaks is not known. It is speculated that during an outbreak, certain *C. perfringens* strains have a competitive advantage over other *C. perfringens* strains in the broiler gut.

In the present study, strains isolated from healthy broilers and strains isolated from broilers suffering from necrotic enteritis were compared with respect to their capacity of intra-species growth-inhibition in an *in vitro* inhibition assay.

2. MATERALS AND METHODS

2.1. BACTERIA

Fifty *C. perfringens* type A strains belonging to different genotypes, as analyzed by PFGE, were included. Thirty-five strains were isolated from broiler chickens in Belgium: 26 strains from clinically healthy broiler chickens and 9 strains from broilers suffering from necrotic enteritis (Gholamiandehkordi et al., 2006). Fifteen Danish *C. perfringens* isolates from necrotic enteritis cases were kindly provided by Dr. L. Bjerrum (Nauerby et al., 2003).

Strains of *C. perfringens* were grown on Colombia agar (Oxoid, Basingstoke, UK) containing 5% defibrinated sheep blood. Plates were incubated at 37 °C in an anaerobic working cabinet (invivo$_2$500, Ruskinn Life Sciences, Bridgend, UK), in an atmosphere of 8%H_2:8%CO_2:84%N_2. Strains were stored at -80 °C in lyophilisation medium (LYM) containing 7% glucose, 23% Brain Heart Infusion broth (BHI, Oxoid) and 70% defibrinated horse serum (Invitrogen, Merelbeke, Belgium).

2.2. *IN VITRO* GROWTH-INHIBITION ASSAY

The 50 *C. perfringens* strains were used in a checkerboard test for intra-species growth-inhibition. Each strain was cultured anaerobically in BHI broth for 24 h at 37 °C. The overnight cultures were diluted in Phosphate Buffered Saline (PBS) to a density of McFarland No. 0.5, and 200 µl of these suspensions were spread with a sterile swab on the whole surface of BHI agar plates to obtain a bacterial lawn (12cm × 12cm). A single colony of each *C. perfringens* isolate was transferred with a sterile toothpick to the agar plates seeded with the different *C. perfringens* strains. Absence of growth of the bacterial lawn around a colony results in an inhibition zone (Figure 1). After overnight incubation under anaerobic conditions, diameters of inhibition zones were measured in mm. The tests were performed in triplicate.

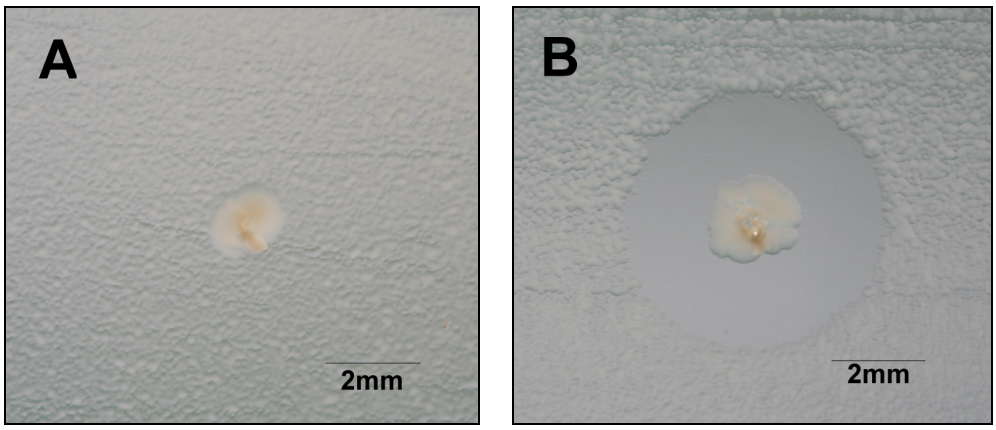

Figure 1: Result of the radial diffusion assay. A colony of a *C. perfringens* isolate was stabbed through a lawn of another *C. perfringens* strain and partially through the agar beneath. A: No inhibition; B: Clear inhibition zone

2.3. STATISTICAL ANALYSIS

The data were analysed with SPSS 16 software using the chi-square test to compare the number of strains of the normal microbiota group with the number of strains of the necrotic enteritis group that were able to inhibit other strains. The student's t-test was used to compare the mean inhibition zones of the healthy animal strains and the necrotic enteritis strains. Significance was determined at $P < 0.05$.

3. RESULTS

Sixty percent of all tested strains inhibited growth of at least one other strain of *C. perfringens* (i.e., there was a zone of clearing around the stabbed colony). While some strains inhibited growth of many other strains, others had a very limited inhibitory spectrum. Fifteen (58%) of the 26 healthy animal strains and 4 (17%) of the 24 clinical outbreak strains were not able to inhibit the growth of any other strain. In contrast, 46% (11/24) of the clinical outbreak strains were able to inhibit more than 90% (more than 45/50) of the other strains while this was only the case for 15% (4/26) of the healthy animal strains (Figure 2). The number of strains that were able to inhibit any number of other strains was significantly higher for the clinical outbreak strains than for the healthy animal strains ($P < 0.05$). If strains were able to inhibit the growth of other *C. perfringens* strains, they were able to inhibit both healthy animal strains and necrotic enteritis outbreak strains.

In the strains that were able to inhibit, the zones of inhibition varied for the strains from healthy broilers between 3 mm and 7 mm and for the necrotic enteritis strains between 3.5 mm and 5.5 mm. There was no significant difference in the average sizes of the inhibition zones: 4.9 mm and 4.3 mm for healthy animal strains and clinical outbreak strains, respectively.

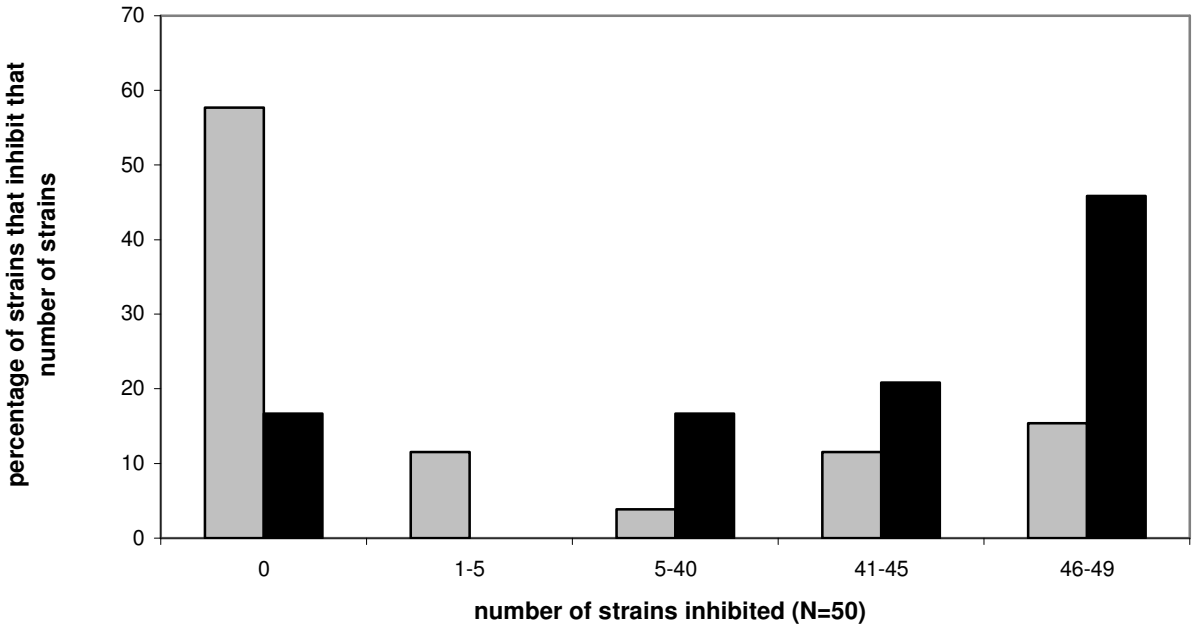

Figure 2: Percentage of *C. perfringens* strains secreting intra-species inhibitory substances. The percentage of healthy animal strains (grey) and clinical outbreak strains (black) of *C. perfringens* that were able to inhibit a number of *C. perfringens* strains (N = 50), as indicated on the X-axis, is shown.

