A toxic approach to beta2-toxigenic *Clostridium perfringens*

Een toxische benadering van beta2-toxigene *Clostridium perfringens* (met een samenvatting in het Nederlands)

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Proefschrift

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Chapter 1

Introduction

Clostridium perfringens

Clostridium perfringens is a gram-positive oxygen-tolerant anaerobic bacterium. Although widely present in the environment and in the intestine of healthy humans and animals, *C. perfringens* is one of the most important causes of intestinal disease (Songer, 1996). The bacterium exists in two different forms: a vegetative form and a spore form. Spore formation occurs under nutritionally deprived conditions. The heat resistant spores of *C. perfringens* can survive in the environment for years (Mueller-Spitz et al., 2010). After uptake by a suitable host the spores germinate and return into active growth (Paredes-Sabja et al., 2008). Transmission from animals to humans is possible, especially via food which is contaminated with large numbers of *C. perfringens* (Songer, 2010). The production of toxins is essential for the development of disease. A wide range of exotoxins has been described for *C. perfringens* but the role of the different toxins in the development of disease is still under investigation (Petit et al., 1999; Songer, 1996). The genes encoding the different toxins are located either on the chromosome or extrachromosomally on plasmids which can be transferred from one strain to another (Brynestad et al., 2001; Songer, 1996). The bacterium is classified into five toxin types (A-E), based on the four major toxins it can produce*:* alpha, beta, epsilon, and iota toxin (Songer, 1996). The gene encoding for the alpha toxin, *cpa,* is present in all *C. perfringens* strains (Songer, 1996).

The toxins of *Clostridium perfringens*

The alpha toxin is a phospholipase C, which hydrolyses membrane phospholipids resulting in cell lysis (Sakurai et al., 2004; Titball et al., 1999). The role of the toxin in the development of intestinal disease remains unclear (Keyburn et al., 2006; Petit et al., 1999; Titball et al., 1999). There is a relationship between the administration of alpha toxin or the presence of alpha toxin in the intestine and intestinal lesions (Al-Sheikly and Truscott, 1977a; Al-Sheikly and Truscott, 1977b; Coursodon et al., 2010; Fukata et al., 1988; McCourt et al., 2005; Truscott and Al-Sheikly, 1977). On the other hand, no relationship between the amount of alpha toxin produced by *C. perfringens* strains type A *in vitro* and the ability of these strains to cause intestinal disease was identified (Timbermont et al., 2009). Moreover, a *cpa*-deficient *C. perfringens* mutant, constructed from a virulent chicken isolate, showed full virulence in a chicken disease model (Keyburn et al., 2006). Beside the alpha toxin other virulence factors seem to

play an important role in the development of intestinal disease caused by *C. perfringens* type A strains (Coursodon et al., 2010; Keyburn et al., 2006; Keyburn et al., 2008; Timbermont et al., 2009).

The beta toxin, encoded by *cpb,* forms cation-selective pores in bilayer lipid membranes resulting in cell swelling and lysis (Nagahama et al., 2003; Shatursky et al., 2000, Tweten, 2001). The toxin binds to intestinal vascular endothelial cells causing hemorrhagic necrosis in the intestine (Miclard et al., 2009; Vidal et al., 2008). The gene which encodes for the beta toxin is present in both type B and type C strains (Smedley et al., 2004). Type B strains are associated with lamb dysentery and hemorrhagic enteritis in lambs, calves, foals, and adult sheep. The presence of type C strains is associated with necrotic or hemorrhagic enteritis in lambs, piglets, fowls, goats, adult sheep, and humans (Songer, 1996).

The epsilon toxin, encoded by *etx*, crosses the mucosal barrier and moves through the bloodstream to target organs (Smedley et al., 2004). After binding to the cell membrane the toxin forms large protein complexes with membrane components which cause large pores in the cell membrane resulting in cell swelling and cell lysis (Petit et al., 1997; Petit et al., 2001). In the heart, lung, liver, and kidney the formation of pores in endothelial cells causes an increase in blood vessel permeability resulting in oedema and haemorrhage (Petit et al., 2003). The toxin can cross the blood-brain barrier and damage cerebral blood vessels and adjacent cells in the neuropil (Miyamoto et al., 1998; Petit et al., 2001; Soler-Jover et al., 2007). The epsilon toxin is present in type B and D strains. Type D strains are associated with enterotoxaemia in lambs and calves and enterocolitis in neonatal and adult goats (Songer, 1996).

The iota toxin is a binary toxin composed of an enzyme component (Ia encoded by *iap*) and a binding component (Ib, encoded by *ibp*). Ib binds to the cell membrane and facilitates the entry of the enzyme component into the cytosol of the cell. Ia ADP-ribosylates actin, resulting in cell rounding and cell death (Nagahama et al., 2004; Sakurai et al., 2009; Stiles et al., 2000). The gene encoding the iota toxin is present in type E strains, which are associated with enteritis in rabbits (Songer, 1996).

In addition to the four major toxins, all types of *C. perfringens* can carry several other toxin genes, including *cpe,* the gene which encodes the enterotoxin (Songer, 1996), *netB,* the gene which encodes the NetB toxin (Keyburn et al., 2008), and *cpb2*, the gene which encodes the beta2 toxin (Gibert et al., 1997).

The enterotoxin binds to enterocytes and forms a series of protein complexes in plasma membranes resulting in membrane permeability alterations and cell lysis. Tight junction components of the enterocytes are included in the protein complexes which causes paracellular permeability changes (Singh et al., 2000; Sonoda et al., 1999; Veshnyakova et al., 2010). The enterotoxin plays a role in the development of intestinal disease in several species including humans (McClane, 1996; Songer, 1996).

The NetB toxin is a pore-forming toxin which causes cell lysis of intestinal cells and herewith necrotic enteritis (Keyburn et al., 2008). The gene encoding the toxin is present in a subset of avian *C. perfringens* type A strains (Keyburn et al., 2008). The NetB toxin seems to be an important virulence factor in the development of necrotic enteritis in chickens (Keyburn et al., 2008).

The beta2 toxin

The 28 kDa protein has been isolated and purified from a *C. perfringens* type C strain of a piglet with neonatal, haemorrhagic, necrotic enteritis (Gibert et al., 1997). The beta2 toxin is encoded by *cpb2*. Two allelic subpopulations have been demonstrated which are called the consensus *cpb2* gene and the atypical *cpb2* gene (Fisher et al., 2005; Jost et al., 2005; Lebrun et al., 2007). Up to now all known *cpb2* sequences can be assigned to one of these two gene variants. The variants have a sequence-identity of only 70.2–70.7% resulting in a 62.3% identity and an 80.4% similarity at amino-acid level (Jost et al., 2005). The atypical gene product reacts in Western blot analysis with a polyclonal antiserum and with a monoclonal antibody both raised against the consensus gene product (Jost et al., 2005; Lebrun et al., 2007).

Differences in expression rate have been found between the two variants. In a study among field isolates only the consensus *cpb2* gene was translated *in vitro* (Jost et al., 2005). Another field study among enterotoxaemic calves showed no differences between the *in vitro* expression rate of the consensus *cpb2* gene and the atypical *cpb2* gene. However, only the translation of the consensus *cpb2* gene *in vitro* was related to intestinal disease (Lebrun et al., 2007). A field study among human isolates showed a higher toxicity of the consensus *cpb2* gene product compared to the atypical *cpb2* gene product (Fisher et al., 2005).

The mode of action and the exact role of the beta2 toxin in the development of intestinal disease are still unknown (Smedley et al., 2004). The

amino acid sequence of the beta2 toxin has no significant homologies with other known proteins. The toxin is lethal to mice after i.v. injection of 3 µg of toxin (Gibert et al., 1997). *In vitro* studies show a cytotoxic effect on several cell lines. The toxin is cytotoxic to CHO cells, I407 cells, and Caco-2 cells as determined by cell rounding, membrane bleb formation, nuclei condensation, and detachment of cells shown by phase contrast microscopy (Fisher et al., 2005; Gibert et al., 1997; Smedley et al., 2004). The toxin causes a decreased viability of CHO cells and I407 cells resulting from a decrease in mitotic cell division as shown by lower thymidine incorporation (Gibert et al., 1997). A decreased viability was observed performing a neutral red viability assay as a declined incorporation and binding of the supravital dye neutral red in the lysosomes of the Caco-2 cells (Fisher et al., 2005). Staining of the actin cytoskeleton did not reveal any changes. In the guinea-pig ligated intestinal loop test the beta2 toxin induced hemorrhagic necrosis of the intestinal mucosa. The cleavage of the protein by trypsin into two polypeptides (13 and 15 kDa) results in a complete loss of biological activity (Gibert et al., 1997).

The prevalence of *cpb2*

Cpb2 is located on plasmids, which suggests the potential for transfer of the gene among different *C. perfringens* strains. *Cpb2* has been demonstrated in *C. perfringens* isolates originating from humans, sheep, cows and calves, chicken and other birds, horses, goats, dogs, pigs, an African elephant, a baboon, felidae, camelids, and in *C. perfringens* strains isolated from the environment (Bacciarini et al., 2001; Bacciarini et al., 2003; Bueschel et al., 2003; Crespo et al., 2007; Dray, 2004; Fisher et al., 2005; Garmory et al., 2000; Gibert et al., 1997; Herholz et al., 1999; Jost et al., 2005; Klaasen et al., 1999; Lebrun et al., 2007; Manteca et al., 2002; Mueller-Spitz et al., 2010; Nikolaou et al., 2008; Siragusa et al., 2006; Thiede et al., 2001; Waters et al., 2003). Both *cpb2*-positive and *cpb2*-negative strains can be present within the intestine of a single healthy or diseased animal (Dray, 2004; Manteca et al., 2002). The consensus *cpb2* variant is mainly present in *C. perfringens* strains isolated from pigs, while the atypical *cpb2* variant is almost exclusively present in non-porcine strains (Jost et al., 2005).

Transcription and translation of *cpb2*

The presence of *cpb2* does not necessarily mean that the beta2 toxin is produced. Several studies showed a high percentage of frameshift mutations in field isolates (Jost et al., 2005; Vilei et al., 2005) and a variation among strains in their ability to produce the beta2 toxin *in vitro* (Bueschel et al., 2003; Fisher et al., 2005; Jost et al., 2005; Lebrun et al., 2007). In two different studies a 30-fold difference in the amount of transcripts was found between strains producing a high level of beta2 toxin and strains producing a low level of beta2 toxin (Harrison et al., 2005; Waters et al., 2005). The levels of *cpb2* mRNA were positively correlated to the production of the beta2 toxin, which suggests that the transcription of *cpb2* is immediately followed by translation of *cpb2* mRNA without regulation on translational level (Harrison et al., 2005; Waters et al., 2005). No differences in promoter region or *cpb2* gene sequence could be determined which could explain the difference in transcription rates of *cpb2*. As long as the underlying causes of this variation have not been clarified, no prediction of the ability of *cpb2*-harbouring *C. perfringens* strains to produce the beta2 toxin can be made without any further phenotyping by Western blot analysis.

The transcription of *cpb2* is positively regulated by a two-component system, the VirR/VirS system which consists of two genes *virR* and *virS* (Ohtani et al., 2003). The two genes form an operon resulting in a simultaneous translation of both genes (Ba-Thein et al., 1996). *VirS* encodes a transmembrane protein which acts as a receptor protein. *VirR* encodes a protein which acts as a respons regulator (Rood and Lyrisitis, 1995). Little is known about environmental circumstances which influence the *in vivo* toxin production by *C. perfringens* via the VirR/VirS system. Contact with enterocyte-like Caco-2 cells causes a rapid upregulation of beta2 toxin production by *C. perfringens* type C strains (Vidal et al., 2009). The upregulating effect was again blocked by inactivation of *virR*, confirming external regulation of toxin production mediated by the VirR/VirS system (Vidal et al., 2009). This result suggests that the level of *in vivo* production of the beta2 toxin may be different from the *in vitro* production of the beta2 toxin by *cpb2*-harbouring *C. perfringens* strains. A lack of correlation between *in vitro* and *in vivo* beta2 toxin production makes it difficult to predict the *in vivo* beta2 toxin production by *cpb2*-harbouring *C. perfringens* strains by means of *in vitro* studies.

Sampling, genotyping and phenotyping of *cpb2*-harbouring *C. perfringens* strains

Since *cpb2* was detected for the first time a wide range of sampling, genotyping, and phenotyping methods have been developed to demonstrate the presence of *cpb2*-harbouring *C. perfringens* strains in the intestine and the ability of these strains to produce the beta2 toxin. For the estimation of the prevalence of *cpb2*-harbouring *C. perfringens* strains among humans and animals, bacteriological culturing followed by polymerase chain reaction in the genome of isolates is the most frequently used diagnostic method. In some prevalence studies only a single isolate of each animal was tested for the presence of *cpb2* (Bueschel et al., 2003; Crespo et al., 2007; Garmory et al., 2000; Jost et al., 2005; Siragusa et al., 2006; Thiede et al., 2001), while in other prevalence studies more than one *C. perfringens* isolate per animal was tested for the presence of *cpb2* (Dray et al., 2004; Herholz et al., 1999; Klaasen et al., 1999; Lebrun et al., 2007; Manteca et al., 2002). In early studies only one primer set was used for the detection of *cpb2* (Dray et al., 2004; Garmory et al., 2000; Herholz et al., 1999; Klaasen et al., 1999; Manteca et al., 2002; Thiede et al., 2001; Waters et al., 2003). Since the identification of two different allelic subpopulations of *cpb2* most studies use two different primer sets or combine two different forward primers with a common reverse primer to demonstrate the presence of both *cpb2* alleles (Fisher et al., 2005; Jost et al., 2005; Lebrun et al., 2007). PCR amplified DNA carrying *cpb2* can be sequenced to determine whether the *cpb2*-harbouring *C. perfringens* isolates carry an intact open reading frame. However not all isolates carrying an intact open reading frame are able to produce the beta2 toxin *in vitro* as shown by Western blotting (Harrison et al., 2005; Waters et al., 2005). The phenotyping of strains by culturing and Western blotting is carried out in only a part of the field studies. At least some of the *cpb2*-harbouring *C. perfringens* strains which have been found in these field studies were tested for the production of the beta2 toxin *in vitro* (Bueschel et al., 2003; Crespo et al., 2007; Fisher et al., 2005; Jost et al., 2005; Lebrun et al., 2007; Waters et al., 2005). Immunohistochemical staining can be carried out after histopathological examination to determine the presence of the beta2 toxin in the intestine of animals from which *cpb2*-harbouring *C. perfringens* strains have been isolated. Only very few immunohistochemical studies show the presence of the beta2 toxin in the intestine after post-mortem examination (Bacciarini et al., 2001; Bacciarini et al., 2003; Nikolaou et al., 2008; Vilei et al., 2005).

The relationship between *cpb2*-harbouring *C. perfringens* strains and intestinal disease

Although isolated from many different animal species, the relationship between *cpb2*-harbouring *C. perfringens* strains and intestinal disease has only been established in pigs, ruminants, and horses. Most evidence for a relationship between the presence of *cpb2*-harbouring *C. perfringens* strains in the intestine and diarrhoea has been established in pigs (Bueschel et al., 2003; Garmory et al., 2000; Waters et al., 2003). However, a recent study demonstrated that *cpb2* was equally devided between *C. perfringens* strains isolated from diarrhoeic and healthy piglets (Farzan et al., 2013). In two different prevalence studies all surveyed *cpb2*-harbouring *C. perfringens* strains from pigs with gastro-intestinal disease were able to produce the beta2 toxin *in vitro* (Bueschel et al., 2003; Waters et al., 2003).

No association between *cpb2*-harbouring *C. perfringens* type A strains and bovine enterotoxaemia could be found in two studies (Garmory et al., 2000; Manteca et al., 2002). In another study on 8 animals with bovine enterotoxaemia and 14 healthy calves the authors distinguished between atypical and consensus *cpb2*-harbouring *C. perfringens* type A strains and between strains which were able to produce the beta2 toxin *in vitro* and strains which were not able to produce the beta2 toxin *in vitro*. Consensus *cpb2*-harbouring *C. perfringens* type A strains which produce the beta2 toxin *in vitro* were mainly present in calves with enterotoxaemia, while atypical *cpb2*-harbouring *C. perfringens* type A strains which produce the beta2 toxin *in vitro* were only present in healthy cattle. It was hypothesized that only consensus *cpb2*-harbouring type A strains play a role in the development of bovine enterotoxaemia (Lebrun et al., 2007).

A study on the relationship between the presence of *cpb2*-harbouring *C. perfringens* type A and typhlocolitis in horses showed a relationship between the presence of the bacterium and intestinal disease (Herholz et al., 1999). A positive relationship was found between the use of prophylactic gentamycin among horses which were tested positive on the presence of *cpb2*-harbouring *C. perfringens* and lethal progression of the typhlocolitis. The severity of typhlocolitis was significantly reduced after abandoning gentamycin from the standard disease prophylaxis protocol (Herholz et al., 1999; Vilei et al., 2005). Sequencing of *cpb2* of the *cpb2*-harbouring *C. perfringens* strains isolated from these horses revealed a stop codon in the gene. No beta2 toxin was produced

in vitro after culturing the strains for 13 hours, while the addition of gentamycin to the growth culture lead to production of the beta2 toxin (Vilei et al., 2005). The addition of gentamycin to the growth culture causes most likely ribosomal frameshifting and beta2 toxin production. The administration of gentamycin to horses may have caused the onset of beta2 toxin production in the intestinal tract by *cpb2*-harbouring *C. perfringens* strains and hereby a fatal progression of the disease (Vilei et al., 2005).

Cpb2 was abundantly present among *cpe*-positive *C. perfringens* type A isolates from humans with antibiotic-associated diarrhoea or sporadic diarrhoea. Out of 35 *cpb2*-harbouring enterotoxigenic *C. perfringens* type A strains 34 isolates were able to produce the beta2 toxin *in vitro* (Fisher et al., 2005). The presence of *cpb2* was less common among *C. perfringens* strains isolated from humans suffering from food poisoning (Fisher et al., 2005).

Scope and outline

Scope

The presence of *cpb2*-harbouring *C. perfringens* has been verified in many animal species and in some cases a relationship has been established between the presence of the bacterium and intestinal disease, but the exact role of the beta2 toxin in the development of intestinal disease is still unknown. Beside the beta2 toxin itself predisposing factors seem to play an important role in the development of disease (Schotte et al., 2004). The aim of this thesis is to gain insight into the role of the beta2 toxin in the development of clostridial intestinal disease and to study predisposing factors which play a role in the development of intestinal disease related to *cpb2*-harbouring *C. perfringens* strains.