4. DISCUSSION

Intra-species inter-strain growth-inhibition can explain the presence of a single clone of *C. perfringens* in a broiler flock suffering from necrotic enteritis. Our results show that inhibition of other *C. perfringens* strains is a trait that is significantly more developed in clinical outbreak strains, compared with healthy animal strains. A minority of healthy animal strains were able to inhibit other *C. perfringens* strains isolated from healthy birds and necrotic enteritis strains. In a recent study of Barbara et al. (2008), none of the normal flora strains (n = 17) could inhibit the growth of other *C. perfringens* strains. Also a minority of necrotic enteritis outbreak strains did not have the intra-species growth-inhibitory phenotype. Possibly in these strains other virulence traits may compensate for the lack of inhibitory capacity. Another possible explanation for the lack of *in vitro* inhibitory phenotype is that expression of the gene encoding for the growth-inhibiting phenomenon can be down-regulated by certain signal transduction systems or regulators (Dupuy and Mamouros, 2006). The gene for the growth-inhibiting factor might also be located on a plasmid and the absence

of growth-inhibition may be caused by the loss of this plasmid after *in vitro* cultivation (Rood and Cole, 1991).

The results of the radial diffusion assay suggest that the intra-species inter-strain growth-inhibition is caused by a substance secreted by the bacterium into its micro-environment. The nature of this substance is hitherto unclear. It is known that *C. perfringens* is able to produce bacteriocins capable of lysing other *C. perfringens* strains. Watson et al. (1982) observed that 79% of strains implicated in food poisoning outbreaks produced bacteriocins; however, only 18% of 322 isolates from the feces of healthy persons, human and animal infections, various foods, and the environment produced bacteriocins suggesting a relationship between bacteriocin production and the ability to cause food poisoning.

In this study, secretion of intra-species growth-inhibitory substances by *C. perfringens* is proposed to be an additional virulence trait that can possibly contribute to the pathogenesis of necrotic enteritis by selective proliferation of one single clone in the broiler gut. Indeed, when certain environmental predisposing factors, such as a coccidial infection, are present, nutrients utilizable by *C. perfringens* are leaking in the lumen (Collier et al., 2008). Intra-species inter-strain growth-inhibition may constitute an important advantage for certain strains in the competition for nutrients in the intestinal tract. It is well documented that *C. perfringens* requires at least 11 different essential amino acids for its multiplication (Petit et al., 1999). Suppressing its competitors may allow unlimited access to the nutrients and thus the possibility of explosive multiplication. The massive production of specific toxins and enzymes, such as the NetB toxin and proteolytic enzymes (Olkowski et al., 2006; Olkowski et al., 2008; Keyburn et al., 2008) at sites of multiplication of these bacteria in the gut would then lead to necrotic lesions, and thus the release of more nutrients, completing the cycle.

In conclusion, *C. perfringens* outbreak isolates are significantly more capable of intra-species growth-inhibition compared to strains isolated from healthy birds. It is proposed that intra-species growth-inhibition by *C. perfringens* is a possible virulence trait in necrotic enteritis in broilers.

ACKNOWLEDGEMENTS

We would like to thank Veerle Flama for her skilful technical assistance. Dr. L. Bjerrum is acknowledged for providing strains. This work was supported by the Institute for Science and Technology, Flanders (IWT). F. Van Immerseel is supported by a Postdoctoral Research Grant of the Research Foundation - Flanders (FWO) and by the Research Fund of Ghent University (BOF).

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Chapter 3.3:

Purification and partial characterization of a novel antimicrobial peptide from *Clostridium perfringens* strain 56

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ABSTRACT

Necrotic enteritis in broiler chickens is associated with *Clostridium perfringens* type A. Single strain dominance is found in the gut of broiler chickens suffering from necrotic enteritis: within an outbreak, all affected animals carry the same clonal *Clostridium perfringens* strain in the affected tissue. It is known that *Clostridium perfringens* is capable of secreting factors inhibiting growth of other *Clostridium perfringens* strains and this characteristic is more prevalent in outbreak strains compared to normal microbiota strains. This characteristic could lead to extensive and selective presence of a strain that contains the genetic make-up enabling to secrete toxins that cause gut lesions. This report describes the purification and the characterization of a bacteriocin-like inhibitory substance (BLIS) from a necrotic enteritis associated *Clostridium perfringens* strain exhibiting antibacterial activity against other *Clostridium perfringens* strains. Amino acid sequence analysis indicated that the BLIS is an 11.5 kDa fragment of a 22 kDa protein and that it constitutes the C-terminal part of this protein. The 22 kDa protein is a cytoplasmic membrane protein without signal peptide. The antibacterial activity of the purified BLIS was abolished by proteolytic enzymes trypsin and proteinase K and by heat treatment (10 min at 80 °C). The purified BLIS showed inhibitory activity over a wide pH-range (4.0 to 10.0). Since the antibacterial activity against other *Clostridium perfringens* strains of the purified BLIS had a narrower spectrum than the crude supernatant of the secreting strain, most probably different of these bacteriocins are produced.

Key words: Clostridium perfringens; Necrotic enteritis; Broiler; Bacteriocin

1. INTRODUCTION

Clostridium perfringens type A is widely prevalent in the environment and in the intestinal tract of animals and men. Necrotic enteritis in poultry is associated with a specific subpopulation of *C. perfringens* type A, i.e. those carrying the NetB toxin (Keyburn et al., 2008; Van Immerseel et al., 2009). As a consequence, strains isolated from outbreaks of necrotic enteritis are able to induce necrotic enteritis in an experimental model using predisposing factors, in contrast to strains isolated from the gut of healthy broilers (Timbermont et al., 2009a). In *C. perfringens* isolates from healthy birds, a high degree of genetic diversity is found, even between isolates within the gut of a single animal. In contrast, in a flock suffering from a clinical outbreak, different isolates are generally of the same pulsed-field gel electrophoresis (PFGE) type, regardless of which animal in the flock or which part of the intestine the strain was isolated from (Nauerby et al., 2003; Gholamiandehkordi et al., 2006). Recent data show that *C. perfringens* is capable of secreting factors inhibiting growth of other *C. perfringens* strains. The intra-species inhibitory phenotype was shown to be more prevalent in outbreak strains compared to normal microbiota strains (Barbara et al., 2008; Timbermont et al., 2009b). This characteristic could lead to extensive and selective presence of a single strain in the gut, and if it contains the genetic make-up enabling to secrete toxins, it could consequently cause gut lesions.

It is known that many *C. perfringens* strains are able to produce antibacterial proteins, called bacteriocins (Tagg et al., 1976). Bacteriocins are proteinaceous toxic compounds produced by bacteria that generally inhibit the growth of closely related strains (Jack et al., 1995), and are thus potential candidates involved in the intra-species inhibitory phenotype of *C. perfringens*. The nature of the inhibitory component that causes intra-species growth-inhibition in broiler outbreak strains was hitherto unknown. Therefore, in the present study, the purification and partial characterization of a novel antimicrobial peptide from a *C. perfringens* outbreak strain is described.

2. MATERIALS AND METHODS

2.1. STRAINS

Fifty *C. perfringens* type A strains belonging to different genotypes, as analyzed by PFGE, were included. Thirty-five strains were isolated from broiler chickens in Belgium: 26 strains from clinically healthy broiler chickens and 9 strains from broilers suffering from necrotic enteritis. Fifteen Danish *C. perfringens* isolates from necrotic enteritis cases were kindly provided by Dr. L. Bjerrum (Nauerby et al., 2003; Gholamiandehkordi et al., 2006; Timbermont et al., 2009b). Strain 56 was isolated from the intestine of a broiler chicken with severe necrotic gut lesions. It was selected from the strains because it is shown to be a virulent strain (Timbermont et al., 2009a) and inhibits the growth of 41 of the 50 strains included (Timbermont et al., 2009b). *C. perfringens* strain 6 was isolated from the normal gut microbiota of a healthy broiler chicken and was used as indicator strain because it is not able to inhibit other *C. perfringens* strains and its growth is inhibited by strain 56 (Timbermont et al., 2009b).

2.2. AGAR SPOT TEST

For the agar spot test, 20 µl drops of the fractions to be tested were spotted on the lawns of bacteria. Lawns were prepared by resuspension of colonies in PBS to a density of McFarland 1, and 100 µl of these suspensions were spread with a sterile swab on the surface of agar plates.