Outline

A reliable method for the detection of *cpb2*-harbouring *C. perfringens* strains in faecal samples is of high importance for prevalence studies on the occurrence of *cpb2*-harbouring *C. perfringens* strains among different animal species. The second chapter describes the development of a new PCR and the comparison of different strategies for the detection of *cpb2*-harbouring *C. perfringens* strains in faecal samples. The aim of this study is to improve the

detection level of *cpb2*-harbouring *C. perfringens* isolates in samples and to simplify the distinction between different subtypes.

The prevalence of *cpb2*-harbouring *C. perfringens* strains and its relationship with intestinal disease has only been established for a limited number of animal species. Chapter three describes a prevalence study on *cpb2*-harbouring *C. perfringens* strains among roe deer. The fourth chapter deals with a field study among commercial layer flocks suffering from subclinical necrotic enteritis whereas bacteriological examination frequently revealed large numbers of *cpb2*-harbouring *C. perfringens*.

Although it is known that the beta2 toxin is cytotoxic for different intestinal cell lines, the exact role of the beta2 toxin in the development of intestinal disease is still not clear. In chapter five the effect of human and porcine beta2 toxin on two different intestinal cell lines is studied.

The normal intestinal microflora plays an important role in the prevention of clostridial intestinal disease. The growth and adherence of *C. perfringens* in the intestine is reduced by the normal intestinal flora by means of competition for nutrients, exclusion by competition in adhesion factors, lowering of the pH and the production of specific antibacterial substances. Nothing is known about the effect of the normal intestinal microflora on the production of toxins by *C. perfringens*. Chapter six describes the effect of the probiotic strain *Lactobacillus fermentum* on beta2 toxin production by *cpb2*-harbouring *C. perfringens* strains.

Besides the normal intestinal flora a wide range of risk factors seem to play a key-role in the occurrence of *C. perfringens*-associated enteritis. Chapter seven gives an overview of predisposing factors which influence the development of intestinal disease due to *C. perfringens*.

Finally, in chapter eight the results of the previous chapters are discussed and summarized.

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Chapter 2

Development and application of a new PCR followed by MboI digestion for the detection of all variants of the *Clostridium perfringens cpb2* gene

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Application of PCR-based detection of *Clostridium perfringens cpb2* in faecal samples
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Abstract

Clostridium perfringens which is a causative agent of several diseases in animals and humans is capable of producing a variety of toxins. Isolates are typed into five types based on the presence of one or more of the four major toxins genes *cpa*, *cpb*, *etx*, and *iap*. A decade ago another toxin termed beta2 (β2) and its gene (*cpb2*) were identified. Two alleles of *cpb2* are known and a possible link between differences in gene expression and allelic variation has been reported. A correlation between the level of expression and the origin of the isolates has also been suggested. The demonstration and typing of the *cpb2* gene in the genome of isolates can be seen as a vital part of research on the role of the beta2 toxin in the pathogenesis of disease. This study describes a PCR with a single primer set which in contrast to published primer sets recognizes both alleles. Subsequent restriction enzyme analysis of the PCR product enables typing of the alleles. Applying this protocol on a total of 102 isolates, a sub-variant was found which occurred only in *C. perfringens* isolates from pigs and appeared to be the predominant variant found in *C. perfringens* isolates from this species.

A comparison was made between three different sampling strategies for testing faecal samples for the presence of *cpb2* by the new developed PCR. Pooling colonies after culturing faecal samples on blood agar plates appeared to be the most efficient and sensitive sampling strategy for the detection of *cpb2*.

Introduction

The Gram-positive anaerobic bacterium *Clostridium perfringens* is part of the normal intestinal flora and is ubiquitous in the environment. However, it is also an important cause of enteric diseases in animals and humans. Type and severity of the disease are correlated with the toxin(s) produced by the bacterium (Songer, 1996). Based on the four major toxins (alpha, beta, epsilon, and iota) the strains of *C. perfringens* are classified into five toxin types: types A–E, by demonstrating the presence of the encoding genes by PCR.

Another toxin produced by a *C. perfringens* strain was described in 1997 (Gibert et al., 1997). This toxin was designated beta2 since its biological activities were comparable to those of the beta toxin. The gene encoding the beta2 toxin was termed *cpb2* and was shown to be located on a plasmid (Gibert et al., 1997). The *cpb2* gene has been demonstrated in isolates originating from a variety of animals, i.e. pigs (Waters et al., 2003; Klaasen et al., 1999), horses (Herholz et al., 1999), ruminants (Bueschel et al., 2003; Dray, 2004; Garmory et al., 2000; Lebrun et al., 2007; Manteca et al., 2002), chickens (Siragusa et al., 2006; Crespo et al., 2007), dogs (Thiede et al., 2001), and from humans (Fisher et al., 2005).

It was shown that 88.5% of the *cpb2*-harbouring non-porcine isolates (n = 78) and 2 out of 76 (2.6%) *cpb2*-harbouring porcine isolates examined carried a variant of *cpb2*. This variant had a sequence-identity of only 70.2–70.7% with the original *cpb2* (GenBank accession number L77965) resulting in a 62.3% identity and a 80.4% similarity at amino-acid level (Jost et al., 2005). The original *cpb2* was termed the consensus gene/allele whereas the variant was termed the atypical gene or atypical allele (Jost et al., 2005; Jost et al., 2006). Up to now all known *cpb2* sequences can be assigned to one of the two variants with some ambiguities within each variant. In order to demonstrate the presence of the atypical or the consensus variant by PCR two different primer combinations are being used (Fisher et al., 2005; Jost et al., 2005; Lebrun et al., 2007). Using BLAST (Basic Local Alignment Search Tool; http://www.ncbi.nlm.nih.gov/BLAST/) none of the published primer combinations recognized all *cpb2* sequences in the Genbank/EMBL/DDBJ databases.

A wide range of sampling and genotyping strategies has been used to establish the presence of *cpb2*-harbouring *C. perfringens* strains in faecal samples. Only a single isolate of each clinical case was tested for the presence

of *cpb2* in a number of these studies (Bueschel et al., 2003; Crespo et al., 2007; Waters et al., 2003; Jost et al., 2005; Siragusa et al., 2006; Thiede et al., 2001). In other studies more isolates per clinical case were tested (Dray, 2004; Herholz et al., 1999; Klaasen et al., 1999; Lebrun et al., 2007; Manteca et al., 2002). It was shown that both *cpb2*-harbouring and *cpb2*-negative strains can be present in one single sample, which demonstrated the limitation of testing only a single isolate per sample (Dray, 2004; Manteca et al., 2002).

The aim of this study was to design primers recognizing all published *cpb2* sequences and to find ways to discriminate between the PCR products originating from either the atypical or the consensus gene (allele). Subsequently three test strategies for determining the presence of *cpb2* by PCR in samples i.e. PCR on single colonies, PCR on pooled colonies, and PCR on DNA directly isolated from feces were compared for their sensitivity and efficiency.

Materials and methods

Bacteria

Sixty *C. perfringens* strains used in the development of the PCR were from the collection of the Veterinary Microbiological Diagnostic Center (VMDC), Division of Clinical Infectiology, Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University. Faecal samples used in the evaluation of the protocol for the demonstration and typing of *C. perfringens* in clinical samples originated from pigs, sheep, cows, horses, dogs and cats as well as from a variety of other (exotic) species. Isolation of *C. perfringens* from these samples was performed using standard procedures (VMDC).

Primers and PCR conditions

Primers used are given in Table 1. For sample preparation one *C. perfringens* colony (single colony PCR) or up to ten *C. perfringens* colonies (pooled colony-PCR) were resuspended in 25–50 µl of distilled water and heated for 10 min at 95 °C. Fifty microliters of PCR reaction mix consisted of 1 µl of this heated bacterial suspension, 100 ng of each primer (Isogen, The Netherlands), 0.2 mM of each dNTP (Fermentas, Germany), 1 U recombinant Taq DNA polymerase (Fermentas), 1.5 mM $MgCl_2$, 5 µl of 10x Taq buffer, and distilled

water. The PCR program consisted of 3 min at 95 °C followed by 40 cycles of 30 s at 95 °C, 30 s at the annealing temperature (Table 1) and amplification at 72 °C during the appropriate time (1 kb/min). A final amplification step of 7 min at 72 °C completed the PCR reaction. PCR was performed using either the GeneAmp PCR system 2400 (Perkin Elmer, USA) or the iCycler (BioRad, England). As a sample preparation control, all samples were subjected to a PCR for the *cpa* gene which is present in all *C. perfringens* strains (Titball et al., 1999). After performing the PCR reaction, the total reaction volume was subjected to gel electrophoresis.

Sequence and restriction enzyme analysis

PCR products obtained with the CpB2total primer set were extracted from gel using the QIAEX II Gel Extraction Kit (Qiagen) and subsequently sequenced (BaseClear, The Netherlands). The resulting sequences were mapped for restriction sites using the NEBcutter software (www.neb.com).

PCR on several separated colonies isolated from a single feces sample

As a first test strategy 33 faecal samples were anaerobically cultured on sheep blood agar for *C. perfringens* and of each sample, up to 10 *C. perfringens*-like colonies were separately subcultured on sheep blood agar. Subsequently, on each colony a PCR with the new CpB2total primer set.was performed. Obtained products were digested with FastDigest MboI (Fermentas) and subjected to gel electrophoresis.

Table 1 Primers used

name primer	target gene	sequence (5′-3′)	T_{an}	product length	name primer set
Cpbeta2F[a]	consensus *cpb2*	CAAGCAATTGGGGGAGTTTA	53°C	200bp	consensus-primer set
Cpbeta2R[a]	consensus *cpb2*	GCAGAATCAGGATTTTGACCA	53°C		
CpEEB2F[b]	atypical *cpb2*	AACATAATAAATCCTATAACCC	50°C	1239bp	atypical-primer set
CpEEB2R[b]	atypical *cpb2*	ATAAATATAATTCTCTAAAACC	50°C		
CpB2totalF2[c]	consensus/atypical *cpb2*	AAATATGATCCTAACCAAM[e]AA	48°C	548bp	total-primer set
CpB2totalR[c]	consensus/atypical *cpb2*	CCAAATACTY[f]TAATYGATGC	48°C		
CPAF[d]	*cpa*	GCTAATGTTACTGCCGTTGA	53°C	325bp	CPA-primer set
CPAR[d]	*cpa*	CCTCTGATACATCGTGTAAG	53°C		

[a]: Baums et al, [b]: Jost et al., 2005, [c]: this paper, [d]: Meer et al.
[e]: M = A or C
[f]: Y = C or T

PCR on pooled colonies isolated from a single feces sample

Up to ten C. *perfringens*-like colonies of each of the 33 faecal samples used with the single colony strategy were pooled and 1 µl of this mixture was used as a template in a PCR with the new CpB2total primer set. Five micrograms of bovine serum albumine (BSA, Sigma, The Netherlands) and a double amount of primers were added to improve sensitivity of the PCR. Obtained PCR products were digested with FastDigest MboI (Fermentas) and subjected to gel electrophoresis.

PCR on DNA directly isolated from feces

As a third test strategy DNA was directly isolated using QIAmp DNA Stool Mini Kit (Qiagen, The Netherlands) from 18 faecal samples. Five microliters of the isolated DNA was used as a template in a PCR with the new CpB2total primer set. The same faecal samples were anaerobically cultured on sheep blood agar for *C. perfringens* and of each sample up to 10 *C. perfringens*-like colonies were tested both separately and pooled in a PCR with the new CpB2total primer set and a PCR with the CPA primer set. PCR products obtained with the CpB2total primers were digested with FastDigest MboI (Fermentas) and subjected to gel electrophoresis.

Results

PCR with the newly designed primers

Sixty strains of the collection of the VMDC were tested with the consensus primer set, the atypical primer set, and the new CpB2total primer set for the presence of the *cpb2* gene. A total of 43 strains were negative with all primer sets. Eight strains were positive with the consensus primer set but negative with the atypical primer set. Nine strains were positive with the atypical-primer set but negative with the consensus primer set. However, these seventeen strains were all positive with the newly designed CpB2total primer set.

Sequence results and restriction enzyme analysis

A PCR product of each variant generated by the CpB2total primer set was sequenced. Alignment of the sequence obtained from the consensus

primers positive strain (accession number EU085382) showed a 98–100% identity with known sequences of this variant using the BLAST software. Analyzing the sequence of the PCR product from the atypical primer set positive strain (accession number EU085383) resulted in a 94–99% identity with the available sequences of the atypical variant.

In silica digestion of both generated sequences with the enzyme MboI resulted in two clearly different restriction patterns; the consensus strain product would result in fragments with sizes of 6, 114, 164, and 264 bp whereas the atypical strain product would generate two 6 bp fragments, one fragment of 193 bp, and one of 343 bp. Subsequently all PCR products obtained with the CpB2total primer set were purified from gel using the QIAEX II kit (Qiagen) and digested with (FastDigest) MboI (Fermentas) according to the manufacturers instructions. Indeed MboI digestion and subsequent electrophoresis of all nine PCR products obtained with the atypical primer set positive strains showed bands of the predicted sizes (Fig. 1). However, MboI digestion of only four out of the eight PCR products originating from consensus primer set positive strains resulted in fragments of the expected sizes (termed con3 pattern, Fig. 1). The other four PCR products showed only two visible bands: one band of 164 bp and one of approximately 390 bp (termed con2 pattern, Fig. 1). Partly sequencing of one of these PCR products (accession number EU085384) revealed a difference in nucleotide 270 (TATC versus GATC) between this sequence and seq EU085382 resulting in a difference in the number of MboI restriction sites. Indeed in silica restriction analysis indicated three fragments: one 6 bp fragment, one fragment of 164 bp, and one fragment of 378 bp. Strikingly the PCR products with a con2 pattern were generated from strains originating from pigs whereas the PCR products resulting in one of the two other MboI patterns were from non-porcine origin.

In order to study the possible correlation between MboI pattern and allelic variant as well as the possible correlation between MboI pattern and sample origin, 36 atypical sequences and 37 *cpb2* consensus sequences in the Genbank/EMBL/DDBJ databases were analyzed for the MboI restriction pattern. The atypical sequences all originating from non-porcine sources showed the predicted atypical pattern. Analysis of 24 consensus sequences originating from sheep (n = 4), cattle (n = 7), horses (n = 6), humans (n = 5), and other sources (n = 2) resulted in the con3 pattern. Thirteen consensus sequences all originating from pig isolates (including the firstly described *cpb2*

gene with accession number L77965) resulted in the con2 pattern. Analyzing 19 submitted partial consensus sequences showed that 17 harboured the MboI site at position 270 downstream the first nucleotide of the CpB2total F2 primer. These sequences all originated from non-porcine sources like food, poultry, etc., whereas two sequences which were devoid of this MboI site were of porcine origin.

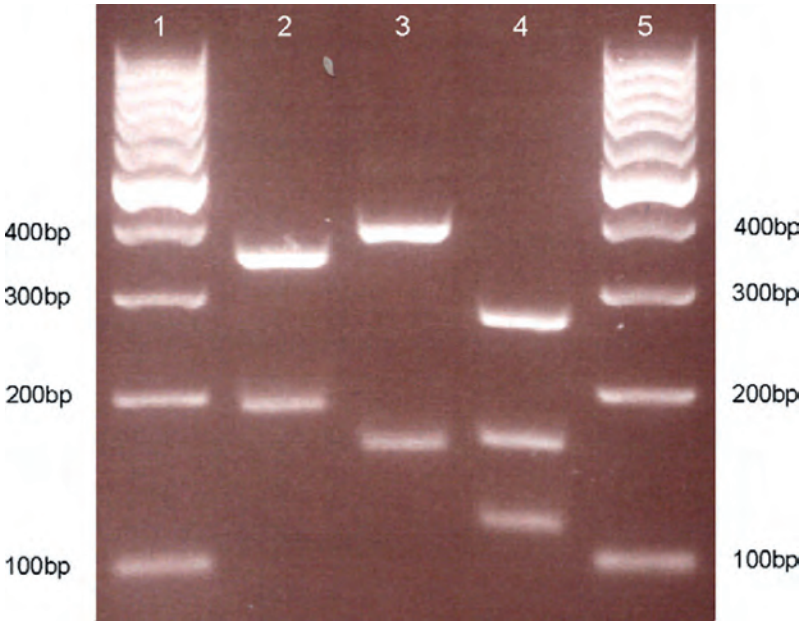

Fig. 1. Agarose gel (3%) electrophoresis of MboI digests of PCR products obtained with the total-primer set on an atypical sample (lane 2) or consensus samples (lanes 3 and 4). Lanes 1 and 5: size marker (MassRuler Express DNA ladder Mix, Fermentas). The presence of the 6 bp bands cannot be demonstrated in this gel under given conditions.

Use of the new total-primer set and MboI digestion in routine screening

Next, a protocol for the demonstration and typing of *cpb2* containing *C. perfringens* in clinical samples was formulated. At the VMDC, *C. perfringens*-like colonies from clinical samples were streaked on blood agar for single colonies. Subsequently, one colony was used in a PCR with the new CpB2total primer set and a PCR with the CPA primer set. PCR products obtained with the CpB2total primer set were digested with FastDigest MboI (Fermentas) without any purification and the resulting digest was electrophoresed on a 3 % agarose

gel. Forty-two isolates were typed accordingly: all were *cpa* positive, 24 were positive for *cpb2*. MboI digestion patterns of PCR products obtained from isolates from all types of animals other than pig resulted in either the atypical pattern (17) or in the con3 pattern (5). Both products amplified from isolates from pigs showed a con2 pattern. To confirm these findings all positive strains were also tested with the corresponding primer set. Results with the consensus primer set were in agreement with the MboI restriction patterns. However, three isolates which were typed atypical with the CpB2total primer set did not result in a PCR product when using the atypical primer set and the advised protocol (Jost et al., 2005). Changing PCR conditions like annealing temperature or annealing time did result in a PCR product from only one of these isolates. The remaining two isolates were also negative with the consensus primer set.

Comparison of three different sampling methods

To establish an efficient and sensitive sampling method for the presence of *cpb2* in faecal samples three different sampling methods were compared. First up to 10 single *C. perfringens*-like colonies from faecal samples were tested separately for the presence of *cpa* and *cpb2*. All tested colonies were *cpa*-positive. In 24 out of 33 faecal samples *cpb2*-positive *C. perfringens* strains were demonstrated. In 11 of these samples also *cpb2*-negative *C. perfringens* strains were demonstrated. Two of these samples harboured strains with different *cpb2* variants as determined by MboI digestion.

To validate a more efficient way of sampling, ten colonies of each sample were pooled and this pool was tested for the presence of *cpb2*. All pooled samples that harboured *cpb2* carrying *C. perfringens* colonies gave a positive PCR result for *cpb2* and the subsequent MboI digestion of the PCR product enabled detection of the *cpb2* variants.