2.3. ANTIMICROBIAL PROTEIN PURIFICATION AND IDENTIFICATION

C. perfringens strain 56 was grown in Tryptic Soy Broth (TSB, Oxoid, Basingstoke, England) for 24 h at 42 °C (500ml). Culture supernatant was obtained by centrifugation at 17000 g for 15 min at 4 °C. The supernatant was filter sterilized (0.22 µm) and supernatant proteins precipitated by overnight incubation in 50% (w/v) $(NH_4)_2SO_4$ at 4 °C followed by centrifugation at 17000 g for 2 h at 4 °C. The precipitate was resuspended in 5 ml of phosphate buffered saline (PBS) and dialysed against 10 mM Tris-HCl buffer, pH 8.5 for 24 h at 4 °C. This concentrated supernatant was loaded onto a SP-sepharose cation exchange

column in 10 mM NaOAc buffer, pH 4. Proteins were eluted stepwise from the column, with increasing concentrations of NaCl (up to 1 M). To determine the antimicrobial activity of the fractions, two-fold dilutions of the fractions were spotted on a lawn of strain 6 in the agar spot test. The most active fractions in the antimicrobial assay were pooled and loaded onto a butyl-sepharose hydrophobic interaction column in 50 mM Sodium phosphate buffer with 1 M $(NH_4)_2SO_4$, pH 7.4. Proteins were stepwise eluted from the column, with decreasing concentrations of $(NH_4)_2SO_4$. Again, the activity of the fractions was determined and the most active fraction was concentrated and analyzed by SDS-PAGE. Precision Plus Protein Standard, All Blue (Bio-Rad Laboratories, Hercules, USA) was used as protein marker. The protein band was cut from the gel and subjected to in-gel protein digestion with trypsin (Devreese et al., 2002) followed by mass spectrometric characterization. After mixing of 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 was done with the obtained amino acid sequences (http://blast.ncbi.nlm.nih.gov/Blast/). The protein fragment sequences were also compared with the incomplete EHE-NE18 sequence, kindly provided by Prof. J. Rood (Monash University). Prediction of transmembrane helices was done with TMHMM (Krogh et al, 2001).

2.4. CHARACTERIZATION OF THE ANTIMICROBIAL PEPTIDE

Thermal, pH and protease stability of the purified bacteriocin-like inhibitory substance (BLIS) was tested. The remaining antibacterial activity after treatment was determined by the agar spot test and compared with the activity of a corresponding control. Dilutions of treated and untreated (control) BLIS were spotted on lawns of strain 6. First, thermal stability of the growth-inhibiting factor was investigated by determination of the residual antibacterial activity after incubation at 4, 24, 37, 42, 60, 80 and 100 °C for 10, 30 and 60 min. To evaluate the influence of pH on activity, the BLIS sample was adjusted to a pH of 2.0, 4.0, 6.0, 8.0, 10.0 and 12.0 with HCl or NaOH, mixed, incubated at room temperature for 1 h, neutralized to pH 7, and tested for activity. Effect of trypsin and proteinase K on bacteriocin activity was tested. Each enzyme was prepared at a concentration of 10 mg/ml and added to the purified BLIS at a final concentration of 1 and 0.1 mg/ml. After incubation for 1 h at 37 °C, inhibitory

activity was tested. The spectrum of the purified BLIS was analyzed by the agar spot test by spotting 20 µl drops on lawns of the 50 *C. perfringens* strains.

3. RESULTS

3.1. PURIFICATION AND IDENTIFICATION OF THE ANTIMICROBIAL PEPTIDE PRODUCED BY *CLOSTRIDIUM PERFRINGENS* STRAIN 56

The molecular mass of the purified BLIS was approximately 12 kDa, according to SDS-PAGE (Figure 1).

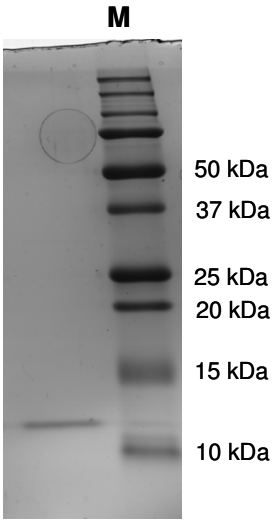

Figure 1: SDS-PAGE analysis of purified BLIS from *C. perfringens* strain 56

Mass spectrometry of this band identified five protein fragment sequences (Table I). No corresponding protein sequence was found in NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast/). A search in the incomplete EHE-NE18 sequence showed identical amino acid sequences in a 22,91 kDa protein (207AA) (Table I). The hypothetical protein has no signal sequence and is a basic protein with a pI of 10.36. Analysis of the amino acid sequence showed that the protein was composed of 4 transmembrane helices (Figure 2). All amino acid sequences found were situated in the last 106 amino acids of the protein. They all start after a trypsin cleavage site except for the one starting at amino acid 101 (Table I).

Table I: Amino acid sequences found by mass spectrometry and their start position in the 22 kDa protein

Sequence	Start position
YPYNSTSNVKR	AA101
KSLSEWTWTSLLAGFNA	AA130
TGAFLGALTALYGYGVNT	AA149
NLSVPLTAANA	AA167
ASGLTLSYTYVLQQTTNLGSFENTR	AA178

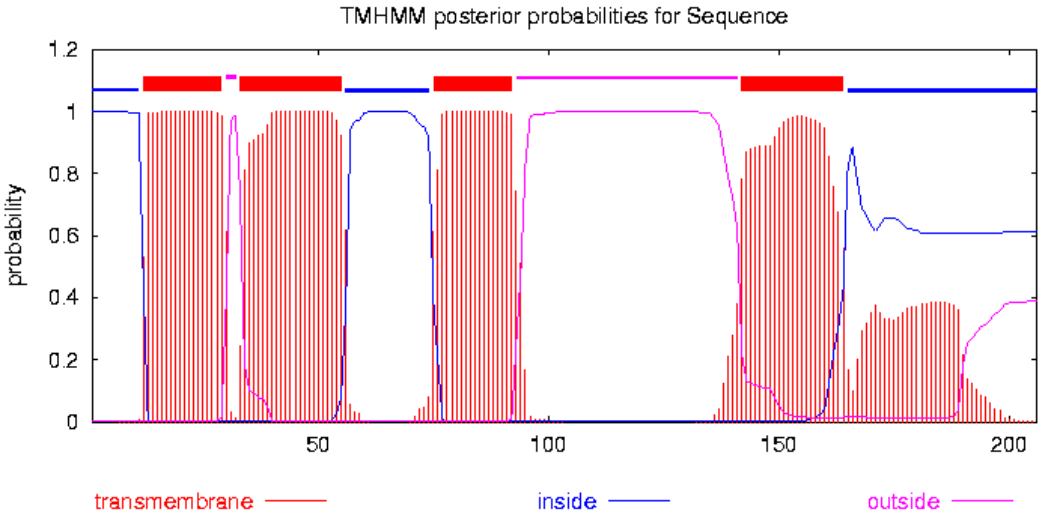

Figure 2: Protein profile analysis of the 22 kDa protein

3.2. CHARACTERIZATION OF THE ANTIMICROBIAL PEPTIDE

After 30 min at 80 °C and after 10 min at 100 °C, no antimicrobial activity against strain 6 was present anymore. Antimicrobial activity was most stable at neutral pH and decrease was detected at pH 2 and pH 12. Complete inactivation or significant reduction in antimicrobial activity was observed after treatment with trypsin and proteinase K (Table II). The purified antimicrobial peptide was able to inhibit the growth of 15 of the 50 *C. perfringens* strains. It did not inhibit the growth of the producer strain, strain 56.

Table II: Residual activity of the purified BLIS after incubation at different temperatures for 10, 30 and 60 minutes, at different pH-values ranging from pH 2 to 12, and after addition of trypsin and proteinase K at 0.1 and 10 mg/ml compared to the corresponding control.

Treatment	**Residual activity compared to the corresponding control (%)**
Temperature	
4 °C; 10 min – 30 min – 60 min	100
24 °C; 10 min – 30 min – 60 min (control)	100
37 °C; 10 min – 30 min – 60 min	100
42 °C; 10 min – 30 min – 60 min	100
60 °C; 10 min – 30 min – 60 min	100
80 °C; 10 min	25
80 °C; 30 min – 60 min	0
100 °C; 10 min – 30 min – 60 min	0
pH	
2	25
4	50
6	75
7 (control)	100
8	75
10	50
12	25
Enzymes	
0 mg/ml (control)	100
Trypsin, 0,1 mg/ml	25
Trypsin, 1 mg/ml	0
Proteinase K, 0,1 mg/ml	0
Proteinase K, 1 mg/ml	0

4. DISCUSSION

Bacteriocin production might be involved in the pathogenesis of necrotic enteritis, since in outbreaks of necrotic enteritis single clone dominance is found (Nauerby et al., 2003; Gholamiandehkordi et al., 2006) and intra-species inter-strain growth-inhibition was found to be more prevalent in outbreak strains from broilers compared to normal microbiota strains (Barbara et al., 2008; Timbermont et al., 2009b).

The antibacterial peptide identified in this study is a novel peptide without any homology with currently known bacteriocins or antimicrobial proteins. No sequence similarity was found with any genes or proteins in database searches. Even more, no similarity was found with gene or protein sequences of any of the 3 genome sequenced *C. perfringens* type A strains (Shimizu et al., 2002; Myers et al., 2006). The sequenced strains are a gangrene strain and a food poisoning strain isolated from humans and a gangrene strain isolated from soil. The antimicrobial peptide may be specific for animal and even for broiler necrotic enteritis strains, since a match was found in the sequence of EHE-NE18, a strain isolated from a chicken suffering from necrotic enteritis (Keyburn et al., 2008).

The newly identified bacteriocin-like peptide is hypothesized to be released by proteolytic activity from a 22 kDa protein without signal peptide that is located in the cytoplasmic membrane. The amino acid sequence of the protein indicates the presence of four transmembrane helices. An extracellular loop is located between the third and the fourth transmembrane helix (Figure 2). Protease activity may cleave the protein at this position, releasing a 12 kDa protein fragment extracellullarly. All amino acid sequences found by mass spectrometry, were situated in the last 106 amino acids of the 22 kDa protein. Remarkably, all amino acid sequences started after a trypsin cleavage site except the one starting at amino acid 101 (Y101; Table I). This result suggests that the purified fragment starts at Y101 since an in-gel trypsin digestion was performed before mass spectrometry. A fragment starting at Y101 would have a molecular mass of 11,538 kDa which is in accordance with the estimated molecular mass of the purified antimicrobial peptide (Figure 1). The extracellular loop starts around amino acid at position 100 and a cleavage site of proteinase K is located before Y101.

Bacteriocins from Gram-positive bacteria are usually produced as precursors with N-terminal signal peptides (Jack et al., 1995). The signal peptides serve as recognition signals for the export of the bacteriocins out of the cells, and they also protect the producers against their own bacteriocin by making them biologically inactive inside the cell. Concomitant with the

export from the cell, bacteriocins and antimicrobial peptides are processed and activated. However, it has been shown that antimicrobial peptides can be produced from the degradation of larger proteins. For example, Faye et al. (2002) showed that *Propionibacterium jensenii* secretes an inactive proprotein which is proteolytically activated in the environment. To our knowledge, this is the first description of a bacteriocin-like peptide that may be formed by an external protease from a bacterial putative cytoplasm membrane associated protein.

The transmembrane helix at the C-terminal part of the 12 kDa part of the protein, might suggest that the antimicrobial peptide acts by pore formation. This is a feature similar to those of many characterized antimicrobial peptides from Gram-positive bacteria (Jack et al., 1995). It has indeed been shown that the C-terminal region of class IIa bacteriocins forms one or two helices and they penetrate the cell membrane, thereby inducing leakage and cell death (Moll et al., 2000; Morisset et al., 2004).

The *C. perfringens* strain 56 most likely secretes or produces more bacteriocins in addition to the protein identified in this work. Indeed, the inhibitory spectrum of *C. perfringens* strain 56 and that of the purified BLIS are not identical. It is not unusual that a *C. perfringens* strain produces more than 1 bacteriocin simultaneously. As an example, Higa et al. (1991) showed that *C. perfringens* SN-17 produced two bacteriocins in succession. Moreover, it was also shown that these bacteriocins have different inhibitory spectra. To detect the other antimicrobial factor(s), screening of the protein fractions derived from the supernatant must be done using other indicator strains. *C. perfringens strain* 56 is not sensitive to its own bacteriocin. Genes encoding membrane-associated molecules that confer a degree of specific protection upon the producer strain have been found. For example, Diep et al. (2007) showed that an immunity protein prevents *Lactococcus lactis* from being killed by its permeabilizing peptide-bacteriocin, lactococcin A, by formation of a strong complex with the receptor proteins and the bacteriocin. Moreover, it was shown that the immunity protein is only expressed if mature bacteriocin is produced or if it is present in the environment (Diep et al., 2007).

In conclusion, a new antimicrobial peptide derived from a necrotic enteritis inducing *C. perfringens* strain was purified and partly characterized. Further studies are necessary to determine the mode of action and the target of the bacteriocin-like peptide and to elucidate its role in the pathogenesis of necrotic enteritis.

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We would like to thank J. Rood for searching the EHE-NE18 sequence and translating the protein sequence into a DNA sequence. This work was supported by the Institute for Science and Technology, Flanders (IWT). F. Van Immerseel is supported by a Postdoctoral Research Grant of the Research Foundation - Flanders (FWO) and the Research Council of Ghent University (BOF).

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Chapter 4

General Discussion

Virulent and avirulent *Clostridium perfringens* strains

Not alpha toxin but NetB is important for virulence

Growth-inhibition

New insights in pathogenesis, a model

Implications: improvement of treatments

Alpha toxin as vaccine

Other antigens as vaccine

GMO probiotics expressing bacteriocins

Conclusion

CHAPTER 4:
GENERAL DISCUSSION

1. VIRULENT AND AVIRULENT *CLOSTRIDIUM PERFRINGENS* STRAINS

For over 30 years, it was believed that alpha toxin was the key virulence factor in necrotic enteritis in broiler chickens caused by *C. perfringens*. As a consequence, it seemed logical that, given appropriate conditions in the chicken intestinal tract, all strains of *C. perfringens* were able to produce necrotic enteritis lesions. However, using a gene knock-out mutant it was shown that alpha toxin is not essential for virulence (Keyburn et al., 2006). Moreover, it is now known that only specific strains are able to produce necrotic enteritis in broilers, i.e. those strains that are isolated from outbreaks of the disease (CHAPTER 3.1). Chicken normal microbiota strains or strains from enteric disease in other species are not virulent for broiler chickens in an experimental infection model. Thus not only predisposing factors are necessary to induce necrotic enteritis, but the genetic make-up of the strain is also a crucial factor. Therefore, key virulence determinants specific for necrotic enteritis outbreak isolates were searched by researchers in the last few years.

1.1. NOT ALPHA TOXIN BUT NETB IS IMPORTANT FOR VIRULENCE

Recently, a novel toxin has been described that is associated with necrotic enteritis in broilers (Keyburn et al., 2008). The toxin has amino acid sequence similarity to beta toxin from *C. perfringens* (38% amino acid sequence identity) and therefore, it is called NetB (Necrotic Enteritis Toxin B-like). The toxin was identified using two complementary approaches. The supernatant of *C. perfringens* cultures was screened *in vitro* for proteins that were cytotoxic for a chicken leghorn male hepatoma cell line (LMH) and the *netB* structural gene was identified by sequencing the genome of a necrotic enteritis outbreak isolate and by comparing