To find an even more efficient way of testing, DNA was directly isolated out of 18 faecal samples and used in a PCR for *cpb2*. The same faecal samples were tested with the single and pooled colony strategy. Only 9 out of the 15 samples positive for *cpb2* as demonstrated by the simultaneously performed single and pooled colony methods tested positive for *cpb2* using the direct DNA isolation method. The use of a more sensitive polymerase (FastStart High Fidelity PCR System, Roche, The Netherlands) did not improve this result.

Discussion

C. perfringens strains harbouring *cpb2* have frequently been associated with enteritis in several animal species and humans (Waters et al., 2003; Klaasen et al., 1999, Herholz et al., 1999; Bueschel et al., 2003; Garmory et al., 2000; Lebrun et al., 2007; Fisher et al., 2005). The actual presence of the beta2 toxin in affected tissue suggesting an association between the toxin and the post-mortem findings has been demonstrated by immunohistochemistry in an African elephant (*Loxodonta africana*) with ulcerative enteritis (Bacciarini et al., 2001), in a hamadryas baboon (*Papio hamadryas*) with enteritis (Nikolaou et al., 2009), and in horses that died from typhlocolitis (Bacciarini et al., 2003; Vilei et al., 2005).

In order to study the role of the beta2 toxin in various animals, a rapid screening method for the presence of the beta2 toxin would be helpful. Although the presence of *cpb2* in the genome of the bacterium does not necessarily mean that the toxin is produced, a PCR for the toxin gene can be seen as a first screening.

Several sets of primers for the demonstration of *cpb2* have been published, however, using the BLAST software none of these primers (fully) aligned with all complete *cpb2* sequences in the Genbank/EMBL/DDBJ databases. Therefore, primers that recognize all known (complete) *cpb2* sequences were designed and tested.

Recent studies indicate that there might be a difference in the toxin production between the consensus and atypical variants (Bueschel et al., 2003; Jost et al., 2005; Lebrun et al., 2007). Furthermore, the level of expression of the consensus *cpb2* gene seems to correlate with the origin of the isolates, with the genotype–phenotype correlation being the highest in porcine isolates (Bueschel et al., 2003; Jost et al., 2005; Vilei et al., 2005). The MboI restriction pattern of the PCR product generated with our CpB2total primer set discriminates between atypical and consensus variants and between porcine and non-porcine origin and might therefore correlate with the level of beta2 toxin production.

The application of the new PCR on faecal samples confirmed the earlier demonstrated limitations of testing a single or few *C. perfringens* colonies of one faecal sample for the presence of *cpb2*-harbouring *C. perfringens* (Dray, 2004; Manteca et al., 2002). Application of the new PCR on pooled colonies increased

the observed prevalence of *cpb2* in faecal samples creating a more sensitive test strategy for prevalence studies on the presence of *cpb2*-harbouring *C. perfringens* in faecal samples.

In conclusion, the described combination of a PCR using the newly designed CpB2total primer set recognizing all known alleles of *cpb2* and the pooled colony strategy can be seen as an efficient and sensitive first step in studying the role of beta2 toxin in intestinal disease.

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Chapter 3

Occurrence of *cpb2*-harbouring *Clostridium perfringens* in wild roe deer (*Capreolus capreolus*) in The Netherlands

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In preparation

Abstract

The occurrence of *cpb2*-harbouring *Clostridium perfringens* type A in diarrheic and non-diarrheic roe deer was studied. *C. perfringens* type A carrying either the atypical or the consensus 3 allele of *cpb2* was isolated from both groups. No beta2 toxin could be demonstrated in tissues from animals of either group. Only two strains originating from a non diarrheic animal produced beta2 toxin *in vitro*. No definite conclusion about the role of beta2 toxin in enteric disease of roe deer could be drawn. This study underlines the importance of demonstrating both *in vitro* and *in vivo* toxin production in understanding the role of *C. perfringens* in enteric disease.

An adult female roe deer (*Capreolus capreolus*) showing signs of diarrhoea was found dead and submitted for post mortem diagnostic investigation to the Dutch Wildlife Health Centre at the Department of Pathobiology of the Faculty of Veterinary Medicine, Utrecht University, The Netherlands. The cecum content was watery, mixed with blood, and the rectum contained soft faecal material. Culturing of the intestinal content resulted in innumerable bacterial colonies surrounded by a double hemolytic zone typical for *Clostridium perfringens*.

Isolates of *C. perfringens* are classified in one of five toxin types (type A-type E) based on the toxin(s) produced by the isolate (Songer, 1996). Besides the four major toxins which underlie this classification scheme (alpha, beta, epsilon, and iota), the various toxin types may produce additional toxins, including enterotoxin and beta2 toxin. The toxin types are each associated with specific diseases in animals (Gibert et al., 1997; Songer, 1996).

To determine the toxin type(s) present in this case, nine single *C. perfringens*-like colonies were picked for toxin-gene typing by multiplex PCR (van Asten et al., 2009). All isolates appeared to be *C. perfringens* type A, while seven of the isolates also harboured the atypical allele of *cpb2*, the gene encoding the beta2 toxin (van Asten et al., 2008).

C. perfringens type A, although being a member of the normal intestinal flora, has been associated with enteric disease in certain domestic ruminant species, and often only for given age categories (Songer, 1996). *C. perfringens* type A has been detected in roe deer found dead in both Germany and Norway (Aschfalk et al., 2002, Sting, 2009), and in roe deer with enteritis in Norway (Aschfalk et al., 2002). The role of beta2 toxin in enteric diseases of ruminants is still unclear (Uzal et al., 2008, van Asten et al., 2010) and no data are available on the occurrence of *C. perfringens* type A-harbouring *cpb2* in roe deer. Therefore the relevance of finding a *C. perfringens* type A carrying *cpb2* with regard to the observed diarrhoea in this roe deer was unclear. The aim of this study was to gain more insight into the occurrence of *C. perfringens* type A with *cpb2* in roe deer in The Netherlands, in relation to the occurrence of diarrhoea.

For this reason, a total of 15 roe deer submitted for post mortem diagnostic investigation were sampled for *C. perfringens*, and toxin types were determined as described above. Two of these sampled animals suffered from diarrhoea and 13 animals, mainly from traffic accidents, did not show any signs of intestinal disorder. Samples of two animals without enteritis had

no *C. perfringens* isolated, whereas all the remaining animals harboured *C. perfringens* type A either with or without *cpb2* or a combination of the two. MboI digestion of the PCR product generated with *cpb*2 primers (van Asten et al., 2008) revealed that the various samples positive for *cpb2* carrying *C. perfringens* harboured isolates with the atypical *cpb2* allele, isolates with the consensus 3 variant of *cpb2*, or a mixture of these isolates (Table 1).

Immunohistochemistry (IHC) was performed on formalin fixed paraffin embedded sections of the intestine from diarrheic and non diarrheic animals using a polyclonal anti-beta2 toxin antibody, in order to further determine the role of beta2 toxin in diarrhoea in roe deer. No beta2 toxin could be demonstrated in any of the intestinal tissues from either the diarrheic animals or non diarrheic animals.

Table 1 Data on examined animals and C. *perfringens* isolates.

Animal	Date of death	Sex	Age	Signs of enteritis	Toxin type(s) isolated	*cpb2* allele
1	30-03-2010	F[1]	≥ 1year	clinical	A, A-*cpb2*	atyp[2]*
2	01-06-2010	M[3]	≥ 1year	absent	A, A-*cpb2*	atyp
3	04-06-2010	F	≥ 1year	absent	-	-
4	04-06-2010	M	≥ 1year	absent	A-*cpb2*, A-*cpe*[4]	atyp
5	07-06-2010	F	≥ 1year	absent	A, A-*cpb2*	atyp, con3[5]
6	08-06-2010	F	≥ 1year	histological	A, A-*cpb2*	atyp, con3
7	19-07-2010	F	≥ 1year	absent	A, A-*cpb2*	atyp, con3*
8	19-07-2010	M	< 1year	macroscopical	A-*cpb2*	con3
9	29-07-2010	M	≥ 1year	absent	-	-
10	02-08-2010	M	≥ 1year	histological	A-*cpb2*, E-*cpe*	con3
11	03-08-2010	M	< 1year	absent	A	-
12	05-08-2010	F	< 1year	absent	A, A-*cpe*	-
13	05-08-2010	F	≥ 1year	histological	A, A-*cpb2*	atyp, con3
14	06-08-2010	F	≥ 1year	absent	A, A-*cpb2*, A-*cpb2-cpe*	atyp, con3*
15	17-09-2010	F	≥ 1year	clinical	A-*cpb2*	atyp
16	09-02-2011	F	<1year	clinical/ macroscopical	A, A-*cpb2*	atyp, con3

[1]: female, [2]: atypical allele, [3]: male, [4]: the gene encoding the enterotoxin, [5]: consensus 3 allele, *: one (animal no. 1) or more (animal no. 7, 14) isolates lost before allele typing.

It has been shown that not all isolates of *C. perfringens* carrying *cpb2* are capable of producing beta2 toxin (Bueschel et al., 2003; Uzal et al., 2008). Therefore all *cpb2*- (7 atypical and 4 consensus 3 alleles) harbouring isolates from two animals with signs of diarrhoea were tested *in vitro* for beta2 toxin

expression. Also, 10 *cpb2*-harbouring *C. perfringens* isolates of non diarrheic animals, 6 carrying the consensus 3 allele and 4 carrying the atypical allele, were tested. Pellets of overnight *cpb2*-harbouring *C. perfringens* cultures were resuspended in an equal volume of fresh Schaedler broth (Oxoid) and re-incubated for 6 hours. The cultures were centrifuged again and supernatants were run on a sodium dodecyl sulphate-polyacrylamide gel. After Western blotting, immunochemical detection of beta2 toxin was performed using the polyclonal anti-beta2 toxin antibody. Only 2 consensus 3-harbouring *C. perfringens* type A isolates produced a detectable amount of beta2 toxin *in vitro*. Both isolates originated from a thin but healthy animal.

In conclusion, results of this study show that *C. perfringens* toxin type A-harbouring either the consensus 3 *cpb2* allele or the atypical *cpb2* allele was abundantly present in the intestines of both diarrheic and non diarrheic roe deer. The presence of both the consensus 3 and the atypical allele in isolates from roe deer is consistent with the observation that both alleles are frequently observed in *C. perfringens* isolated from cattle (van Asten et al., 2010). No *C. perfringens* with the consensus 2 allele was isolated from roe deer, which is in agreement with the earlier observation that the consensus 2 allele is exclusively found in porcine samples (van Asten et al., 2008).

No conclusions on the role of beta2 toxin-producing *C. perfringens* in enteric disease of roe deer could be drawn from this study, since none of the isolates tested from diseased animals produced beta2 toxin *in vitro* and no beta2 toxin was demonstrated in tissue of diseased or healthy animals by IHC. Clearly the present study emphasizes the importance of demonstrating toxin production by *C. perfringens in vitro* as well as in situ when studying the role of clostridial toxin(s) in disease.

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Chapter 4

NetB-producing and beta2-producing *Clostridium perfringens* associated with subclinical necrotic enteritis in laying hens in The Netherlands

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Abstract

Since 2006 increasing numbers of laying hen flocks with decreased production were reported in The Netherlands. At necropsy, animals from affected flocks showed multifocal areas of necrosis in the duodenum. Histologically the duodenum had moderate to marked villus atrophy and fusion with crypt hyperplasia and a mixed inflammatory infiltrate within the lamina propria underlying focal areas of degenerative epithelium. Multifocally, free within the intestinal lumen and associated with epithelial necrosis, were marked numbers of large rod-shaped bacteria. Anaerobic culturing and subsequent toxin typing revealed, in 19 out of 73 affected animals, the presence of *Clostridium perfringens* strains, either type A- or type C-harbouring the atypical allele of *cpb2* and *netB*. Eighteen out of these nineteen animals carried *C. perfringens* strains capable of producing beta2 toxin *in vitro* and all of these animals harboured *C. perfringens* strains capable of producing NetB toxin *in vitro*. In contrast, specific pathogen free (SPF) animals lacked gross or histological lesions in their duodenum, and *C. perfringens* type C was isolated from 4 out of 15 SPF animals tested. One of these isolates harboured the consensus 3 allele of *cpb2* that produced beta2 toxin *in vitro*. None of the *C. perfringens* isolates originating from SPF animals harboured *netB*. These findings might indicate that the NetB toxin produced by *Clostridium perfringens* is associated with subclinical necrotic enteritis (SNE) in layers whereas the involvement of beta2 toxin in SNE, if any, might be variant dependent.

Introduction

Since 2006, increasing numbers of laying hen flocks of different breeds, diets and geographical locations that failed to reach peak production and had a persistent poor laying were reported in The Netherlands. In addition to impaired production, animals showed extremely high feed intake and poor body condition (mainly muscular atrophy). Moreover, hens showed exaggerated feather loss without feather renewal. Necropsies on animals from these affected flocks sent for post mortem examination to the Animal Health Service, Deventer, The Netherlands, frequently revealed multifocal, macroscopic areas of duodenal necrosis that were several millimetres in diameter and from which *Clostridium perfringens* was repeatedly isolated.

C. perfringens is the causative agent of necrotic enteritis in poultry, affecting predominantly young animals (Songer, 1996; Van Immerseel et al., 2004; Gholamiandekhordi et al., 2006). Along with the acute form of necrotic enteritis (NE), a subclinical form of necrotic enteritis (SNE) with focal necrosis of the intestinal mucosa has also been described in the upper intestinal tract of broilers (Kaldhusdal & Hofshagen, 1992). Whereas NE and SNE are common diseases in broilers, NE has been reported only incidentally in adult poultry (Broussard et al., 1986; Dhillon et al., 2004), and no data are available on the occurrence of SNE in adult poultry.

C. perfringens toxin type A is the toxin type most frequent isolated from chickens with NE (Engström et al., 2003; Keyburn et al., 2006; Crespo et al., 2007), although type C is also occasionally found (Opengart, 2008). Little is known about the virulence factors involved in the development of NE. The alpha toxin produced by all *C. perfringens* toxin types has been considered to be a major virulence factor in NE for many years (Al-Sheikhly & Truscott, 1977; Fukata et al., 1988). However, experiments with an alpha toxin mutant of *C. perfringens* indicated the limited role of the alpha toxin in NE (Keyburn et al., 2006). Recent studies suggest that an additional toxin, the NetB toxin encoded by *netB*, might be a significant virulence factor in the development of both NE and SNE since there is a strong association between necrotic lesions and presence of NetB-producing *C. perfringens* strains in birds with NE and SNE. Furthermore, *C. perfringens* carrying *netB* lost its virulence in a chicken disease model when *netB* was knocked out. Virulence was fully restored upon complementation with a wild type *netB* (Keyburn et al., 2008; Cooper & Songer,

2009; Abildgaard et al., 2010; Johansson et al., 2010; Keyburn et al., 2010; Smyth & Martin, 2010).

Beta2 toxin, encoded by *cpb2*, which is another toxin produced by many *C. perfringens* isolates (Gibert *et al*., 1997), has been associated with necrotic enteritis in horses (Bacciarini et al., 2003), ruminants (Lebrun et al., 2007), and pigs (Bueschel et al., 2003; Waters et al., 2003). However, it has been suggested that beta2 toxin is not involved in NE in poultry (Gholamiandekhordi et al., 2006; Crespo et al., 2007). A potential association between *cpb2* and SNE has never been reported.

Here we present a study on the possible association of NetB toxin and beta2 toxin of *C. perfringens* and subclinical necrotic enteritis in layer hens.

Materials and methods

Birds

Seventy-three hens from seven commercial layer flocks were submitted to the Animal Health Service, Deventer, The Netherlands. Farms originated from scattered geographic locations within The Netherlands. Flocks were of five different commercial layer breeds (coded A to E) with different origins and variable ages from 36 to 69 weeks. Flock size differed from 6,000 to 35,000 birds. Housing systems included cage housing (n=1), aviary housing (n=2), free-range housing (n=1), and floor housing without free-range access (n=3) (Table 1). Feed was obtained from different commercial feed mills. The flocks suffered from very slightly to moderately impaired egg production levels (1-5% decrease), low peak production and poor laying persistence. Severe loss of feathers at a very young age was consistently seen, with presence of only a few new feather follicles. Feathers were rapidly eaten by the birds, resulting in complete absence of loose feathers on the floor of farms with floor housing systems. Additional signs and problems present in some flocks included higher feed consumption, weight-loss and aggressive behaviour. Wet litter or diarrhoea was not reported. All flocks suffered increased mortality rates, up to 22 % at the end of the laying period, that on post mortem examination predominantly related to peritonitis caused by *Escherichia coli*. Animals of several flocks showed signs of external trauma due to cannibalism. From each farm 8 to 15 live chickens submitted for diagnostic necropsy were stunned

using a mixture of CO_2 and O_2, exsanguinated and a general pathological examination was performed. Control animals were 15 specific pathogen free (SPF) layers of 50 weeks of age, delivered for routine diagnostic purposes.

Histology

From each animal, the last centimeter of the duodenum descendens before the flexura duodeni caudalis was sampled, irrespective of the presence of gross necrotic lesions, and fixed in 4% neutral buffered formalin, paraffin embedded, sliced in 4 µm thick sections, and haematoxylin & eosin stained for histopathological examination.

Isolation, culturing, and toxin typing of *C. perfringens*

Duodenal contents collected at post-mortem examination were cultured on sheep blood agar under anaerobic conditions at 37 °C. Subculturing of *C. perfringens*-like colonies was limited to a minimum to prevent loss of plasmid. If present, up to 10 *C. perfringens*-like colonies of each duodenal sample were tested for the presence of *cpa, cpb, cpb2, etx, iap, cpe*, and *netB* as described before (Keyburn et al., 2008; van Asten et al., 2008; van Asten et al., 2009).

Demonstration of *in vitro* toxin production

All *cpb2*- and *netB*-harbouring *C. perfringens* isolates were tested for the *in vitro* production of beta2 toxin and NetB toxin. Each isolate was grown overnight in 4 ml Schaedler broth (Oxoid) under anaerobic conditions at 37 °C. Next day the cultures were centrifuged for 15 min at 3000 *g*, after which the bacterial pellets were resuspended in 4 ml prewarmed Schaedler broth and incubated for another 6 hours. The cultures were centrifuged again, supernatants were run on sodium dodecyl sulphate-polyacrylamide gels, and proteins were blotted onto a PVDF membrane (BioRad). The presence of the beta2 toxin was demonstrated using a polyclonal rabbit anti-beta2 toxin antibody recognizing the consensus as well as the atypical variant of the beta2 toxin (kindly provided by Prof. J. Frey, University of Bern, Switzerland). The presence of the NetB toxin was demonstrated using a polyclonal rabbit anti-NetB antibody (kindly provided by Dr. R. Moore, CSIRO Livestock Industries, Australia). A swine anti-rabbit antiserum coupled to peroxidase was used as secondary antibody (DakoCytomation). ECL Plus Western blotting detection reagent (GE Healthcare) was used for visualizing a positive reaction.