the genome of the necrotic enteritis isolate with the known genomes of other *Clostridia*. Both native and recombinant NetB caused cell rounding and lysis of LMH cells, and it was shown that small hydrophilic 1.6 - 1.8nm pores were formed. Moreover, a *netB* mutant of *C. perfringens* failed to cause necrotic lesions in the gut of experimentally infected broilers, but a mutant complemented with the wild-type *netB* gene was as virulent as the wild-type strain (Keyburn et al., 2008). Confirmation for the role of NetB in disease came from the finding that most necrotic enteritis outbreak strains carry the *netB* gene, whereas non-necrotic enteritis derived *C. perfringens* isolates lack this gene (Keyburn et al., 2008). Several groups screened *C. perfringens* isolates derived from different countries for the occurrence of *netB*. In a Canadian study, 95% (39/41) *C. perfringens* isolates from broilers with necrotic enteritis were positive for *netB* while only 35% (7/20) isolates from healthy boilers were *netB* positive (Chalmers et al., 2008). A screening of isolates from the US reported a carriage rate of 58% (7/12) for the necrotic enteritis isolates while only 8.75% (7/80) normal microbiota strains carried the *netB* gene (Martin and Smyth, 2009). The isolates used in CHAPTER 3.2 were also screened. Of the 26 isolates from healthy broilers from Belgium, only 1 (3.8%) was *netB* positive while 20 of the 24 (83%) Belgian and Danish necrotic enteritis isolates were *netB* positive (unpublished data). This is in accordance with NetB being important for necrotic enteritis. However, all these studies have also found some isolates from diseased birds that do not carry the *netB* gene. A plausible explanation is that these isolates may possess a sequence divergent of the published toxin gene sequence. Another possibility is that the toxin gene is lost during culture. Such possible instability of the virulent phenotype might occur if the *netB* gene is on a plasmid that is readily lost during the colony purification process. Loss of virulence following *in vitro* culture is not an uncommon occurrence in pathogenic bacteria. Also the isolation protocol is an important issue here. *Clostridium perfringens* strains are always present in the gut of animals and isolation of strains should be performed with care, preferentially within the lesions, to ensure picking the causal strains.

To determine if *netB* is essential for virulence, it is important to determine the disease producing capability of both *netB* positive strains recovered from healthy chickens, and *netB* negative strains recovered from chickens suffering from necrotic enteritis. In CHAPTER 3.1, strain 56 and strain 61 were able to induce necrotic enteritis lesions in 48.15% and 55.56% of infected chickens, respectively. They both carry the *netB* gene. Strain 48 was able to induce disease in 11.11% of the animals while it is a *netB* negative strain. However, the genotype of

the isolates recovered from lesions was not compared with the genotype of the test strain. Therefore, it can not be ruled out that the small amount of lesions is caused by an environmentally derived organism, especially since the chickens in this experimental model are exposed to a lot of predisposing factors (Gholamiandehkordi et al., 2007) and are thus more prone to developing necrotic enteritis. Strain 23, the only *netB* positive strain isolated from healthy broilers used in CHAPTER 3.2 was tested in our *in vivo* model and it was able to induce lesions in 36% infected broiler chickens. Moreover, a necrotic enteritis isolate without the *netB* gene and a *C. perfringens* isolate from a healthy broiler with the *netB* gene but with a premature stop codon were also tested in our *in vivo* model. Both strains were unable to induce necrotic enteritis lesions in the infected animals (own unpublished data). These results support the hypothesis that NetB plays a central role in necrotic enteritis. A recent unpublished study, using a large number of NetB positive and negative *C. perfringens* strains to inoculate broiler chickens, showed a perfect match between necrotic enteritis induction and the ability to produce NetB (Keyburn et al., personal communication).

1.2. GROWTH-INHIBITION

1.2.1. BACTERIOCIN OR ANTIBIOTIC?

The peptide identified in CHAPTER 3.3 is a bacteriocin and not an antibiotic. Indeed, bacteriocins are often confused in the literature with antibiotics. All classes of bacteriocins are ribosomally synthesized and are encoded by one structural gene. Only class I bacteriocins, the lantibiotics, are post-translationally modified to produce the active form. In contrast, antibiotics are generally considered secondary metabolites and they are not ribosomally synthesized. Antibiotics may be composed of amino acids but they are enzymatically synthesized. Peptide antimicrobials are nonribosomally synthesized by the multiple-carrier thiotemplate mechanism. In this template-driven assembly, peptide synthetases assemble amino acids to form the antibiotic molecule (Hancock and Chapple, 1999). Generally, antibiotics are considered to have a wide spectrum while most bacteriocins have a narrow spectrum by killing closely related bacteria. Also the mode of action distinguishes bacteriocins from antibiotics. Bacteriocins act mostly by pore formation while most antibiotics inhibit RNA, DNA or protein synthesis and cell wall synthesis. Antibiotic resistance is usually associated with a genetic determinant, facilitating the transfer of

resistance between cells, strains and species. Unlike most antibiotic resistance, bacteriocin resistance usually results from a physiological change in the target cell membrane (Cleveland et al., 2001; Eijsink et al., 2002; Joerger, 2003; Gillor et al., 2008). The main differences between bacteriocins and antibiotics are summarized in Table I.

Based on these differences, it can be concluded that the antimicrobial peptide isolated in CHAPTER 3.3 is a bacteriocin. Indeed, the purified antimicrobial factor is produced by a bacterium, it is a peptide, has a narrow spectrum, is not toxic to the producer strain, and sequence analysis suggest that the peptide acts by pore formation. Since it is heat labile, it can be classified as a class III bacteriocin although it is not a large peptide.

Table I: Bacteriocins versus antibiotics (Adapted from Cleveland et al., 2001)

Characteristic	**Bacteriocins**	**Antibiotics**
Producer organism	Bacteria	Microorganisms including fungi and protozoa
Synthesis	Ribosomal	Secondary metabolite
Activity	Narrow spectrum	Varying spectrum
Mode of action	Mostly pore formation, sometimes interference with cell wall biosynthesis	Cell membrane or intracellular targets
Interaction requirements	Sometimes docking molecules	Specific target
Mechanism of target cell resistance or tolerance	Usually adaptation affecting cell membrane composition	Usually genetically transferable determinant affecting different sites depending on the mode of action

1.2.2. SIGNIFICANCE IN PATHOGENESIS

In natural cases of necrotic enteritis, mostly one clone of *C. perfringens* is found in the gut of the animals while in healthy birds different clones are present (Nauerby et al., 2003; Engström et al., 2003; Gholamiandehkordi et al., 2006). The fact that this single strain dominance is described in several studies indicates that it is an essential step in the pathogenesis of necrotic enteritis. Moreover, this dominance of one virulent *C. perfringens* isolate could be reproduced in an experimental infection model after administration of a combined inoculum comprising

different necrotic enteritis strains or a combination of virulent and avirulent *C. perfringens* strains (Barbara et al., 2008). Finally, the secretion of factors inhibiting growth of other *C. perfringens* strains is shown to be more prevalent in outbreak strains than in normal microbiota strains *in vitro* (CHAPTER 3.2.; Barbara et al., 2008). Taken together, these results suggest that this is a trait associated with virulence. Although there is no information on *in vivo* bacteriocin production by *C. perfringens*, production of bacteriocins by a virulent strain in the gut would allow this strain to displace the *C. perfringens* strains present. Inhibiting the growth of other *C. perfringens* strains in the gut is also important as part of the competition for nutrients. Indeed, *C. perfringens* is not able to produce 13 amino acids and is thus dependent on environmental presence of these molecules.

It is pertinent to note that there are also examples of other antimicrobial factors of Gram-positive bacteria that are associated with virulence. In persistent endodontic infections where *Enterococcus faecalis* is dominant, a low number of other species is found. It is therefore suggested that AS-48 and other bacteriocincs of *E. faecalis*, although they are not strictly acting as virulence factors, contribute to its dominance in persistent endodontic infections and may serve as a means to obtain ecological advantages which can result in disease (Kayaoglu and Ørstavik, 2004).

2. NEW INSIGHTS IN PATHOGENESIS, A PRESUMED MODEL

After Keyburn et al. (2006) showed that alpha toxin is not an essential virulence factor in necrotic enteritis in broiler chickens, new *C. perfringens* virulence factors have been proposed and new insight was gained into the mechanisms by which *Eimeria* predispose to *Clostridium perfringens* induced necrotic enteritis. The current knowledge is summarized in Figure 1.