Results

Post-mortem examination

In general at necropsy, all 73 birds from 7 flocks with impaired production had a poor body condition with muscular atrophy predominantly of the pectoral muscles, in contrast to normally sized to relatively large abdominal fat pads. Ventriculi were normal, but the lumens contained large numbers of feathers. Only 4 to 5 follicles were present in the ovary of each animal indicating impaired production. Furthermore, animals had slightly pale livers with reduced consistency and a few small haemorrhages, indicating (fatty) degeneration. Intestinal examination revealed three categories of gross duodenal findings: A: normal contents in 14 birds (19.2%), B: abnormal liquid contents in 41 birds (56.2%), and C: abnormal liquid contents combined with multifocal intestinal necrotic lesions of several millimetres in diameter (Figure 1A) in 18 birds (24.7%). No abnormalities were found in the necropsy of 15 SPF birds.

Histology

In contrast to the different categories of gross duodenal findings, histological abnormalities were similar and comparable in all birds from the flocks with impaired production. Most characteristic were diffuse marked crypt deepening (hyperplasia) and multifocal marked villus blunting and fusion. The lamina propria contained coalescing, moderate to marked infiltrates of lymphocytes and plasma cells, indicating hyperplasia of the gut associated lymphoid tissue (GALT). Multifocal mild to moderate heterophillic infiltrates were present in the lamina propria of the apical part of the villi (Figure 1D), mainly underlying areas that contained increased numbers of necrotic or apoptotic clusters of enterocytes. Increased numbers of intraepithelial lymphocytes (IEL) were present in the villus epithelium. The intestinal lumen contained scattered multifocal small and larger clusters of large rod- shaped bacteria; in several sections associated with both intact and necrotic enterocytes (Figure 1B). The SPF birds in contrast, had only multifocal, very mild crypt deepening and occasional mild blunting and fusion of villi (Figure 1C). The lamina propria contained multifocal, mild lymphocytic infiltrates, without indication for GALT hyperplasia. Within the epithelial layer, no lesions or increased numbers of IELs were present. The intestinal lumens of a few animals sporadically contained few large rod-shaped bacteria.

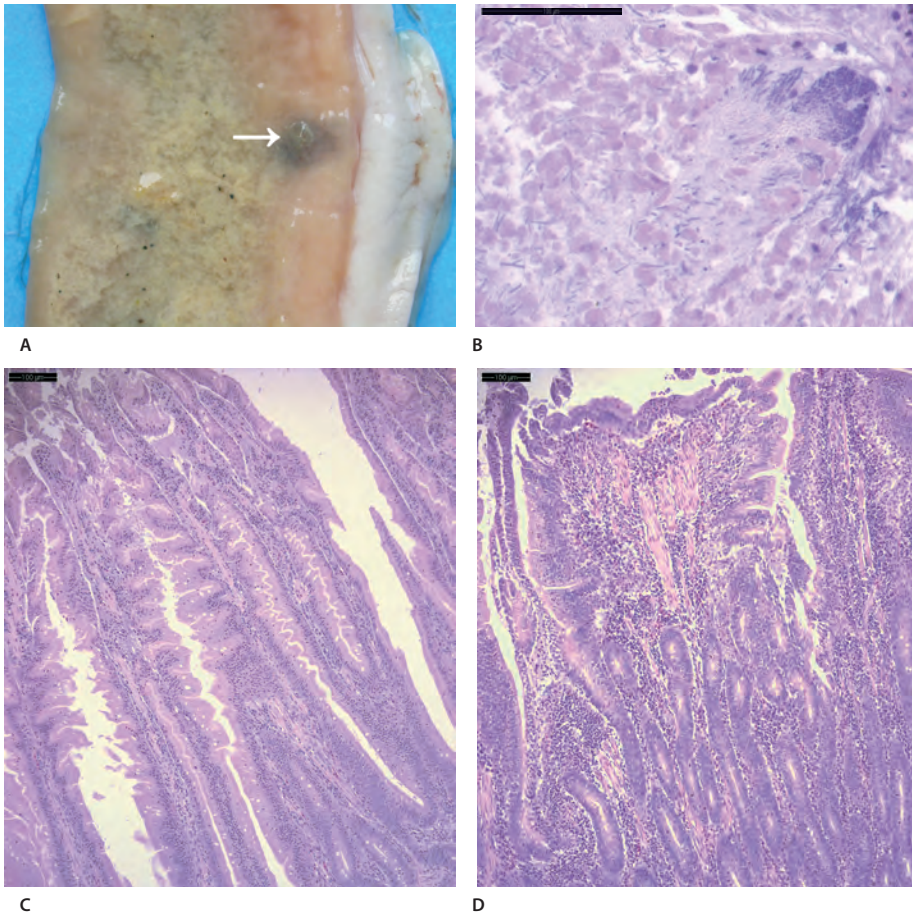

Figure 1. 1A: Focal necrotic lesion in the duodenum (arrow). 1B: Duodenum of a bird from an affected flock: necrotic debris at villus tips with large numbers of large rod-shaped bacteria. 1C: Duodenum of an SPF bird: no abnormalities. 1D: Duodenum of a bird from an affected flock: marked crypt hyperplasia with atrophy and fusion of the villi and a mixed inflammatory infiltrate in the lamina propria. Bar represents 100 µm.

Isolation, culturing, and toxin typing of *C. perfringens* and *in vitro* toxin production

Anaerobic culturing of duodenal contents demonstrated *C. perfringens* in 19 out of 73 (26 %) of the birds from 6 farms (Table 1). No *C. perfringens* was isolated from animals originating from one farm. *C. perfringens* was isolated from 6 out of the 14 birds with normal duodenal contents (cat. A), from 8 out of 41 birds with abnormal liquid duodenal contents (cat. B), and from 5 out of 18 animals with an abnormal liquid duodenal contents combined with multifocal intestinal necrotic lesions (cat. C).

Table 1. Flock characteristics and the pathology and laboratory results of examined specimens

Flock	Flock size (animals)	Age (wks)	Housing system	Breed	Number of hens examined	Number of hens with abnormal duodenal contents	Number of hens with gross focal necrosis	Number of hens harbouring *C. perfringens*	Toxin type	Presence Beta2	Presence NetB
838	12500	36	Cage	B	10	10	0	0	-	-	-
652	30000	52	Aviary	B	8	8	0	2	C	+	+
799	35000	69	Aviary	A+D	10	10	2	4	A&C	+	+
709	12000	56	Free range	C	15	8	4	5	A&C	+	+
651	10000	48	Floor Housing	A	10	4	0	4	C	+	+
965	6000	54	Floor housing	E	10	10	2	2	A&C	+	+
966	14000	54	Floor housing	E	10	10	10	2	C	+	+
398	350	50	SPF Floor housing	SPF	15	0	0	4	C	+	-

Table 2. Detailed PCR and Western blot results on *C. perfringens* isolates per *C. perfringens* carying animal

			Toxin type A							**Toxin type C**						
Animal	cat.[1]	isolates typed[2]	number isolates[3]	*cpb2* pos.[4]	Beta2 pos.[5]	*netB* pos.[6]	NetB pos.[7]	*cpb2/netB* pos.[8]	Beta2/ NetB pos.[9]	number isolates[10]	*cpb2* pos.[4]	Beta2 pos.[5]	*netB* pos.[6]	NetB pos.[7]	*cpb2/ netB* pos.[8]	Beta2/ NetB pos.[9]
652-4	B	10	0	0	0	0	0	0	0	10	10	2	10	7	10	2
652-6	B	10	0	0	0	0	0	0	0	10	10	4	10	10	10	4
799-1	B	10	10	10	10	9	1	9	1	0	0	0	0	0	0	0
799-2	B	10	10	10	10	9	1	9	1	0	0	0	0	0	0	0
799-4	C	10	0	0	0	0	0	0	0	10	10	1	10	7	10	1
799-8	B	10	0	0	0	0	0	0	0	10	10	3	8	7	8	3
709-2	C	10	10	10	4	10	1	10	1	0	0	0	0	0	0	0
709-5	A	3	1	1	1	1	1	1	1	2	2	0	2	2	2	0

Table 2 continued

			Toxin type A							Toxin type C						
Animal	cat.[1]	isolates typed[2]	number isolates[3]	*cpb2* pos.[4]	Beta2 pos.[5]	*netB* pos.[6]	NetB pos.[7]	*cpb2/netB* pos.[8]	Beta2/ NetB pos.[9]	number isolates[10]	*cpb2* pos.[4]	Beta2 pos.[5]	*netB* pos.[6]	NetB pos.[7]	*cpb2/ netB* pos.[8]	Beta2/ NetB pos.[9]
709-11	A	3	2	2	1	2	2	2	1	1	1	1	1	1	1	1
709-12	A	10	1	1	0	1	1	1	0	9	9	5	3*	4	3*	1
709-13	B	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0
651-4	B	10	0	0	0	0	0	0	0	10	9	2	9	7	9	0
651-5	A	6	0	0	0	0	0	0	0	6	6	2	6	3	6	2
651-7	A	10	0	0	0	0	0	0	0	10	10	0	10	9	10	0
651-10	A	9	0	0	0	0	0	0	0	9	9	3	9	4	9	0
965-1	B	10	0	0	0	0	0	0	0	10	10	8	9	9	9	7
965-7	C	10	10	10	1	10	1	10	1	0	0	0	0	0	0	0
966-1	C	8	0	0	0	0	0	0	0	8	8	5	8	8	8	5
966-2	C	10	0	0	0	0	0	0	0	10	10	6	10	10	10	6
SPF-1	A	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0
SPF-4	A	1	0	0	0	0	0	0	0	1	1	1	0	0	0	0
SPF-6	A	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0
SPF-10	A	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0

[1]: category of gross findings in the duodenum of the animal: A: normal contents, B: abnormal liquid contents, C: abnormal liquid contents combined with multifocal necrotic lesions. [2]: number of *C. perfringens* isolates that were toxin typed. [3]: number of isolates that were typed as toxin type A. [4]:number of isolates that reacted positively in the PCR detecting *cpb2*. [5]: number of isolates that reacted in a Western blot using polyclonal anti-beta2 antibody. [6]: number of isolates that reacted positively in the PCR detecting *netB*. [7]: number of isolates that reacted in a Western blot using polyclonal anti-NetB antibody. [8]: number of isolates that reacted positively in the PCR detecting *cpb2* and in the PCR detecting *netB*. [9]: number of isolates that reacted in a Western blot using polyclonal anti-beta2 antibody and in a Western blot using polyclonal anti-NetB antibody. [10]: number of isolates that were typed as toxin type C. *: Two isolates that reacted in a Western blot using polyclonal anti-NetB antibody did not react positively in the PCR detecting *netB*.

The majority of animals (11 out of 19) from any gross duodenal category carried solely *C. perfringens* type C (Table 2). Five animals carried solely *C. perfringens* type A. At necropsy, these five animals showed abnormal liquid duodenal contents, both with and without gross visible necrotic foci (cat. B and C). The remaining 3 animals carried both *C. perfringens* type C and *C. perfringens* type A, and did not show any gross abnormalities at necropsy. *C. perfringens* type C was the only type isolated from animals originating from 3 farms. *C. perfringens* type A was always isolated from animals originating from flocks that also contained *C. perfringens* type C carrying animals. All animals carrying both *C. perfringens* type A and *C. perfringens* type C originated from the same farm.

All 19 animals carried at least one *C. perfringens* isolate capable of producing NetB in *vitro*. From all 19 animals, *C. perfringens* was isolated harbouring the atypical variant of *cpb2*; 18 of these animals carried a *C. perfringens* isolate capable of producing the beta2 toxin *in vitro*. C. *perfringens* was isolated from 4 out of 15 SPF chickens. These isolates were typed as type C and did not harbour *netB*. One of these strains harboured the consensus 3 variant of *cpb2* (Van Asten et al., 2008) and produced the beta2 toxin *in vitro*. Laboratory tests revealed no indications for underlying viral or parasitic infections or the presence of other known pathogenic bacteria.

Discussion

Infection models inducing low levels of gut necrosis in broilers are used in studying subclinical necrotic enteritis (SNE) (Gholamiandehkordi et al., 2007). Parameters used in these studies as well as in SNE diagnosis are body weight gain, growth rate, and feed conversion ratio. These parameters are typically used for broilers (Gholamiandehkordi et al., 2007). Based on the observation that the laying hens with impaired production showed high feed intake, weight loss, and poor body condition together with the fact that at necropsy only 24,7% of the birds examined showed intestinal necrotic lesions, it was concluded that these hens suffered from SNE. Furthermore, the combination of a lack of gross necrotic enteritis with the presence of histologic abnormalities found in all affected animals resembled the results of experiments in which broilers were orally challenged with *C. perfringens*. These experiments resulted

in an intestinal disorder described as a sub-clinical form of necrotic enteritis (Olkowski et al., 2006).

C. perfringens is regarded as the causative agent of both necrotic enteritis (NE) and SNE (Engström et al., 2003; Van Immerseel et al., 2004). In this study, all examined animals from affected flocks showed histologically large rod-shaped bacteria associated with the epithelium as well as with areas of epithelial necrosis, whereas a few SPF animals contained very little numbers of large rod-shaped bacteria in the intestinal lumens. However, *C. perfringens* was isolated from the duodenal contents of 26% (19 out of 73) of the birds from flocks with impaired production and from the duodenal contents of 27% (4 out of 15) of the SPF animals. The discrepancy between the histology and culture results might be explained by the presence of one or more large rod-shaped bacterial species other than *C. perfringens* in the intestine of the conventionally kept animals that are absent in the intestine of animals kept under SPF conditions. *C. perfringens* was isolated from 43% of the affected birds with a normal duodenal contents (cat A.) and from 22% of birds with an abnormal liquid duodenal contents (cat. B and cat. C). The fluidity of the duodenal contents might (partly) explain why *C. perfringens* was isolated from only 26% of examined birds of affected flocks. Also variances in sample size or exact location of sampling might have influenced the isolation of *C. perfringens*.

C. perfringens is a normal inhabitant of the chicken intestine (Gholamiandekhordi et al., 2006) and the capability of inducing disease is believed to lie in the possession of accessory virulence factors. The presence of NetB-producing *C. perfringens* isolates in laying hens with SNE and the absence of NetB-producing *C. perfringens* in laying hens of the SPF group might be an indication for a possible role of NetB-producing *C. perfringens* in SNE in adult animals. The fact that all flocks with signs of SNE as well as the SPF flock carried *C. perfringens* capable of producing the beta2 toxin *in vitro* again argues against the role of the beta2 toxin in enteritis in chickens (Crespo et al., 2007). However, the role of beta2 toxin in SNE might be variant dependent since all *cpb2* carrying *C. perfringens* isolates from SNE animals carried the atypical allele, and the *cpb2* carrying *C.perfringens* isolate from the SPF animal carried the consensus 3 allele. The role of the variants of beta2 toxin in intestinal disorders in various animal species is still under discussion (Jost et al., 2005; Lebrun et al., 2007; van Asten et al., 2008). The difference in expression of *cpb2* between the various atypical *cpb2* allele harbouring *C. perfringens* isolates from the same animal is

in line with what has been demonstrated in another study (Jost et al., 2005).

In conclusion, this is the first study demonstrating the presence of *C. perfringens* capable of producing both the NetB toxin and the beta2 toxin in laying hens with subclinical necrotic enteritis. Infection experiments with *C. perfringens* expressing both *netB* and either *cpb2* allele or expressing one of these toxins and isogenic non-expressing mutants as well as immunohistochemistry on affected and non- affected tissue should be performed in order to further determine the exact role of NetB toxin and beta2 toxin in SNE in adult chickens.

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Chapter 5

Beta2 toxin is not involved in *in vitro* cell cytotoxicity caused by human and porcine *cpb2*-harbouring *C. perfringens*

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Abstract

Clostridium perfringens is a common cause of intestinal disease in animals and humans. Its pathogenicity is attributed to the toxins it can produce including the beta2 toxin. The presence of *cpb2,* the gene encoding the beta2 toxin, has been associated with diarrhoea in neonatal piglets and humans. However, the exact role of the beta2 toxin in the development of diarrhoea is still unknown. In this study we investigated the level of cytotoxicity to porcine IPI-21 and human Caco-2 cell-lines caused by porcine and human *cpb2*-harbouring *C. perfringens* and the significance of the beta2 toxin for the induction of cell cytotoxicity. Supernatants of porcine *cpb2*-harbouring *C. perfringens* strains were cytotoxic to both cell lines. Cell cytotoxicity caused by supernatant of human *cpb2*-harbouring *C. perfringens* strains was variable among strains. However, removal by anti-beta2 toxin antibodies or degradation by trypsin of the beta2 toxin from supernatant of both porcine and human *cpb2*-harbouring *C. perfringens* cultures did not reduce the cytotoxic effect of the supernatants. These data suggest that beta2 toxin does not play a role in the development of cell cytotoxicity and subsequently diarrhoea.

Introduction

Clostridium perfringens is one of the most important causative agents of intestinal disease in animals and humans. The bacterium species is divided into five different toxin types, based on the four major toxins it can produce: alpha, beta, epsilon, and iota toxin (Songer, 1996). Besides the four major toxins, *C. perfringens* can produce additional toxins, including the beta2 toxin which was first identified from a *C. perfringens* strain isolated from a piglet that died of necrotizing enterocolitis (Gibert et al., 1997). The beta2 toxin has no significant homologies with other clostridial toxins and the mode of action of the beta2 toxin has not been elucidated yet (Gibert et al., 1997; Van Asten et al., 2010). The gene encoding the beta2 toxin, *cpb2*, has been demonstrated among all *C. perfringens* toxin types and among *C. perfringens* strains isolated from a large variety of animal species and humans (van Asten et al., 2010). Three allelic subpopulations of *cpb2* have been demonstrated which are called the consensus 3 allele, the consensus 2 allele (exclusively demonstrated in piglets), and the atypical allele (Fisher et al., 2005; Jost et al., 2005; Lebrun et al., 2007; van Asten et al., 2008). Differences in translation rate between the subpopulations of *cpb2* and differences in the level of toxicity caused by the encoded beta2 toxins have been identified (Bueschel et al., 2003; Fisher et al., 2005; Jost et al., 2005; Lebrun et al., 2007).

The presence of *cpb2* has been associated with diarrhoea in neonatal piglets and *cpb2*-harbouring *C. perfringens* strains from diarrhoeic piglets were able to produce the beta2 toxin *in vitro* (Garmory et al., 2000; Bueschel et al., 2003; Waters et al., 2003). However, the presence of beta2 toxin in the intestinal tract was not investigated in these studies. A recent study reported the presence of *cpb2* in isolates from both healthy and diarrhoeic neonatal piglets, however, no significant difference in the presence of *cpb2* was observed between isolates derived from healthy and diarrhoeic piglets (Kircanski et al., 2012). The presence of the beta2 toxin in the intestinal content of the piglets was determined by a beta2 antibody ELISA (Kircanski et al., 2012). Both healthy and diarrhoeic piglets showed increased intestinal presence of *C. perfringens* beta2 toxin and no significant difference in the presence of beta2 toxin between healthy and diarrhoeic piglets could be determined (Farzan et al., 2013). Reproduction of *cpb2*-harbouring *C. perfringens*-associated diarrhoea in infection models has not been achieved yet

(Springer et al., 2012). As a result the role of the beta2 toxin in the development of diarrhoea in piglets remains unclear.

In humans, 75 % of *C. perfringens* isolates from cases of antibiotic-associated diarrhoea and sporadic diarrhoea harboured *cpb2* and 97 % of these isolates could produce beta2 toxin *in vitro*. However, a negative control group with healthy humans was not included and the presence of beta2 toxin (e.g. in fecal samples) was not examined (Fisher et al., 2005). More research on the association between *cpb2* and diarrhoea in humans has not been performed, which indicates that the role of beta2 toxin in the development of diarrhoea in humans is still under debate.

In vitro studies can be seen as a first step in studying a possible role of beta2 toxin in the induction of diarrhoea. Studies on the effect of beta2 toxin on intestinal cells are scarce (Smedley et al., 2004). Purified beta2 toxin derived from a porcine *C. perfringens* strain was cytotoxic to human intestinal 407 cells (Gibert et al., 1997) and purified beta2 toxin derived from a human *C. perfringens* strain was cytotoxic to human Caco-2 cells (Fisher et al., 2005). However, protein deletion studies on the significance of the beta2 toxin in the development of intestinal cell cytotoxicity induced by *cpb2*-harbouring *C. perfringens* have not been reported to date. Here we report on the level of cytotoxicity to human and porcine cell-lines caused by supernatant of human and porcine *cpb2*-harbouring *C. perfringens* cultures and the significance of the beta2 toxin in the induction of *in vitro* intestinal cell cytotoxicity by *cpb2*-habouring *C. perfringens*.

Materials and methods

Cell culture

Porcine epitheloid intestinal (IPI-21) cells (Kaeffer et al., 1993) and human epithelial colorectal adenocarcinoma (Caco-2) cells (Pinto et al., 1983) were grown in Dulbecco's modified Eagle medium (DMEM, Lonza) supplemented with sodium bicarbonate 7.5% (Merck), gentamicin 50 µg/ml solution (Gibco-BRL), L-glutamine 3% (Sigma), and fetal bovine serum 15 % (FBS, Lonza). The cells were seeded in 24 well plates with a density of 320000 cells per cm^2. The cells were grown to confluence in 5-6 days.

Bacterial strains and growth conditions

A porcine (consensus 2) *cpb2*-harbouring *C. perfringens* type A strain (Cp15, originally designated Jf2251, kindly provided by Prof. J. Frey, Berne, Switzerland), a human (consensus 3) *cpb2*-harbouring *C. perfringens* type A strain (Cp107, kindly provided by Dr. A. Weersink, AZU, Utrecht, The Netherlands), and a human (atypical) *cpb2*-harbouring *C. perfringens* type A strain (Cp147, kindly provided by Dr. M. Scholing, GGD, Amsterdam, The Netherlands) were grown in Schaedler anaerobe broth (Oxoid Limited) anaerobically at 37 ºC for 16 hours (stationary phase). Furthermore, a porcine *cpb2*-negative *C. perfringens* type A strain with a mutation in the gene encoding alpha toxin (Jf2288, kindly provided by Prof. J. Frey, Berne, Switzerland) was grown under the same circumstances. Neither strain harboured *cpe* or *netB*. The cultures were centrifuged for 10 min at 4000 *g* at 37 °C and resuspended in Schaedler anaerobe broth to the same volume. After another two hours of incubation bacterial growth during incubation was evaluated by the decrease of pH of growth medium from 7.0 to 5.5. Cultures were centrifuged again and the pH of the supernatants were then set at pH 7 by the addition of NaOH and kept at 4 °C overnight for cell culture experiments. The presence of beta2 toxin in the culture supernatants was verified by Western blot analysis. The concentration of beta2 toxin in the supernatants of *cpb2*-harbouring *C. perfringens* cultures was estimated by comparison of the intensity of the resulting band in Western blot with the intensity of the band obtained with purified recombinant beta2 toxin with a known concentration as determined by spectrophotometric analysis (Nanodrop ND-1000, Isogen-Lifescience).

Removal of beta2 toxin from supernatant by use of antibodies

Protein A sepharose CL-4B beads (GE Healthcare) were washed three times in 20 mM sodium phosphate, pH 7.0. Rabbit polyclonal anti-beta2 antibody serum or pre-immune serum of the same rabbit was diluted 1:100 in 20 mM sodium phosphate and was added to the protein A sepharose CL-4B beats. After a 5 hours of incubation at 4 °C the loaded protein A sepharose CL-4B beads were washed again. Supernatant of *cpb2*-harbouring *C. perfringens* cultures (set to pH 7) was added to the protein A sepharose CL-4B beads loaded with anti-beta2 antibody and the mixture was incubated overnight at 4 °C. The mixture was then centrifuged at 500 *g* for 2 min and the supernatant was saved at 4 °C for cell culture experiments. Removal of beta2 toxin from supernatants was verified by Western blot analysis.

Degradation of beta2 toxin from supernatant by trypsin treatment

Supernatant of *cpb2*-harbouring *C. perfringens* cultures (set to pH 7) was treated with 1 mg/ml trypsin (BDH Biochemicals) for 5 min at 37 °C or, as a control, incubated for 5 min at 37 °C without trypsin. The supernatants were immediately used for cell culture experiments after incubation. Degradation of beta2 toxin was verified by Western blot analysis.

Cell experiments

All supernatants were applied to confluent Caco-2 and IPI-21 cells which were then incubated at 37 °C for specified periods. The persistence of the beta2 toxin during cell experiments was validated by Western blot analysis. After incubation the supernatants were collected and diluted 1:20 in distilled water in a 96-well microplate (Greiner Bio-one). Lactate dehydrogenase (LDH) levels released by damaged cells were measured by a cytotoxicity detection assay in order to analyze cell damage (LDH, Roche). Percentage of cell death was subsequently determined by measuring absorbance at 490 nm using a Microplate Reader (Biorad). Experiments were performed three times in triplicate.

Purification of beta2 toxin

Purified recombinant His-tagged beta2 toxin was produced as described (Bacciarini et al., 2003) using an *Escherichia coli* strain containing a plasmid encoding *cpb2* (Jf 2128, pJFbeta2M1) kindly provided by Prof. J. Frey, Berne, Switzerland. After purification, the beta2 toxin was stored at -20 °C. The purification of beta2 toxin was evaluated by Coomassie blue staining (Thermo Scientific) and Western blot analysis. Rabbit polyclonal anti-beta2 antibody serum was obtained by immunisation of rabbits with the purified beta2 toxin (Eurogentec).

Western blot

Beta2 toxin was detected by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting with rabbit polyclonal anti-beta2 antibody serum followed by an appropriate porcine secondary antiserum coupled to peroxidase (DakoCytomation). The reaction was visualized using chemiluminescence (ECL Plus Western blotting detection reagents, GE Healthcare).

Statistical analysis

To achieve normality, the outcome percentage cell death of the first experiment in which the beta2 toxin was removed by antibodies and the outcome percentage of IPI-21 cell death of the last experiment were ln-transformed. Explanatory factors (categorical variables) for the first experiment were intervention (s, pi, ab, 88, sb), time, and the interaction between intervention and time. Explanatory factors for the second experiment were intervention (s, 37, tr, 88), time, and the interaction between intervention and time. Explanatory factor for the last experiment was intervention (s, pi, ab, 37, tr, 88). As experiments were performed three times a generalized linear mixed model was applied (Pinheiro et al., 2013) with repetition as random effect to account for the correlated observations within each experiment. The Akaike's Information Criterion (AIC) was used to select the best model (the smaller the better). The statistical package R version 2.15.2 was used for data analysis (R core team, 2012). Significance was considered when $p < 0,05$.

Results

To determine the level of beta2 toxin produced by Cp15, Cp107, and Cp147 during incubation, the purificity and concentration of purified His-tagged beta2 toxin were firstly determined. Coomassie blue staining and Western blot analysis of the purified beta2 toxin showed only one clear band slightly larger than predicted due to the His-tags. The concentration of purified His-tagged beta2 toxin was 20 µg/ml as determined by spectrophotometric analysis. This was roughly 20-fold higher than the beta2 toxin concentration in supernatant of Cp15 and 200-fold higher than the beta2 toxin concentration in supernatant of Cp107 and Cp147 as determined by Western blot analysis. So, the concentration of beta2 toxin produced by Cp15 was roughly 1 µg/ml, while the concentration of beta2 toxin produced by Cp107 and Cp147 appeared to be roughly 0.1 µg/ml.

To study the cytotoxic effect of supernatant produced by *cpb2*-harbouring *C. perfringens,* Caco-2 cells were incubated with supernatant of Cp15, Cp107, or Cp147. The supernatants of Cp107 and Cp147 were not cytotoxic to Caco-2 cells as determined by LDH release (data not shown). In contrast, LDH levels released by Caco-2 cells increased during the first two

hours of incubation with supernatant of Cp15 after which LDH levels remained the same (Fig. 1b). Incubation with supernatant of Jf2288 caused only a slight LDH release similar to the LDH release after incubation with plain Schaedler anaerobe broth (Fig. 1b). Supernatant of Jf2288 was used to verify a baseline level of LDH release in further experiments.

In order to determine the significance of the beta2 toxin in the development of cell cytotoxicity caused by *cpb2*-harbouring *C. perfringens,* Caco-2 cells were incubated with supernatants of Cp15 or supernatant of Cp15 from which the beta2 toxin was removed (Fig. 1a). The percentage of Caco-2 cell death did not decrease after incubation with supernatants of which the beta2 toxin was removed (Fig. 1b).

To confirm this result, the beta2 toxin in the supernatant of Cp15 was digested by trypsin treatment at 37 °C (Gibert et al., 1997). To exclude a potential effect of the incubation temperature, supernatant was simultaneously incubated at 37 °C without trypsin (Fig. 2a). Caco-2 cells were subsequently incubated for 2 hours with the supernatant of Cp15, the supernatant of Cp15 incubated at 37 °C, or trypsin-treated supernatant of Cp15. The percentage of cell death did not decrease after incubation with supernatant of Cp15 from which the beta2 toxin was degraded by trypsin treatment (Fig. 2b).

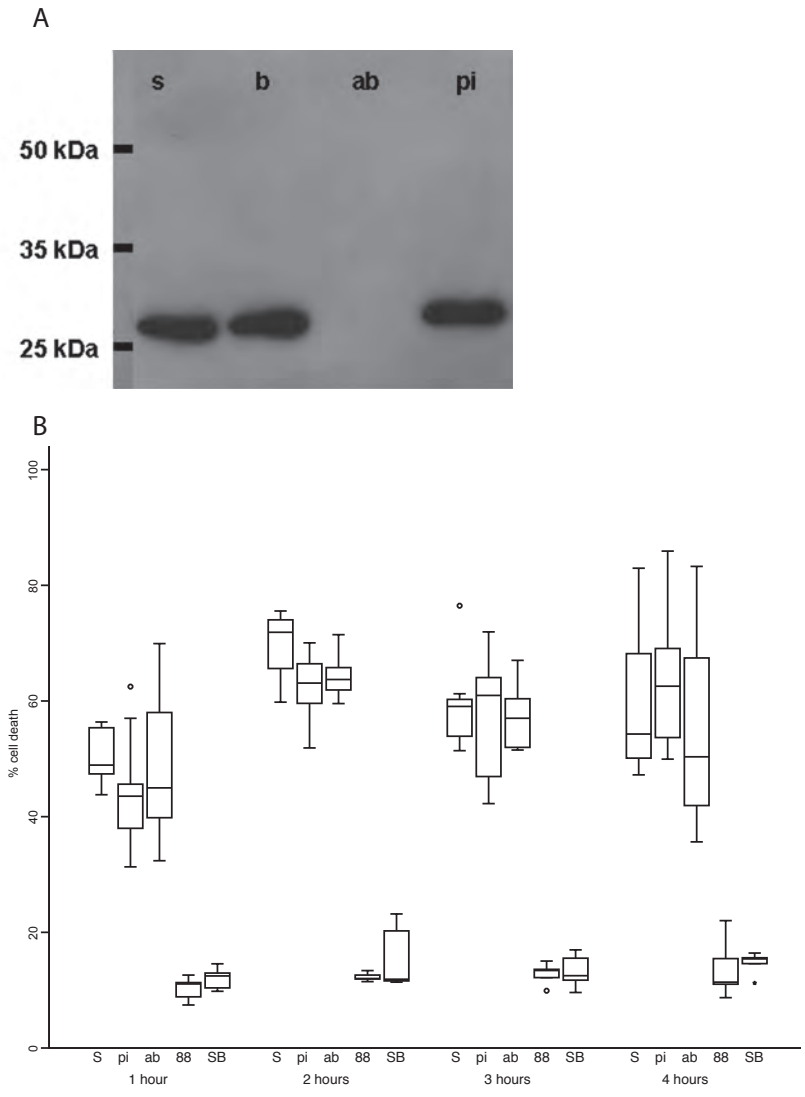

Figure 1:

A: Western blot assay of beta2 toxin in supernatant of Cp15 without further treatment (s); after incubation with plain sepharose beads (b); after incubation with sepharose beads previously incubated with anti-beta2 toxin antibody serum (ab); and, after incubation with sepharose beads previously incubated with pre-immune serum (pi).

B: A boxplot representing the percentage of Caco-2 cell death after incubation with supernatant of Cp15 (s); supernatant of Cp15 after incubation with sepharose beads previously incubated with pre-immune serum (pi); supernatant of Cp15 after removal of beta2 toxin (ab); supernatant of Jf2288 (88); or, Schaedler anaerobe broth (sb) after 1, 2, 3, and 4 hours of incubation. The p value for the difference between the mean percentage of cell death of s and pi, of s and ab, and of pi and ab was > 0,05 and that for the difference between the mean percentage of cell death of s, pi, or ab and 88 or sb < 0,001. Median (horizontal line), interquartile range (rectangle), range (vertical line), outliers (o), and extremes (*) are shown.

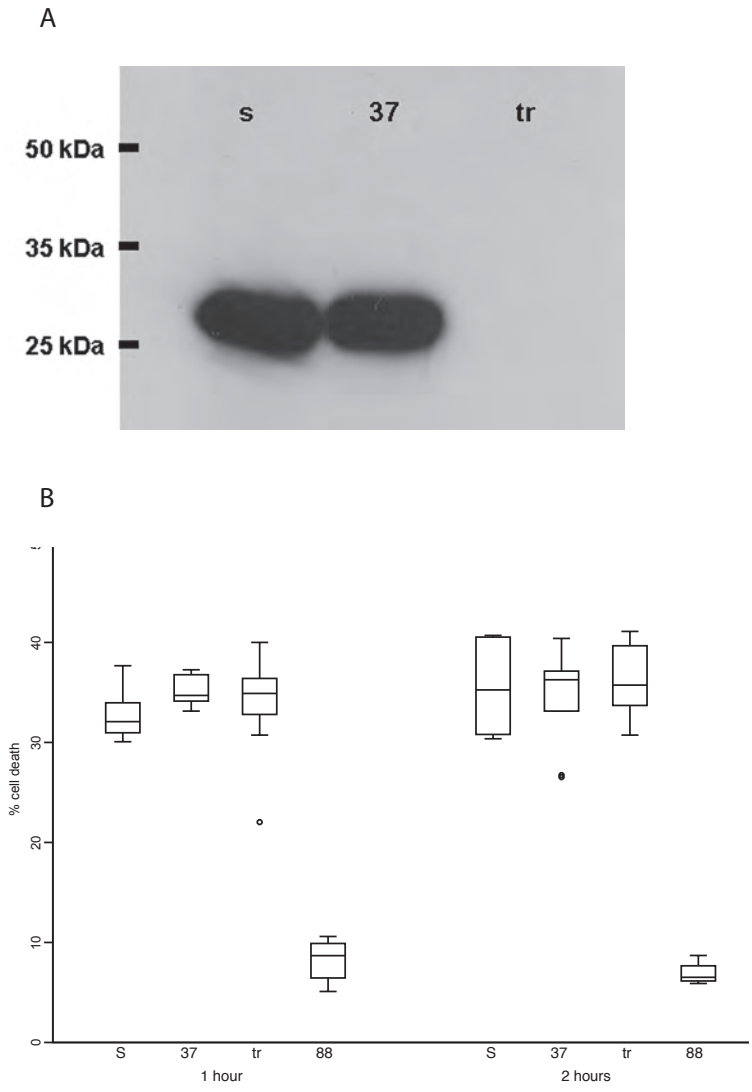

Figure 2:

A: Western blot analysis of toxin in supernatant of Cp15 without further treatment (s); after incubation at 37 °C (37); and, after 1 mg/ml trypsin-treatment at 37 °C (tr).

B: A boxplot representing the percentage of Caco-2 cell death after incubation with supernatant of Cp15 directly used after storage at 4°C (s); supernatant of Cp15 after incubation at 37 °C (37); trypsin-treated supernatant of Cp15 (tr); and, supernatant of Jf2288 (88). The p value for the difference between the mean percentage of cell death of s and 37, s and tr, and 37 and tr was > 0,05 and that for the difference between the mean percentage of cell death of s, 37, or tr and 88 < 0,001. Median (horizontal line), interquartile range (rectangle), range (vertical line), and outliers (*) are shown.