In short, *Eimeria* parasites induce leakage of plasma proteins by killing epithelial cells as a consequence of the intracellular stages of their life cycle. Moreover, according to Collier et al. (2008), a coccidial infection enhances mucus production in the intestine. Both effects provide an increase in available nutrients and create an environment favorable for proliferation of *C. perfringens*. Indeed, *C. perfringens* lacks the machinery to produce 13 amino acids but is able to utilize mucus as a substrate. Finally, Park et al. (2008) suggested that the exacerbated pathological findings after co-infection with *Eimeria* and *C. perfringens* are caused by an altered cytokine response. Taken together, these data show that an *Eimeria* infection aids in

the first stage of pathogenesis: massive proliferation of *C. perfringens* in the gut. The factors involved in the second stage of pathogenesis, possibly are bacteriocins. A significantly higher proportion of virulent *C. perfringens* strains produce bacteriocins compared to normal microbiota *C. perfringens* strains (CHAPTER 3.2). By inhibiting other *C. perfringens* strains, the virulent strain is able to obtain the maximum benefit of the increased nutrient availability due to the *Eimeria* infection. The virulent strain is thus able to proliferate massively and as a consequence it secretes virulence factors playing a role in the final step in pathogenesis: the induction of necrotic lesions. Olkowski et al. (2006; 2008) suggested that the initial pathological changes are due to the activity of collagenolytic enzymes. The nature of the morphological changes seen in the early stages of necrotic enteritis is consistent with the action of collagenolytic enzymes. Indeed, the pathological changes start at the basal and lateral domains of enterocytes and then invade progressively the entire lamina propria. Both host collagenases and collagenolytic enzymes secreted by proliferating pathogens may play a role. It was shown that virulent *C. perfringens* strains are able to secrete collagenolytic enzymes and high levels of different collagenolytic enzymes were found in intestinal tissue of broilers challenged with *C. perfringens*, in comparison to controls (Olkowski et al., 2008). Furthermore, it is shown that the NetB toxin is crucial for the induction of necrotic enteritis (Keyburn et al., 2008). NetB forms pores in a chicken epithelial cell line *in vitro*. Its activity *in vivo* is not shown yet, but it may induce necrotic lesions by forming pores in enterocytes leading to cell death. By inducing necrotic enteritis lesions, more nutrients are leaking into the gut lumen which again can be used by *C. perfringens* strains.

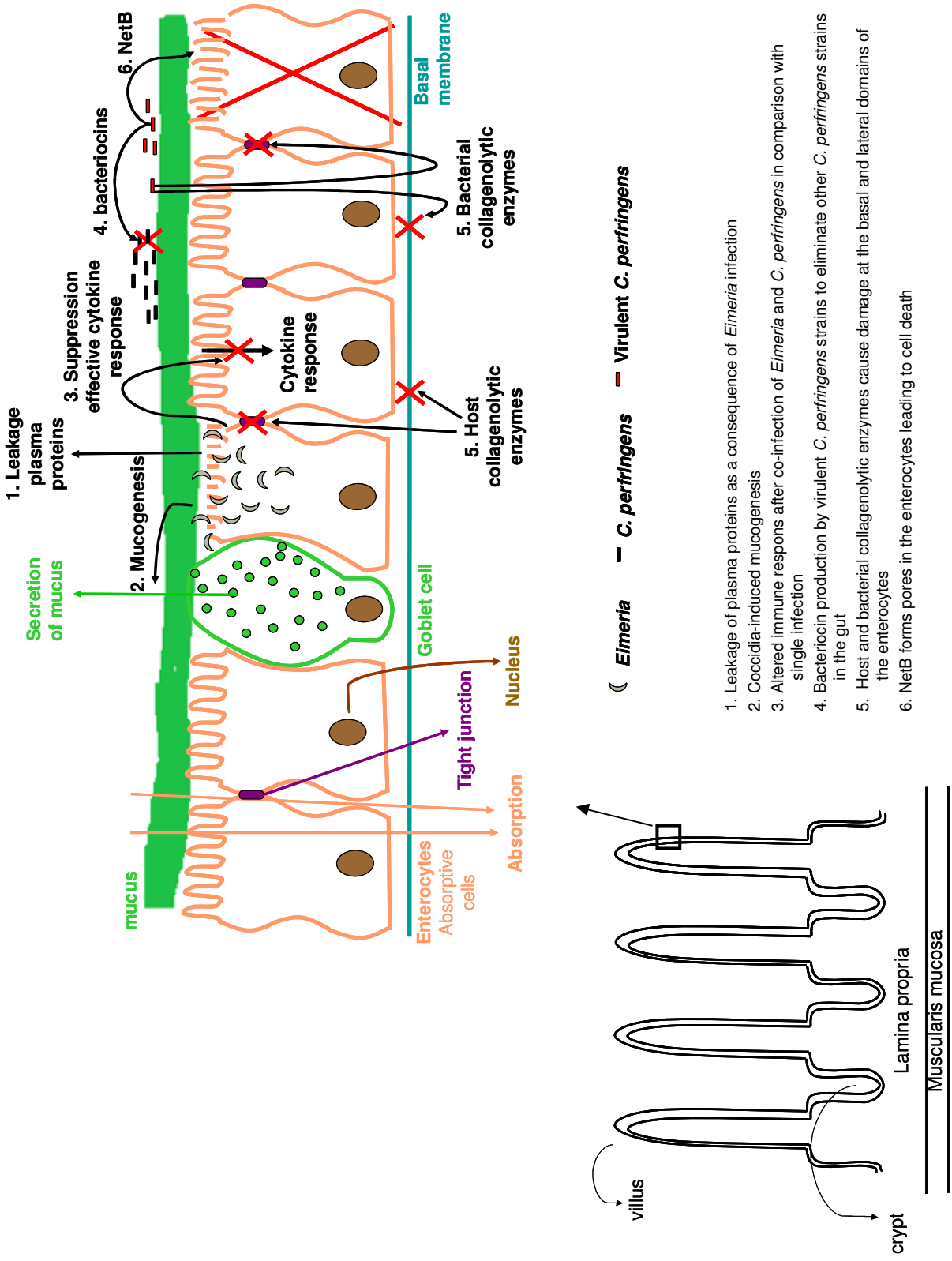

Figure 1: Summary of the pathogenesis of *C. perfringens* induced necrotic enteritis

3. IMPLICATIONS: POSSIBLE IMPROVEMENT OF CONTROL MEASURES

Control of necrotic enteritis has commonly been accomplished by administration of antimicrobials in feed or water. However, concerns are rising about effects on human health of such routine use of antimicrobials because of the emergence of antibiotic resistance. Therefore, the European Union banned the use of antibiotics as antimicrobial growth promoters in animal production (Van Immerseel et al., 2009). Alternative non-antibiotic approaches to control necrotic enteritis are thus searched for. Vaccination is a widely used preventive measure against many infectious diseases in mammals and poultry (Dahiya et al., 2006). Because bacteriocins are commonly found in food and thus have been consumed for centuries, they are considered natural and nontoxic to eukaryotic cells, in contrast to traditional antibiotics (Cleveland et al., 2001; Lin, 2009).

3.1. ALPHA TOXIN AS VACCINE

Despite the fact that it has been shown that alpha toxin is not essential for the development of necrotic enteritis (Keyburn et al., 2006), specific antibodies against alpha toxin protect birds from disease. Vaccines using recombinant alpha toxin in toxoid and active form and live delivery of the C-terminal (non-toxic) domain of alpha toxin by an attenuated *Salmonella enterica* serovar Typhimurium strain have been tested and shown to give partial protection against necrotic enteritis in experimental challenge models (Kulkarni et al., 2007; Zekarias et al., 2008; Cooper et al., 2009). Initially, it was not clear how antibodies against alpha toxin could offer any level of protection if alpha toxin did not play a part in the pathogenesis. However, Zekerias *et al.* (2008) found that serum antibodies, from broilers that had been vaccinated, bound directly to the cell surface of *C. perfringens* and suppressed its growth *in vitro*. Antibodies binding to the membrane-bound preprotein might block protein transport channels and as a consequence, inhibit proliferation of the bacteria (Zekarias et al., 2008). Alpha toxin can thus be used as a protective antigen to vaccinate broilers, but this does not necessarily mean that alpha toxin plays a primary role in the pathogenesis of necrotic enteritis.