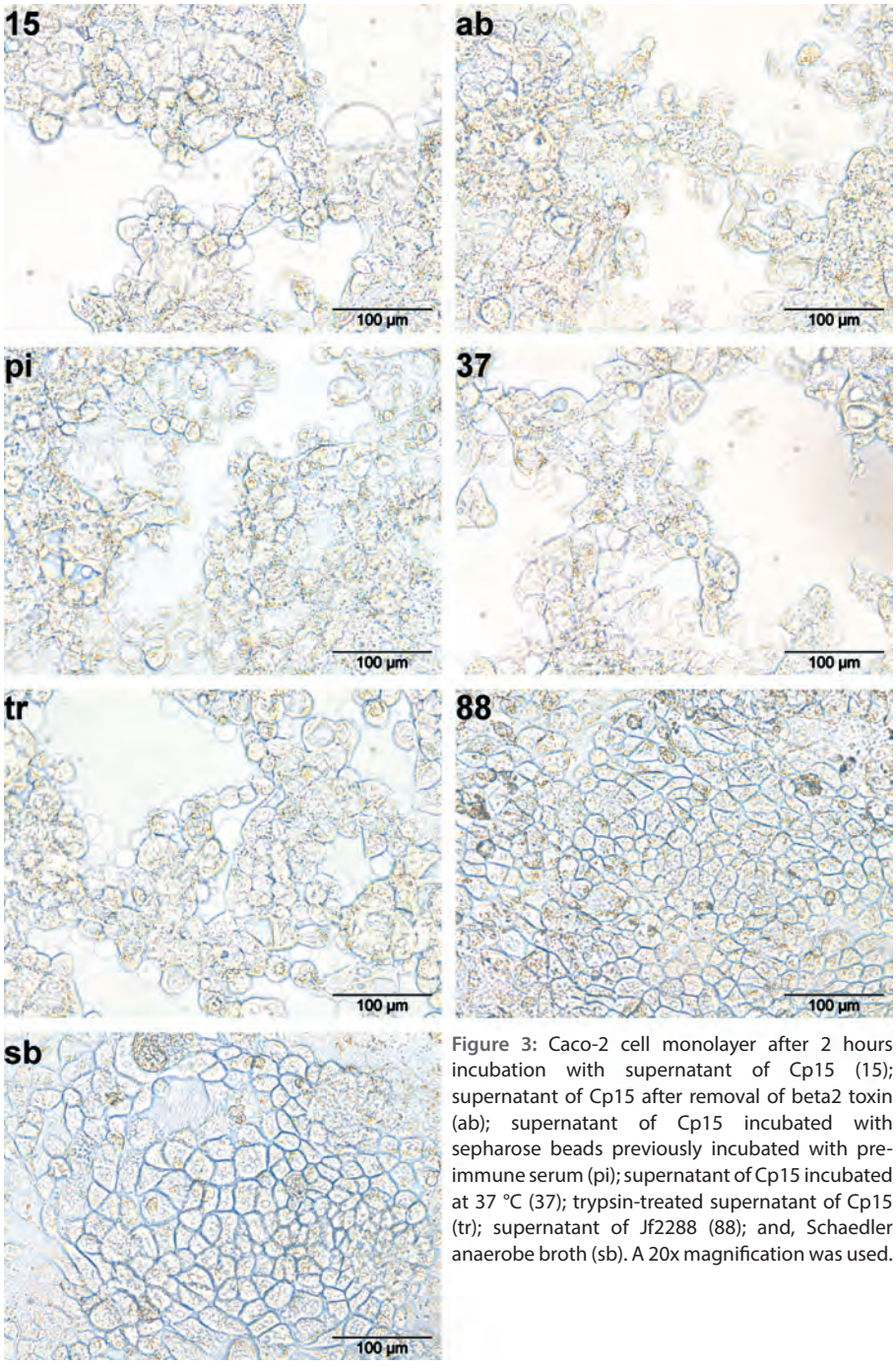

Figure 3: Caco-2 cell monolayer after 2 hours incubation with supernatant of Cp15 (15); supernatant of Cp15 after removal of beta2 toxin (ab); supernatant of Cp15 incubated with sepharose beads previously incubated with pre-immune serum (pi); supernatant of Cp15 incubated at 37 °C (37); trypsin-treated supernatant of Cp15 (tr); supernatant of Jf2288 (88); and, Schaedler anaerobe broth (sb). A 20x magnification was used.

The Caco-2 cell monolayers incubated with treated supernatant of Cp15 still exhibited severe cell damage and detachment (Fig. 3). Plain Schaedler medium containing 1 mg/ml trypsin caused only slight LDH release (data not shown) which was similar to the level of LDH release after incubation with plain Schaedler medium alone or to the level of LDH release after incubation with supernatant of Jf2288.

To exclude the possibility that the lack of an effect of beta2 toxin on Caco-2 cells was strain dependent, the experiments were repeated with other human and porcine *cpb2*-harbouring *C. perfringens* strains. Two other tested human strains (one harbouring the consensus 3 *cpb2* allele and one harbouring the atypical *cpb2* allele) produced a similar level of beta2 toxin compared to Cp107 or Cp147. No increase of LDH release was obtained after incubation of Caco-2 cells with the human strain harbouring the atypical *cpb2* allele. The human strain harbouring the consensus 3 *cpb2* allele induced variable levels of cell cytotoxicity, but removal or degradation of beta2 toxin did not decrease the induction of cell cytotoxicity. Two other tested porcine strains (harbouring the consensus 2 allele of *cpb2*) produced a similar level of beta2 toxin compared to Cp15 and the increased level of LDH release by Caco-2 cells caused by supernatant of these two strains was not influenced by degradation or removal of beta2 toxin from the supernatants.

The effect of the beta2 toxin on intestinal cell cytotoxicity might depend on the cell line used. Therefore, experiments were performed incubating the porcine intestinal cell line IPI-21 with the supernatant of the different strains. The supernatant of Cp15 caused a high percentage of cell death after 2 hours of incubation. No differences in LDH release by IPI-21 cells were identified after 2 hours incubation with either supernatant of Cp15 or supernatant of Cp15 from which the beta2 toxin was removed. The supernatant in which the beta2 toxin was digested by trypsin was slightly less cytotoxic for IPI-21 cells compared to untreated supernatant of Cp15, but not compared to the supernatant of Cp15 incubated at 37 °C (Fig. 4). Incubation with plain Schaedler broth or 1 mg/ml trypsin in plain Schaedler broth caused only a slight LDH release which was similar to the level of LDH release after incubation with supernatant of Jf2288. The IPI-21 experiments were repeated with the human *C. perfringens* strains, which resulted in similar outcomes as obtained in the Caco-2 cell experiments (data not shown). The lack of an effect of the beta2 toxin produced by porcine *C. perfringens* on porcine intestinal cells may be

due to a difference in cell morphology between IPI-21 cells which originate from the ileum of an adult miniature boar (Kaeffer et al., 1993) and intestinal cells of neonatal piglets. However, supernatant of Cp15 increased LDH release by porcine IPEC-J2 intestinal epithelial cells originating from neonatal piglet mid jejunum (Brosnahan and Brown, 2012), which was not counteracted by degradation of beta2 toxin after trypsin treatment (data not shown).

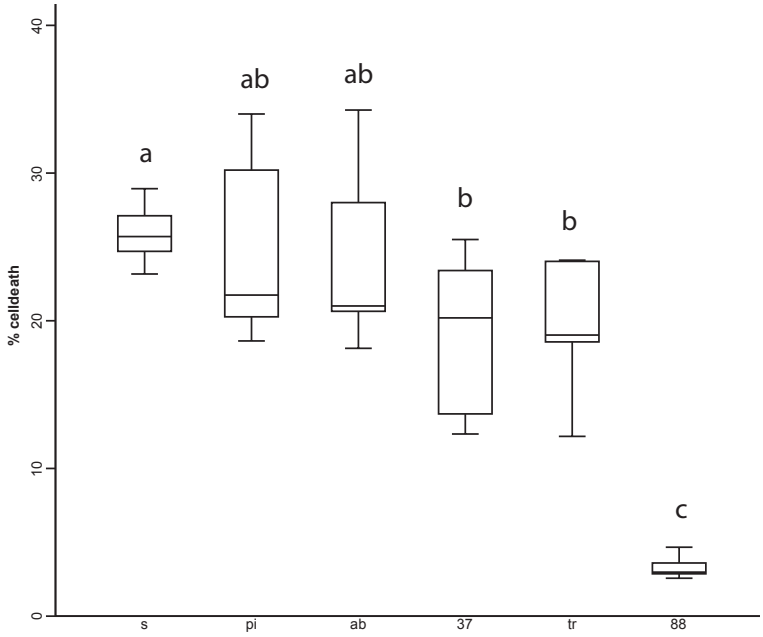

Figure 4: Boxplot representing the percentage of IPI-21 cell death after 2 hours incubation with supernatant of Cp15 (s); supernatant of Cp15 after incubation with sepharose beads previously incubated with pre-immune serum (pi); supernatant of Cp15 after removal of beta2 toxin (ab); supernatant of Cp15 after pretreatment at 37 °C (37); trypsin-treated supernatant of Cp15 (tr); and, supernatant of Jf2288 (88). A different letter indicates significant differences ($p < 0,05$) between groups in the mean percentage of cell death. Median (horizontal line), interquartile range (rectangle), and range (vertical line) are shown.

Discussion

The role of *cpb2*-harbouring *C. perfringens* in the development of diarrhoea has not yet been clarified. *In vitro* studies might represent the first step in clarifying the role of the beta2 toxin in the induction of intestinal disease. For that reason, the cytotoxic effect of supernatant of *cpb2*-harbouring *C. perfringens* strains on intestinal cells was studied. However, the cytotoxicity of the beta2

toxin might be influenced by the diversity in amino acid sequences of the toxin encoded by the various alleles (consensus 2, consensus 3, atypical) (Fisher et al., 2005, van Asten 2008). Protein sequence alignment showed a 63 % identity between the beta2 toxin encoded by either the consensus 2 *cpb2* allele (Gibert et al., 1997) or the consensus 3 *cbp2* allele (Vilei et al., 2005) and the atypical *cpb2* allele (Fisher et al., 2005) and a 92 % identity between the beta2 toxin encoded by the consensus 2 *cpb2* allele (Gibert et al., 1997) and the consensus 3 *cpb2* allele (Vilei et al., 2005). Therefore, the effect of supernatants of *C. perfringens* harbouring the consensus 2 *cpb2* allele, the consensus 3 *cpb2* allele, and the atypical *cpb2* allele were all tested for the induction of intestinal cell cytotoxicity.

Supernatants of one human consensus 3 and two human atypical *cpb2*-harbouring *C. perfringens* strains were not cytotoxic to both Caco-2 and IPI-21 cell lines and removal or degradation of beta2 toxin did not influence cytotoxicity caused by another human consensus 3 *cpb2*-harbouring *C. perfringens* strain. The concentration of beta2 toxin produced by human *cpb2*-harbouring *C. perfringens* strains was lower than the earlier determined cytotoxic concentration of 0.3 µg/ml purified beta2 toxin encoded by the consensus 3 allele of *cpb2* or 4 µg/ml purified beta2 toxin encoded by the atypical allele of *cpb2* of human *C. perfringens* strains (Fisher et al., 2005). Furthermore, the concentration of beta2 toxin produced by human *cpb2*-harbouring *C. perfringens* strains was considerably lower than the cytotoxic concentration of 50 µg/ml beta2 toxin in concentrated culture supernatants (Fisher et al., 2005). These results argue the relevance of studying the cytotoxic effect of higher concentrations of beta2 toxin than the concentration of beta2 toxin actually produced by *cpb2*-harbouring *C. perfringens* and might indicate a limited role for beta2 toxin in the development of cell cytotoxicity and subsequent diarrhoea in human.

The porcine *cpb2*-harbouring *C. perfringens* strains produced a roughly ten times higher level of beta2 toxin than the human strains which is in agreement with earlier study (Harrison et al., 2005). The supernatants of porcine *cpb2*-harbouring *C. perfringens* strains induced a cytotoxic effect on both cell lines; however, this cytotoxic effect did not decrease after removal or degradation of beta2 toxin. This suggests that other substances than beta2 toxin are responsible for the IPI-21 and Caco-2 cell cytotoxicity induced by *cpb2*-harbouring *C. perfringens*. The concentration of beta2 toxin in the supernatant of the porcine *C. perfringens* strains was roughly 1 µg/ml, which is considerably

lower than the earlier described cytotoxic concentration of 20 µg/ml beta2 toxin derived from a porcine *cpb2*-harbouring *C. perfringens* strain to intestinal 407 cells (Henle and Deinhardt, 1957; Gibert et al., 1997). These results, again, argue the relevance of studying the cytotoxic effect of high concentrations of purified beta2 toxin and might be an indication for the absence of a potential role for beta2 toxin in the development of diarrhoea.

Intestinal circumstances may influence the level of beta2 toxin in the intestinal tract and increase the *in vivo* relevance of beta2 toxin in the development of intestinal cell cytotoxicity and subsequent diarrhoea. E.g. nutritional anti-trypsin factors may cause an accumulation of trypsin-sensitive beta2 toxin (Gibert et al., 1997) which may lead to higher concentrations of beta2 toxin which might be cytotoxic and in the end result in diarrhoea. Anti-trypsin in colostrum has been determined as a key-factor in the development of diarrhoea in neonatal piglets associated with *C. perfringens* producing beta toxin, another trypsin-sensitive toxin (Songer and Uzal, 2005). However, beta toxin seems to be considerably more cytotoxic to intestinal cells than beta2 toxin. Earlier experiments determined a cytotoxic effect of 0.4 µg/ml purified beta toxin derived from a porcine *C. perfringens* strain to intestinal 407 cells which concentration was a 50-fold lower than the cytotoxic concentration of purified beta2 toxin derived from a porcine *C. perfringens* strain to intestinal 407 cells (Gibert et al., 1997).

In conclusion, no significant role for the beta2 toxin in the occurrence of *in vitro* cell cytotoxicity caused by porcine and human *cpb2*-harbouring *C. perfringens* could be established using Caco-2 and IPI-21 cell lines. These results seem to argue the hypothesis that beta2 toxin is involved in the development of cell cytotoxicity and subsequent diarrhoea in humans and piglets. If any role for the beta2 toxin in the development of diarrhoea exists, this role might be depending on environmental circumstances which might increase the level of beta2 toxin in the intestinal tract. However, these intestinal circumstances might influence other potential intestinal pathogens too. Therefore, *in vivo* studies investigating a possible role for both beta2 toxin and predisposing factors in the development of intestinal disease are necessary to definitely determine or exclude a potential role for beta2 toxin in the development of cell cytotoxicity and subsequent diarrhoea.

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Chapter 6

The effect of *Lactobacillus fermentum* on beta2 toxin production by *Clostridium perfringens*

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Abstract

Clostridium perfringens although a member of the normal gut flora is also an important cause of intestinal disease in animals and, to a lesser extent, in humans. Disease is associated with the production of one or more toxins and little is known about environmental influences on the production of these toxins.

One of the health promoting effects of lactic acid bacteria (LAB) is the establishment and maintenance of a low pH in the intestine since an acidic environment inhibits the growth of many potentially harmful bacteria. Here the effect of the LAB *Lactobacillus fermentum* on beta2 toxin production by *C. perfringens* is described. Co-culturing *C. perfringens* with *L. fermentum* showed that under *in vitro* conditions *L. fermentum* was capable of silencing beta2 toxin production by *C. perfringens* without influencing bacterial viability. The reduction in toxin production was shown to be most likely as a result of the decline in pH. Quantitative PCR showed that the reduction in beta2 toxin production was due to a decrease in *cpb2*-mRNA. These results suggest that in the intestine the production of beta2 toxin by *C. perfringens* might be regulated by other members of the normal intestinal flora.

Introduction

The normal intestinal flora comprises several hundred bacterial species and is recognized as being beneficial to its host (Nicholson et al., 2005; Tancrède, 1992). The species contribute to this phenomenon in various ways. For instance some species break down undigested or indigestible food, whereas others synthesize vitamins or short chain fatty acids. Additionally, these bacteria prevent the colonization of the gut by potential pathogens (Hooper et al., 1998). Lactic acid bacteria (LAB) such as those belonging to the genera *Bifidobacterium, Lactobacillus*, *Enterococcus*, and *Streptococcus* are members of the normal intestinal flora. They not only prevent colonization by their actual presence, but they also prevent growth and colonization by pathogens through their production of acid (Naidu et al., 1999). Furthermore, in an attempt to maintain the status quo of their environment, it is known that bacteria may influence intra- and inter-special growth and gene expression through a phenomenon called quorum sensing (Miller et al., 2001).

Clostridium perfringens although being a member of the normal intestinal flora, is regarded as one of the most important causes of intestinal disease in farm animals, wild animals, and to a lesser extent in humans (Petit et al., 1999). Strains of *C. perfringens* are classified into one of five toxin types (A-E) based on the four major toxins they can produce (alpha, beta, epsilon, and iota toxin). Each toxin type is associated with specific diseases of various animal species and of humans (Songer, 1996). In addition to the four major toxins, all types of *C. perfringens* may carry other toxin encoding genes, including *cpb2*, the gene which encodes the beta2 toxin (Gibert et al., 1997). The presence of *cpb2*-positive *C. perfringens* strains in the intestine has been associated with intestinal disease in humans (Fisher et al., 2005), ruminants (Lebrun et al., 2007), horses (Herholz et al., 1999), and pigs (Bueschel et al., 2003; Waters et al., 2003). However, *cpb2*-positive *C. perfringens* strains have also been reported in animals and humans without any signs of intestinal disease (Bueschel et al., 2003; Carman et al., 2008; Lebrun et al., 2007).

Most likely, toxin production by *C. perfringens* and the subsequent induction of disease is initiated by intestinal environmental changes (Schotte et al., 2004; Songer, 1996). It is known that antibiotic use can lead to such a change by shifting the percentage composition of the intestinal flora species (Fisher et al., 2005). Such a shift is generally thought to favor a proliferation of *C.*

perfringens with a resultant higher production of toxin(s). Alternatively, it might well be that the shift in the equilibrium of the normal intestinal flora leads to a deregulation of gene expression of the remaining members of the intestinal flora including *C. perfringens*.

This study describes the influence of *Lactobacillus fermentum*, a member of the normal intestinal flora (Dogi and Perdigón, 2006; Kinoshita et al., 2007; Shirkey et al., 2006), on the production of beta2 toxin by *C. perfringens* and unravels the underlying mechanism.

Experimental procedures

Bacterial strains and culture conditions

Cp15, a porcine *C. perfringens* type A strain harbouring *cpb2* (originally designated JF2251, kindly provided by Prof. J. Frey, Berne, Switzerland) was used as a representative of the beta2 toxin-producing *C. perfringens* species. *Lactobacillus fermentum* strain 104R, a porcine strain (Henriksson et al., 1991) was used as the representative strain for the *Lactobacillus fermentum* species.

Unless otherwise stated resuspended stationary overnight cultures of *C. perfringens* strain Cp15 were used in all experiments. After centrifugation (10 min at 4000 *g*) and resuspension of the pelleted bacteria in fresh growth medium to the original volume, the cultures were (metabolically) active as demonstrated by the production of acid, beta2 toxin, and gyrase mRNA. The use of these active stationary *C. perfringens* cultures enabled the study of the actual influence of *L. fermentum* on beta2 toxin production by *C. perfringens* without the confounding influence of changes in numbers of *C. perfringens* on beta2 toxin production.

All experiments were performed in MRS broth (Oxoid) since this provided the best overall growth environment for both species as *L. fermentum* growth was severely impaired in Schaedler broth (Oxoid). Anaerobic conditions were obtained using jars and the Anoxomat gas exchange system (Mart Microbiology). Immediately after opening the jars, tubes were closed in order to maintain the anaerobic condition as best as possible. Manipulations, such as the removal of supernatants and resuspension of the pellets, were performed under aerobic conditions.

Co-culture experiments of *C. perfringens* and *L. fermentum*

Lactobacillus fermentum strain 104R anaerobically cultured in MRS broth (Oxoid) set to pH 7 with NaOH at 37° C for 24 h (stationary phase) was divided in two equal parts. One half was centrifuged, followed by the pellet being resuspended in MRS broth (pH 7) and incubated for another hour (L1). The other half was reincubated for another hour without further treatment, centrifuged, and the pellet resuspended in fresh MRS broth at pH 7 (L). An overnight culture of Cp15 was equally divided over 12 tubes and centrifuged, with the resulting pellets being resuspended in either MRS broth with pH 7 (M), in the *L. fermentum* suspension in fresh MRS broth (pH 7) (L), or in the *L. fermentum* culture that was incubated for one hour in fresh MRS broth (pH 7) (L1). For each combination a tube was incubated for either 1, 2, 3, or 4 hours. At each time point, the number of colony forming units (CFU) of the various cultures was determined and the pH was measured. The cultures were centrifuged and the supernatants were analyzed by Western blot for the presence of beta2 toxin.

Establishing beta2 toxin degradation by *L. fermentum*

An overnight culture of *L. fermentum* was divided in two equal parts. After centrifugation (10 min, 4000 *g*), pellets were resuspended in either a two hour Cp15 culture in MRS broth (pH 7) *or* in the supernatant of a two hour Cp15 culture and incubated anaerobically at 37° C for two hours. The suspensions were then centrifuged (10 min, 4000 *g*) and the supernatants were analyzed by Western blot for the presence of beta2 toxin.

Beta2 toxin production at various pH

A stationary (16 hours) culture of Cp15 in Schaedler broth was equally divided into 12 tubes and centrifuged for 10 min at 4000 *g* at 37° C. Pellets were resuspended in MRS broth set at pH 5, pH 6, or pH 7 using either HCL or NaOH and incubated anaerobically at 37° C for either 1, 2, 3, or 4 h. After each time period the number of CFU's of the various cultures was determined and the pH of each culture was measured. The cultures were again centrifuged 10 min at 4000 *g*, and the supernatants were analyzed for the presence of beta2 toxin by Western blot. Pellets were used for RNA isolation and subsequent quantitative (Q-)PCR analysis.

Co-culture experiments on Caco-2 cells

Human-derived enterocyte-like Caco-2 cells (Pinto et al., 1983) were seeded in 12-well-plates (Greiner) at 80,000 cells/cm^2 and cultured in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) supplemented with 0.03 % L-glutamine (Invitrogen) 1 % non-essential amino acids (Flow Laboratories), 10mM sodium bicarbonate, 25 mM HEPES (Flow Laboratories), 50 µg/ml gentamicin (Gibco), and 20% fetal bovine serum (Lonza) in a humid atmosphere of 5% CO_2 at 37° C. At day 5, the medium was removed and the monolayers were washed twice with 0.01M phosphate buffered saline pH 7.3 (PBS) and incubated with plain DMEM. After 1 h, DMEM was removed and MRS broth (pH 7) with or without bacteria was added to the cells.

A 24 h MRS (pH 7) broth culture of *L. fermentum* was divided into two equal parts. One half was centrifuged and the pellet was resuspended in MRS broth (pH 7) and put on the cells (L1) in a number of wells, while the DMEM on the remaining wells was replaced by MRS broth. Plates were incubated anaerobically for 1 h at 37° C. The other half of the *L. fermentum* culture was reincubated for another hour without further treatment. Equal parts of an overnight culture of Cp15 were centrifuged and resuspended in overlaying MRS broth without lactobacilli (M), in overlaying MRS broth already containing *L. fermentum* for 1 hour (L1), or together with the pellet of the reincubated *L. fermentum* culture resuspended in the MRS broth pH 7 which had overlaid the Caco-2 cells for 1 hour (L). The plates were then incubated anaerobically for either 1, 2, 3, or 4 hours. Anaerobic conditions were obtained using jars and the Anoxomat gas exchange system (Mart Microbiology). After each time period the number of CFU of *C. perfringens* and *L. fermentum* was determined and the pH was measured. Finally the cultures were centrifuged (10 min, 4000 *g*) and supernatants were analyzed by Western blot for the presence of beta2 toxin.

RNA isolation and cDNA synthesis

Bacterial pellets were washed with distilled water and resuspended in STET buffer (0.1M NaCl, 10 mM Tris/HCl pH 8.0, 1mM EDTA pH 8.0, 5 % Triton X100) containing 20 mg/ml lysozyme (Merck) and 100 µg/ml proteinase-K (Merck) and incubated for 4 h at 37° C with continual agitation. Next the suspensions were centrifuged for 5 min at 16000 *g* and the resultant pellets were used for RNA isolation using Trizol (Invitrogen) according to manufacturer's protocol with one modification; after the addition of isopropanol, mixtures were kept

at -20º C for 15 min. RNA concentration was measured with a Nanodrop spectrophotometer (Isogen). Residual DNA was removed with RNase free DNaseI (Fermentas). One µg of RNA was used for cDNA synthesis using the Transcriptor First Strand cDNA Synthesis Kit (Roche).

Quantitative-PCR analysis.

The amount of *cpb2*-mRNA in the samples was measured by Q-PCR. The Q-PCR mix consisted of 5 µl of cDNA, 12.5 µl SYBR Green mix (Bio-Rad Laboratories), 10 µM forward primer (5'-CAAGCAATTGGGGGAGTTTA-3'),10 µM reverse primer (5'-GCAGAATCAGGATTTTGACCA-3'), and 6.5 µl distilled water. For normalization purposes the amount of gyrase-mRNA was determined simultaneously, using 10 µM forward primer (5'- AGATATAGAAGACTTAATACAAG -3') and 10 µM reverse primer (5'- AAAGAATAATAAGTTGAGTGTG-3'). The Q-PCR program consisted of 40 cycles of 30 s denaturation at 95º C, 30 s annealing at 57º C and 15 s extension at 72º C. Results were analyzed using the IQ5 software (Bio-Rad Laboratories) and expressed as C_t *cpb2* - C_t *gyrA*.

Western blot analysis

Ten µl of supernatant was tested for the presence of beta2 toxin by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis with a monospecific polyclonal anti-beta2 toxin antibody (kindly provided by Prof. J. Frey, Berne, Switzerland) followed by a swine secondary antiserum coupled to peroxidase (Dako). The reaction was visualized by a chemiluminescence detection system (ECL Plus Western blotting detection reagents, GE Healthcare). Protein bands were quantified by densitometry using a Bio-Rad GS-700 Densitometer (Bio-Rad Laboratories).

All experiments with the exception of the degradation experiment (n=2) were performed three times. Q-PCR reaction of each cDNA sample was performed twice in triplicate.

Statistics

Due to a low intra class correlation coefficient in both the experiment with lactobacilli and pH, a general linear model (GLM) was used instead of a linear model, with random effects to account for the correlation between observations within a repetition. For the lactobacillus experiment the measured outcome was toxin production which was square-root transformed, with the fixed factors

being lactobacilli (L, L1, and M), time (1h, 2h, 3h, and 4h), repetition (1, 2, and 3) and the interaction between lactobacilli and time. For the pH experiment pH (pH 5, pH 6, pH 7), time (1h, 2h, 3h, and 4h), and repetition (1, 2, and 3) were used as block factors and the interaction between pH and time as explanatory factors. Due to non-consistency of variance, the best GLM model for the experiment with lactobacilli and the pH experiment was again fitted by an iterated re-weighted least squares (IWLS) method (Venables and Ripley, 2002) using the median absolute difference for weighting the measurements. To analyze the data from the experiment on the production of beta2 toxin in the presence of lactobacilli and Caco-2 cells, a mixed model was used with repetition as a random effect (J. Pinheiro, D. Bates, S. DebRoy, D. Sarkar, and the R Core team 2009. nlme: Linear and Nonlinear Mixed Effects Models. R package version 3.1-96). The outcome was square-root transformed and the same explanatory variables were used as for the experiment with lactobacilli. Model selection was based on the Akaikes Information Criterion (AIC). The residuals of the final models were used to check for normality and consistency of variance. For all analyses the statistical package R version 2.11.1 (R Development Core Team, 2010) was used.

Results

Beta2 toxin production by *C. perfringens* cultured in the presence of *L. fermentum*.

To study the effect of *L. fermentum* on beta2 toxin production by *C. perfringens*, Cp15 was co-cultured with *L. fermentum*. The amount of beta2 toxin produced by Cp15 in this co-culture (Fig. 1, L) was lower compared to the amount of beta2 toxin produced by Cp15 in the absence of *L. fermentum* (Fig. 1, M). This effect was enhanced when the *L. fermentum* strain was grown in fresh MRS broth pH 7 for one hour before adding Cp15 to this culture (Fig. 1, L1). Co-culturing *C. perfringens* with *L. fermentum* lowered the pH of the co-culture from 7 to 4.5 after 4 hours, while the *C. perfringens* culture without *L. fermentum* reached a final pH of 5.5 after 4 hours (Fig. 2). The pH of culture L1 had already decreased to pH 6.3 by the time Cp15 was added to the culture. After 4 hours of co-culturing a final pH of 4.5 was reached (Fig. 2). Co-culturing Cp15 with *L. fermentum* did not influence the viability of either bacterial species as the number of CFU's of the various cultures remained stable for each species during the entire experiment (data not shown).

In order to rule out the possibility that the decrease in beta2 toxin was due to a degradation by *L. fermentum, L. fermentum* was added to both a two-hour Cp15 culture and to the supernatant of such a culture and reincubated. No degradation or decrease in total amount of beta2 toxin in either combination was observed by Western blot when compared to the beta2 toxin produced by a plain Cp15 culture (Fig. 3). These experiments also demonstrate that pH did not effect the stability of the toxin since *L. fermentum*, when metabolic active, lowers the pH over time.

Beta2 toxin production by *C. perfringens* cultured at different pH values.

From the above data it could be hypothesized that beta2 toxin production by *C. perfringens* is regulated by the pH of the environment. In order to study this in more detail, Cp15 was grown in MRS broth at pH 5, pH 6, or pH 7 for several time periods. Western blot analysis revealed that at every time-point the amount of beta2 toxin produced was lower when Cp15 was grown at a lower pH compared to the production at pH 7 (Fig. 4). Total amount of beta2 toxin produced in broths that had initial pH 6 or pH 7 increased less than expected after three and four hours of incubation (Fig. 4). This was most likely caused by a decrease in the pH of the medium since the pH of both broths decreased during prolonged incubation. The broth set at pH 7 reached a pH of 5.3 after 4 hours, whereas the broth set at pH 6 already reached the (final) pH of 5.3 after 3 hours. The pH of the broth set at pH 5 remained the same during the entire experiment (Fig. 5). No influence on the viability of Cp15 by the various pH's was observed as the number of CFU's of the various cultures remained stable and no differences in number of CFU's were found between the cultures grown in MRS with a different initial pH (data not shown). Efforts to stabilize the pH of the various media were not successful since the salt concentrations needed to buffer these media at the desired pH influenced bacterial growth/survival.

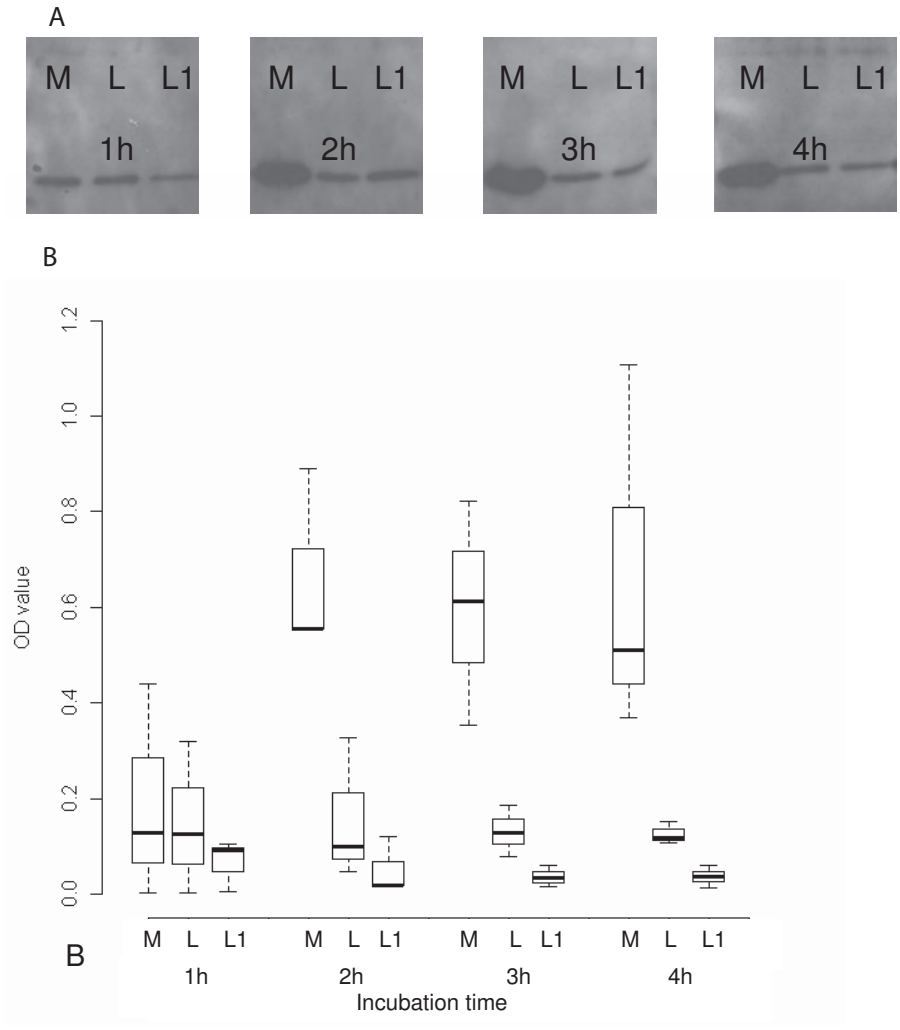

Figure 1. Differences in beta2 toxin production by *C. perfringens* strain Cp15 either alone (M), in the presence of *L. fermentum* (L), or with a 1h pre-incubation step of *L. fermentum* (L1) after 1, 2, 3, and 4 hours of incubation.

A: Western blot of a single experiment, B: A boxplot representing OD values of the various bands of three independent experiments as quantified by densitometry. P value for the differences in mean toxin level between M and L:<0.0001, between M and L1:< 0.0001, between L and L1:< 0.019. Median (horizontal line), interquartile range (rectangle), and range (dashed vertical line) are shown.

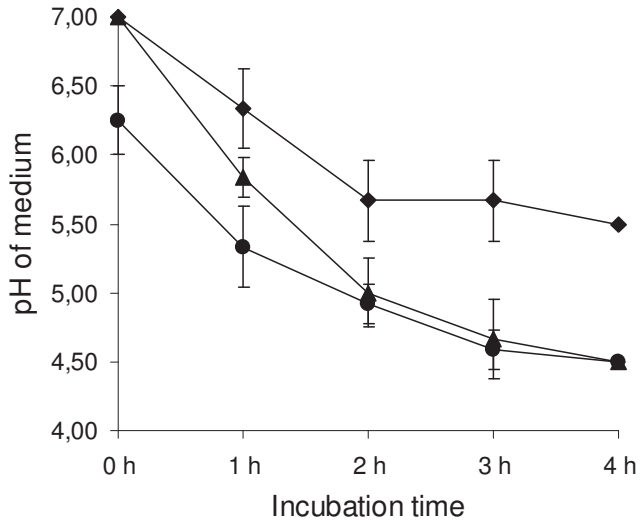

Figure 2. Decline in pH of the medium due to growth of *C. perfringens* strain Cp15 either alone (◆), in the presence of *L. fermentum* (▲), or with an 1h pre-incubation step of *L. fermentum* (●). 0 h = start of the experiment; immediately after the *C. perfringens* cultures were added. Mean of 3 individual experiments, standard deviations are given.

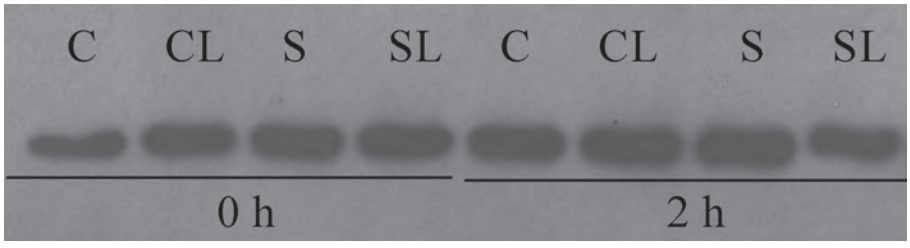

Figure 3. Stability of beta2 toxin in a culture of *C. perfringens* strain Cp15 alone (C), in a Cp15 culture to which *L. fermentum* was added (CL), in the supernatant of a Cp15 culture alone (S), or in the supernatant of a CP15 culture to which *L. fermentum* was added (SL).
0 h = start of the experiment; immediately after the *L. fermentum* cultures were added. 2 h = after 2 hours of incubation starting at the moment the *L. fermentum* cultures were added.