3.2. OTHER ANTIGENS AS VACCINE

Thompson *et al.* (2006) have used alpha toxin deficient mutants to immunize broilers orally and found that these strains protected birds from experimental necrotic enteritis. These results clearly demonstrate that antigens other that alpha toxin can provide immunity against necrotic enteritis. Subsequently, Kulkarni et al. (2006) identified six proteins that reacted to serum antibodies from birds immune to necrotic enteritis in the supernatant of virulent *C. perfringens* strains and not in the supernatant of avirulent *C. perfringens* strains. These proteins are a hypothetical protein (HP, possibly a zinc-binding protease); pyruvate:ferredoxin oxidoreductase (PFOR); Elongation factor G (EF-G); Perfringolysin O; glyceraldehyde-3-phosphate dehydrogenase (GAPDA) and fructose 1,6-biphosphate aldolase (FBA). Recombinant HP, PFOR, GAPDA and FBA were tested as vaccine and all proteins protected broiler chickens against a mild challenge while immunization with HP and PFOR also offered protection against a severe challenge (Kulkarni et al., 2007). Broilers orally immunized with an avirulent *Salmonella enterica* serovar Typhimurium vaccine vector expressing FBA or HP proteins were also significantly protected against challenge (Kulkarni et al., 2008). Also other proteins that have been shown to play a role in the pathogenesis of necrotic enteritis might be good candidates as vaccine antigens. These include the metalloproteinases that are suggested to have a role in early lesion development and the NetB protein (Olkowski et al., 2008; Keyburn et al., 2008). Candidate protective antigens have thus been identified. This does not mean that a solution to the necrotic enteritis problem is found, but the results are promising for an answer in the near future.

3.3. GMO PROBIOTICS EXPRESSING BACTERIOCINS

The use of bacteriocins active against *C. perfringens* could also be a good alternative for antibiotic control of necrotic enteritis. Bacteriocins of *C. perfringens* are naturally present in the intestine, and can be considered as safe. There are two possible ways to deliver the bacteriocins in the intestinal tract: by the administration of bacteriocin-producing bacteria or by the administration of purified bacteriocins (Kirkup, 2006). The purified bacteriocins can be included in the feed. However, sensitivity of bacteriocins to gastric acidity and the presence of proteases in the gastrointestinal tract can potentially result in loss of activity when the

bacteriocins are orally administered. An additional difficulty is the challenge of bulk production of purified bacteriocins.

To circumvent the loss of activity, the genes for bacteriocin production can be cloned and expressed in an appropriate bacterial carrier. This approach not only prevents *in vivo* degradation of the bacteriocin during gastric transit, but it also facilitates continued bacteriocin production at the site of infection. It is important that the bacterial carrier is able to colonize the gut and is not pathogenic to the host. Probiotic strains can be used as bacterial carrier. Probiotics are 'live microorganisms, which, when consumed in adequate amounts, confer a health benefit on the host'. The potential of genetically modified bacteriocin-producing bacteria has already been shown. An avian *E. coli* strain genetically engineered to produce the bacteriocin microcin 24 lowered intestinal *Salmonella* Typhimurium counts in chickens when administered continuously in the water supply (Wooley et al., 1999).

As with any antimicrobial compound, also for bacteriocins it is important to consider the issue of resistance. Although the mechanism of action is not known for all bacteriocins, most of the bacteriocins appear to interact with the bacterial membrane. Therefore, resistance is usually the result of changes in the membrane of bacteria targeted by a bacteriocin (Joerger, 2003). However, in the producer strains, bacteriocins are accompanied by specific immunity proteins that can theoretically be transferred horizontally to pathogens (Kirkup, 2006).

If bacteriocins are going to be used as a strategy for the prevention of necrotic enteritis or other infectious diseases, there are some important requirements, such as stability, lack of toxicity, a specific activity spectrum, lack of development of resistance, and a thorough understanding of their biochemical and genetic properties. A detailed knowledge of the isolated bacteriocin structures and properties is therefore needed. The same goes for the carrier strain.

4. CONCLUSION

The past decade, great progress has been made in understanding broiler necrotic enteritis. It is shown that the single strain dominance found in natural cases of necrotic enteritis is likely to be due to bacteriocin production by *C. perfringens* strains. The assumption that alpha toxin was the major virulence factor in necrotic enteritis is refuted and a novel toxin, NetB, is now associated with necrotic enteritis. Proteolytic enzymes are suggested to have a role in the early

stages of pathogenesis. This insight in the etiology and the pathogenesis of *C. perfringens* infection in the gut provides important information that will be valuable to develop effective disease control strategies.

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Summary

SUMMARY

Necrotic enteritis was first described in 1961. It is a common enteric disease, caused by *Clostridium perfringens* type A. However, *C. perfringens* type A can also be present in the intestine of healthy broilers. Necrotic enteritis is characterized clinically by a sudden increase in flock mortality, often without premonitory signs. Also a sub-clinical form of necrotic enteritis is described. At necropsy, necrotic foci are found in the small intestinal mucosa. The disease usually occurs in broiler chickens at about 4 weeks after hatching and is found in all poultry-growing areas of the world. Despite the clinical and economic importance of necrotic enteritis, the pathogenesis of *C. perfringens* associated disease in poultry is still not fully understood. For a long time, it was believed that alpha toxin was the key virulence factor in necrotic enteritis in broiler chickens caused by *C. perfringens* but, at the start of this thesis, its role in the pathogenesis of necrotic enteritis was called into doubt.

C. perfringens type A is associated with necrotic enteritis but can also be detected in relatively high numbers in the intestinal tract of apparently normal chickens. Consequently, in the first study of this thesis (CHAPTER 3.1), the hypothesis was tested that the necrosis inducing capability would be a characteristic of specific strains. *C. perfringens* isolates from both clinically healthy broilers and broilers suffering from necrotic enteritis were compared for their ability to induce necrotic lesions using an experimental necrotic enteritis model in broiler chickens. High, intermediate and low alpha toxin producing strains were chosen from each isolation source to determine the role of alpha toxin in the pathogenesis of necrotic enteritis. In this experimental model, only the isolates from field outbreaks induced necrotic gut lesions, independent of the amount of alpha toxin produced *in vitro*. It was also shown that alpha toxin producing isolates from calf hemorrhagic enteritis cases were not able to induce necrotic enteritis in poultry. These results suggest that alpha toxin is not important to induce necrotic lesions in broiler chickens. Moreover, the results are indicative of the presence of host specific virulence factors in certain *C. perfringens* strains, isolated from chickens with intestinal necrotic enteritis lesions.

Clinically healthy chickens carry several different *C. perfringens* clones in their intestine. In flocks suffering from necrotic enteritis, however, mostly only one single clone is isolated from the gut of all the diseased animals. Selective proliferation of such clinical outbreak strain in the gut and spread within the flock seems likely. The hypothesis that necrotic enteritis associated *C. perfringens* strains might suppress the growth of normal microbiota *C. perfringens* strains, was therefore tested (CHAPTER 3.2). Twenty-six *C. perfringens* strains isolated from healthy broilers and 24 clinical outbreak isolates were evaluated for their ability to induce intra-species growth-inhibition in an *in vitro* setup. A significantly higher proportion of the *C. perfringens* clinical outbreak strains inhibited the growth of other *C. perfringens* strains compared to *C. perfringens* strains isolated from the gut of healthy chickens. It is proposed that, in addition to toxin production, intra-species growth-inhibition may be a virulence trait that contributes to the ability of certain *C. perfringens* strains to cause necrotic enteritis in broilers. The secretion of factors inhibiting the growth of other *C. perfringens* strains could lead to extensive and selective presence of a strain that contains the genetic make-up enabling to secrete toxins that cause gut lesions.

In CHAPTER 3.3, the purification and the characterization of such a growth-inhibiting factor is described. A bacteriocin-like inhibitory substance (BLIS) was purified from a necrotic enteritis associated *C. perfringens* strain exhibiting antibacterial activity against other *C. perfringens* strains. Amino acid sequence analysis indicated that the BLIS is an 11.5 kDa fragment of a 22 kDa protein and that it constitutes the C-terminal part of this protein. The 22 kDa protein is a cytoplasmic membrane protein without signal peptide. The antibacterial activity of the purified BLIS was abolished by proteolytic enzymes trypsin and proteinase K and by heat treatment (10 min at 80 °C). The purified BLIS showed inhibitory activity over a wide pH range (4.0 to 10.0). Since the antibacterial activity against other *C. perfringens* strains of the purified BLIS had a narrower spectrum than the crude supernatant of the secreting strain, most probably different of these bacteriocins are produced.

In conclusion, this thesis offers new insights in the pathogenesis of *C. perfringens* necrotic enteritis in broiler chickens. It was shown that only a specific subpopulation of *C. perfringens* type A strains are able to induce necrotic enteritis lesions. We suggest that the single clone dominance found in necrotic enteritis is due to the secretion of growth-inhibiting factors, also

called bacteriocins, secreted by these virulent strains. This provides important information that will be valuable to develop effective disease control strategies.

Samenvatting

SAMENVATTING

Necrotische enteritis werd voor het eerst in 1961 beschreven. Het is een vaak voorkomende darmziekte veroorzaakt door *Clostridium perfringens* type A. Hoewel *C. perfringens* dus in staat is om necrotische enteritis te veroorzaken, komt deze bacterie bijna altijd voor in de darm van gezonde vleeskippen. Necrotische enteritis wordt klinisch gekarakteriseerd door een plotse stijging van sterfte in een toom, meestal zonder voorafgaande tekenen. Er is ook een subklinische vorm van necrotische enteritis beschreven. Bij lijkschouwing worden necrotische haarden gevonden in de mucosa van de dunne darm. De ziekte komt meestal voor bij vleeskippen van ongeveer 4 weken oud en komt voor in alle landen waar pluimvee gefokt wordt. Ondanks het klinische en economische belang van necrotische enteritis, is de pathogenese nog niet volledig ontrafeld. Lange tijd werd er gedacht dat alfa toxine de belangrijkste virulentiefactor was van *C. perfringens* die een rol speelt in necrotische enteritis. Bij het begin van deze thesis werd de rol van alfa toxine in de pathogenese van necrotische enteritis echter in twijfel getrokken.

C. perfringens type A wordt geassocieerd met necrotische enteritis maar kan ook in relatief hoge aantallen teruggevonden worden in de darm van gezonde vleeskippen. Daarom werd in het eerste hoofdstuk van deze thesis (CHAPTER 3.1), de hypothese getoetst dat necrotische enteritis zou veroorzaakt worden door welbepaalde stammen van *C. perfringens*. Hiervoor werden *C. perfringens* isolaten van zowel klinisch gezonde vleeskippen als van vleeskippen met necrotische enteritis getest op hun capaciteit om necrotische letsels te induceren in een experimenteel necrotisch enteritis model *in vivo*. Om de rol van alfa toxine in de pathogenese na te gaan werden *C. perfringens* isolaten uit kippen met necrotische enteritis en isolaten uit klinisch gezonde kippen gekozen die *in vitro* hoge, gemiddelde en lage concentraties alfa toxine produceerden. Enkel de isolaten geïsoleerd uit kippen met necrotische enteritis konden necrotische letsels induceren, ongeacht de concentratie alfa toxine die ze produceerden *in vitro*. Er werd ook aangetoond dat alfa toxine producerende isolaten van gevallen van hemorragische enteritis bij het kalf niet in staat waren om necrotische enteritis in vleeskippen te induceren. Deze resultaten suggereren dat alfa toxine niet belangrijk is om necrotische

letsels te induceren bij vleeskippen. Bovendien doen deze resultaten vermoeden dat de *C. perfringens* stammen geïsoleerd uit kippen met necrotische letsels, over gastheer specifieke virulentiefactoren beschikken.

Klinisch gezonde vleeskippen hebben verschillende *C. perfringens* klonen in hun darm terwijl in een toom die necrotische enteritis doormaakt, meestal één en dezelfde kloon geïsoleerd wordt uit de darm van verschillende vleeskippen. Selectieve proliferatie in de darm van één stam en verspreiding in de toom lijkt waarschijnlijk. De hypothese dat necrotische enteritis geassocieerde *C. perfringens* stammen de groei van *C. perfringens* stammen uit de normale flora inhiberen, werd daarom getest (CHAPTER 3.2). Bij 26 *C. perfringens* isolaten uit gezonde vleeskippen en 24 isolaten uit klinische uitbraken werd nagegaan in hoeverre ze over de eigenschap beschikken om elkaars groei te inhiberen *in vitro*. Significant meer *C. perfringens* stammen uit klinische uitbraken inhibeerden de groei van de andere *C. perfringens* stammen in vergelijking met *C. perfringens* stammen geïsoleerd uit de darm van gezonde dieren. Naast toxine productie zou groei-inhibitie een virulentie eigenschap kunnen zijn die bijdraagt tot de eigenschap van bepaalde *C. perfringens* stammen om necrotische enteritis te induceren in vleeskippen. De secretie van factoren die de groei inhiberen van andere *C. perfringens* stammen kan leiden tot proliferatie en selectieve aanwezigheid van een stam die de genetische eigenschappen heeft om toxines uit te scheiden die darmletsels veroorzaken.

In het laatste hoofdstuk (CHAPTER 3.3) wordt de opzuivering en karakterisatie van een groei-inhiberende factor beschreven. De bacteriocine-achtige inhibitorische factor werd opgezuiverd uit het supernatans van een *C. perfringens* stam geïsoleerd uit een kip met necrotische enteritis. Deze stam vertoonde antibacteriële activiteit tegen andere *C. perfringens* stammen. Aminozuur sequentie analyse toonde aan dat de factor een 11.5 kDa groot fragment van een proteïne van 22 kDa en dat de factor het C-terminale deel van dit proteïne is. Het 22 kDa proteïne is een cytoplasmatisch membraan proteïne zonder signaal sequentie. Na incubatie met de proteolytische enzymen trypsine en proteinase K of na hittebehandeling (10 min, 80 °C) werd geen antibacteriële activiteit meer waargenomen. De inhibitorische activiteit was stabiel bij een brede pH range (4.00 tot 10.00). Aangezien de intra-species antibacteriële activiteit van de opgezuiverde bacteriocine-achtige inhibitorische factor een

nauwer spectrum had dan het ruwe supernatans van de producerende stam, kan verondersteld worden dat deze stam meerdere dergelijke bacteriocine-achtige factoren aanmaakt.

De resultaten van deze thesis hebben geleid tot een beter inzicht in de pathogenese van *C. perfringens* geïnduceerde necrotische enteritis bij vleeskippen. Er werd aangetoond dat slechts een subpopulatie van *C. perfringens* type A in staat is necrotische letsels te induceren. Onze resultaten tonen voorts aan dat het feit dat slechts één kloon teruggevonden wordt bij vleeskippen met necrotische enteritis waarschijnlijk te wijten is aan de secretie van groei-inhiberende factoren, ook bacteriocines genoemd, gesecreteerd door *C. perfringens* type A stammen die pathogeen zijn voor de kip. Dit levert belangrijke informatie die waardevol zal zijn om meer efficiënte bestrijdingsstrategieën te ontwikkelen.

Curriculum vitae

CURRICULUM VITAE

Leen Timbermont werd geboren op 10 november 1981 te Dendermonde. Na het beëindigen van haar studies algemeen secundair onderwijs, richting Latijn-Wiskunde, aan het Koninklijke Atheneum Dendermonde begon ze in 1999 aan de studies Bio-ingenieur aan de Vrije Universiteit Brussel. In 2004 behaalde zij met onderscheiding het diploma Bio-ingenieur optie cel- en genbiotechnologie.

Daarna trad zij in dienst bij de vakgroep Pathologie, Bacteriologie en Pluimveeziekten voor onderzoek naar het gedrag van *Salmonella* Enteritidis in leghennen en eieren. In 2005 startte ze haar doctoraatsonderzoek aan dezelfde vakgroep met Prof. Dr. F. Van Immerseel en Prof. Dr. R. Ducatelle als promotoren. Gedurende vier jaar werkte zij aan het onderzoeksproject: "Studie van pathogenese en bestrijding van *Clostridium perfringens* bij pluimvee' dat gefinancierd werd door het IWT, afdeling landbouwkundig onderzoek.

In 2007 behaalde zij aan de Faculteit Psychologie en Pedagogische Wetenschappen (UGent) het diploma van de Academische Initiële Lerarenopleiding (AILO) en in 2008 voltooide zij de doctoraatsopleiding in de Diergeneeskundige Wetenschappen.

Leen Timbermont is auteur of medeauteur van meerdere wetenschappelijke publicaties in nationale en internationale tijdschriften. Zij nam deel aan verschillende congressen en presenteerde de resultaten van haar onderzoek in de vorm van voordrachten en posters.

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