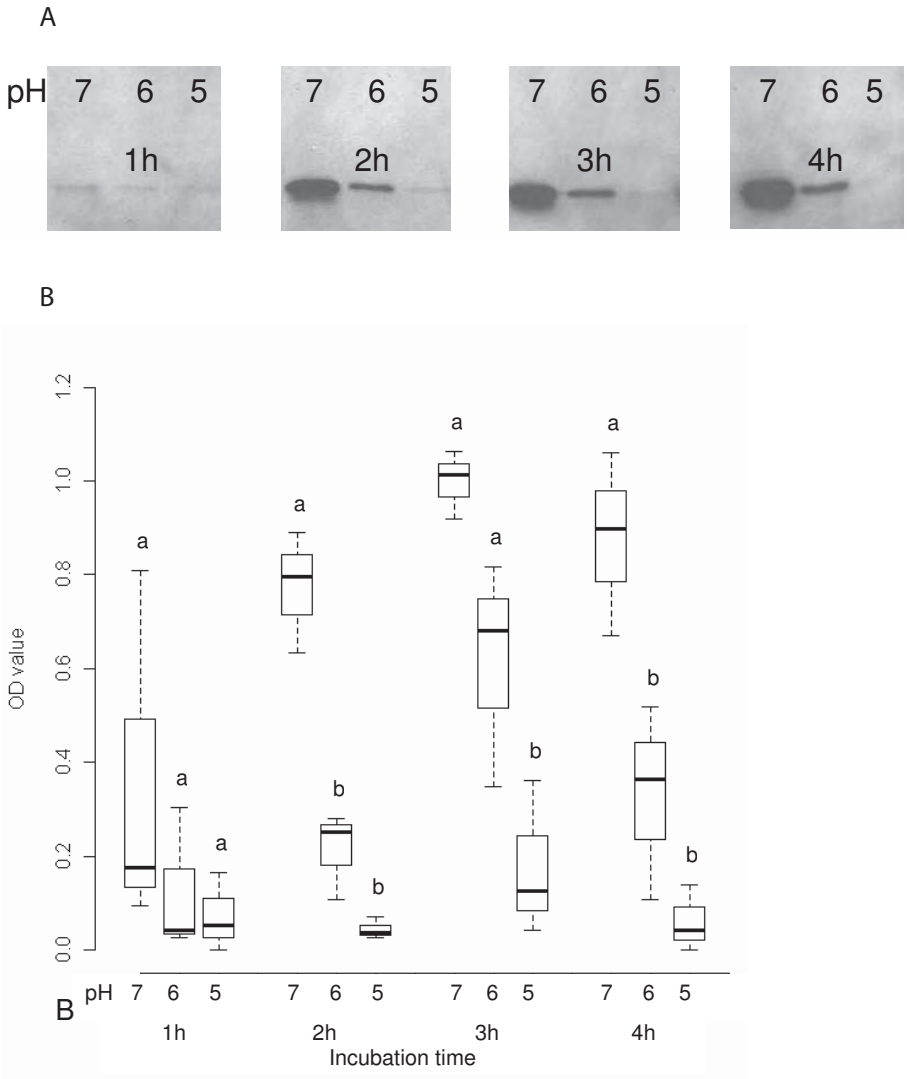

Figure 4. Differences in beta2 toxin production by *C. perfringens* strain Cp15 in MRS broth with either initial pH 7, initial pH 6, or initial pH 5 after 1, 2, 3, and 4 hours of incubation.
A: Western blot of a single experiment, B: A boxplot representing OD values of the various bands of three independent experiments as quantified by densitometry. A different letter indicates a significant difference ($p<0.05$) in mean toxin level between pH levels within time. Median (horizontal line), inter-quartile range (rectangle), and range (dashed vertical line) are shown.

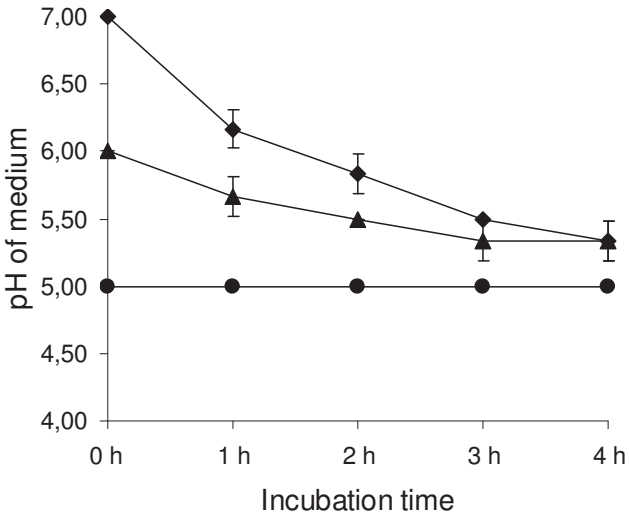

Figure 5. Decline in pH of the medium due to growth of *C. perfringens* strain Cp15 in MRS broth with either initial pH 7 (), initial pH 6 (), or initial pH 5 (). Mean of 3 individual experiments, standard deviations are given.

Q-PCR's were performed in order to address the possibility that the lower amount of beta2 toxin produced in the broth with an initial pH 6 or pH 5 compared to the amount produced at initial pH 7 was due to a lower transcription of *cpb2*. The gyrase mRNA was used as reference mRNA. In each experiment at a given time point the C_t value of gyrase between the various conditions only varied by approximately 1 C_t value, again demonstrating the equal viability of the bacterium under the various conditions. However, in contrast, the amount of *cpb2*-mRNA was greater in samples grown at a higher pH. There was at least an eight-fold difference between cultures grown at pH 7 and pH 5, two-fold difference between pH 7 and pH 6, and a four-fold difference between pH 6 and pH 5 (Fig. 6). The results from the Q-PCR clearly indicated that the pH of the environment influences the transcription of *cpb2*.

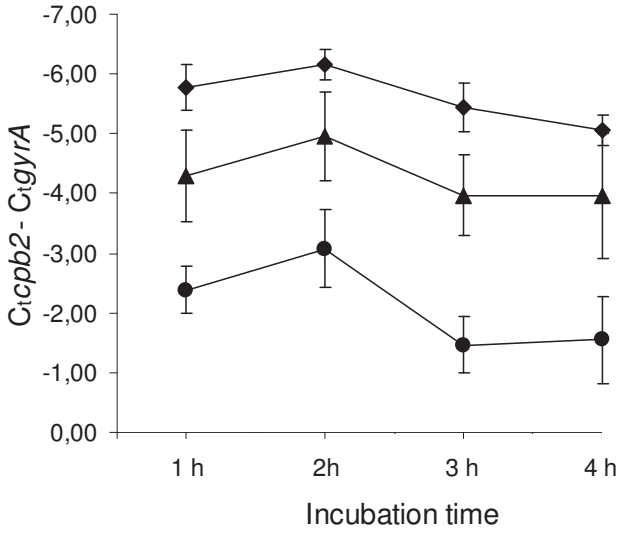

Figure 6. Differences in amount of beta2-mRNA produced by *C. perfringens* strain Cp15 in MRS broth with either initial pH 7 (♦), initial pH 6 (▲), or initial pH 5 (●). The amount of produced beta2-mRNA is relatively expressed to the amount of gyrase-mRNA produced. Mean of 2 individual experiments in triplicate, standard deviations are given.

Beta2 toxin production by *C. perfringens* co-cultured with *L. fermentum* on Caco-2 cells.

Finally we performed co-culture experiments in the presence of Caco-2 cells in order to study whether the effect of *L. fermentum* on the beta2 toxin production by *C. perfringens* seen in *in vitro* experiments is also found in a semi *in vivo* setting. The results were that for Cp15 not co-cultured with *L. fermentum* large amounts of beta2 toxin were produced at all time points, whereas in co-culture little (L) or no toxin (L1) could be detected (Fig. 7). The drop in pH of the various cultures (Fig. 8) was comparable to that in the co-culture experiments without Caco-2 cells. No effects of co-culturing or presence of Caco-2 cells on the viability of *L. fermentum* or *C. perfringens* were observed as the number of CFU's of both species remained stable during the entire experiment (data not shown).

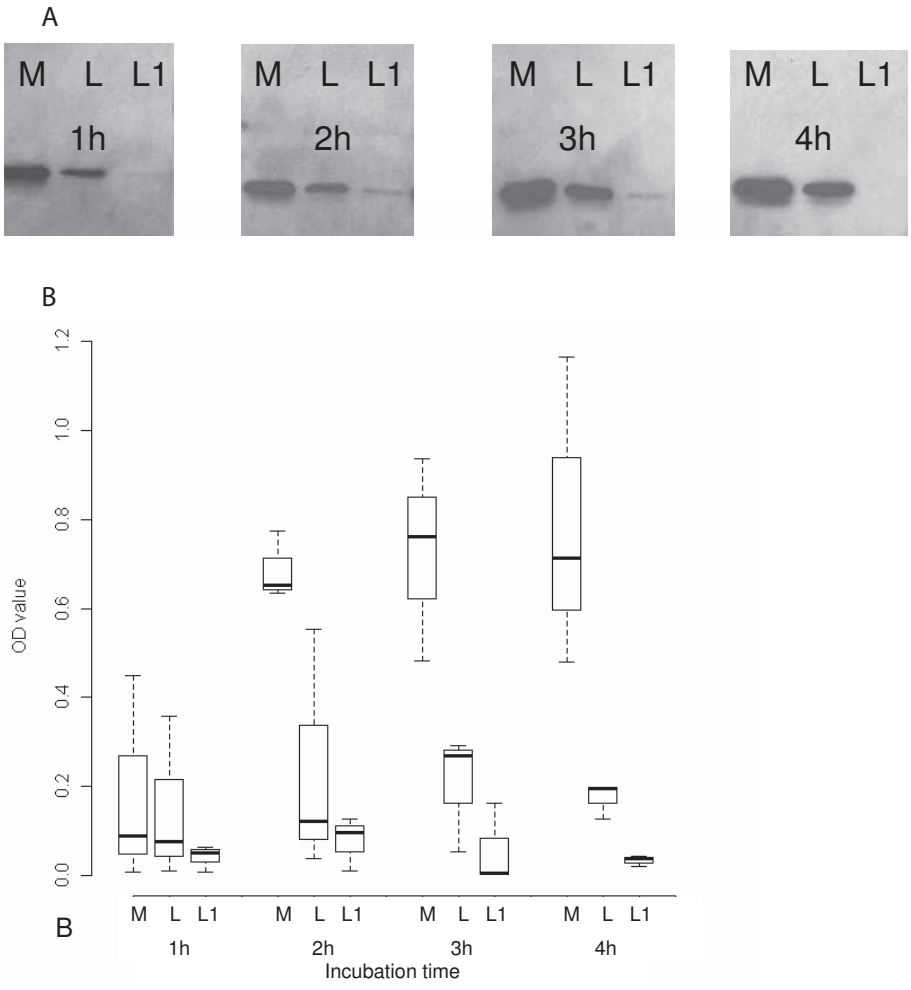

Figure 7. Differences in beta2 toxin production by *C. perfringens* strain Cp15 either alone (M), in the presence of *L. fermentum* (L), or with a 1h pre-incubation step of *L. fermentum* (L1) on a monolayer of Caco-2 cells after 1, 2, 3, and 4 hours of incubation.
A: Western blot of a single experiment, B: A boxplot representing OD values of the various bands of three independent experiments as quantified by densitometry. P value for the differences in mean toxin level between M and L:<0.010, between M and L1:< 0.0001, between L and L1:< 0.067. Median (horizontal line), interquartile range (rectangle), and range (dashed vertical line) are shown.

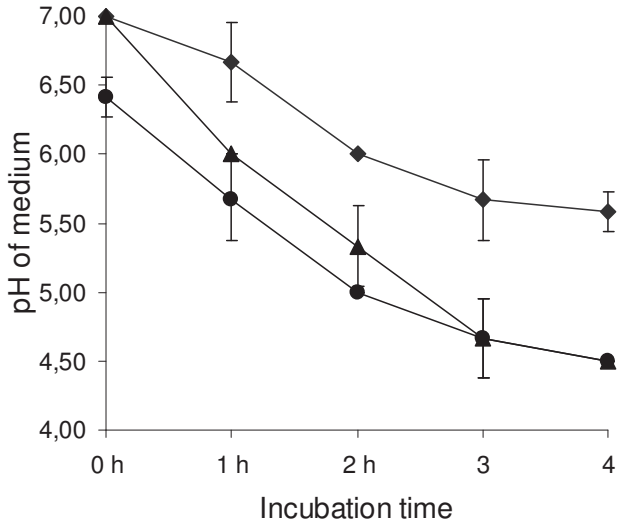

Figure 8. Decline in pH of the medium due to growth of C. *perfringens* strain Cp15 either alone (◆), in the presence of *L. fermentum* (▲), or with an 1h pre-incubation step of *L. fermentum* (●) on a monolayer of Caco-2 cells after 1, 2, 3, and 4 hours of incubation. 0 h = start of the experiment; immediately after the *C. perfringens* cultures were added. Mean of 3 individual experiments, standard deviations are given.

Discussion

Although differing in composition, the resident intestinal flora of both animals and humans contributes to the well-being of the host (Berg, 1996; Holzapfel et al., 1998). This microflora forms a delicate ecosystem in which the different bacterial species may, to a certain extent, regulate each other's growth and gene expression. Regulation is achieved through the phenomenon of quorum sensing in which there is an inter and intra species communication via released signal molecules (Bassler, 1999). It has also been shown that bacteria belonging to the microflora influence each other and other bacteria by their metabolic products (Holzapfel et al., 1998).

Here we present a study on the possible influence of acid produced by *L. fermentum* on the production of beta2 toxin by *C. perfringens*. Results from our initial experiments in which the beta2-producing *C. perfringens* strain Cp15 was co-cultured with *L. fermentum* strain 104R clearly indicate that beta2 toxin production was negatively regulated by *L. fermentum*. However, this regulation could have been achieved through either the bacterium itself or through one of its products.

It is known that the transcription of *cpb2*, the gene encoding the beta2 toxin, is under the control of the two-component VirR/VirS regulatory system (Ohtani et al., 2003). This VirR/VirS system is demonstrated to be sensitive to the signal molecule encoded by *C. perfringens agrBD* (*agrBD*$_{Cp}$), a homologue for *Staphylococcus (S.) aureus agrBD* (*agrBD*$_{Sa}$). In *S. aureus, agrBD* encodes the propeptide AgrD and its modifying protein AgrB. Once AgrD is processed the resulting autoinducing peptide (AIP) is secreted in the environment and acts as a quorum sensing signal molecule (Ohtani et al., 2009). This accessory gene regulator (agr) system is conserved within the phylum *Firmicutes* which comprises amongst others, the orders *Clostridiales* and *Lactobacillales* (Wuster and Babu, 2008). From this it could be hypothesized that beta2 toxin production in the co-culture experiments is regulated via quorum sensing. The production of beta2 toxin might then be influenced by the total amount of AIP produced by *C. perfringens* and *L. fermentum.* However, at each experiment the number of bacteria (either *C. perfringens* or *L. fermentum*) was determined and it was concluded that the viability and the total number of of CFU's of either strain remained stable in all experiments. This finding made regulation of beta2 toxin production via quorum sensing unlikely, and indicated a role for products of *L. fermentum* in the down regulation of beta2 toxin production.

It was already shown in 1964 that the production and stability of toxins produced by *C. perfringens* is influenced by the pH of the growth medium (Pivnick et al., 1964). The various toxins had different optimum pH's for their production and some, for example the alpha toxin, appeared unstable regardless of pH, whereas others, like the beta toxin, were sensitive to pH, while others remained stable regardless of pH. The beta2 toxin was first described in 1997 (Gibert et al., 1997) but there is no data regarding the influence that pH has on its production or stability.

To study the role of the acid produced by *L. fermentum* on beta2 toxin production we performed experiments in which beta2 toxin production was measured in the absence of *L. fermentum* at different pH's (5, 6, and 7) since these values roughly correspond to the normal pH range in piglet (pH 4.6-6.8) (Snoeck et al., 2004), and human intestines (pH 5.7-7.7) (Nugent et al., 2001). From these experiments it became apparent that the pH of the environment influences the production of beta2 toxin indicating that indeed the acid produced by *L. fermentum* had a clear effect on beta2 toxin production by *C. perfringens* at co-culturing. However, the stability of any beta2 toxin that was

produced was not affected by the pH. Q-PCR's performed on mRNA isolated from *C. perfringens* grown at various pH's indicated that the mechanism through which environmental pH influences beta2 toxin production is via effects on gene transcription. However, no conclusion could be drawn from our experiments on a possible influence at translational level.

Recently it has been reported that transcription of *cpb2* is rapidly upregulated when a *cpb2*-harbouring *C. perfringens* type C strain of porcine origin comes in close contact with the enterocyte-like Caco-2 cells (Vidal et al., 2009). In the intestine *C. perfringens* is in close contact with the epithelial layer. This might indicate that the production of beta2 toxin is indeed under the control of an environmental signal since it can be concluded that if this were not the case *C. perfringens* would always produce large amounts of (beta2) toxin in the intestine. To address this hypothesis we performed co-culture experiments of *C. perfringens* and *L. fermentum* in the presence of a monolayer of Caco-2 cells. These experiments clearly demonstrated that lactobacilli are capable of diminishing or even abolishing the promoting effect of cell to cell contact between *C. perfringens* and Caco-2 cells on beta2 toxin production. Based on the results of the former experiments it is likely that this effect was achieved through changes in pH. The rapid upregulation of toxin production by *C. perfringens* after contact with Caco-2 cells is mediated via the VirR/VirS system as demonstrated in experiments using mutants with a non-functional VirR/VirS system (Vidal et al., 2009). VirS, the "signaling component" of the VirR/VirS system is situated in the plasma membrane (Vidal et al., 2009). This makes it unlikely that pH has a direct effect on the binding of the $agrBD_{Cp}$ encoded signaling protein or other molecule(s) involved in the upregulation after cell to cell contact. It has been suggested that, at least in the case of upregulation of toxin production through cell-cell contact, one or more surface factors of *C. perfringens* might be involved (Vidal et al., 2009) and it is tempting to speculate that the binding to these factors is pH dependent.

It is generally accepted that *C. perfringens* related intestinal disease is initiated by the use of antibiotics which allows for *C. perfringens* overgrowth and excess toxin production after die-off of other bacterial species of the normal intestinal flora. Our findings that beta2 production is regulated by environmental pH might also explain the onset of disease after the use of antibiotics by hypothesizing that beta2 production is normally silenced by the acids produced by other members of the normal intestinal flora. Removal of

these other members by the use of antibiotics would result in a rise in local pH and subsequent toxin production by *C. perfringens*.

In conclusion, the results presented here clearly suggest that at least one member of the normal intestinal flora is of great importance in preventing toxin production by *C. perfringens* in the host. Further research should focus on the role of other members of the normal intestinal flora on silencing the toxin production by *C. perfringens*. Also, the underlying mechanism should be a subject for further studies since understanding this mechanism might lead to new ways of preventing disease without the use of antibiotics.

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Chapter 7

Predisposing factors and prevention of *Clostridium perfringens*-associated enteritis: A review

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Abstract

Clostridium perfringens is one of the major causes of intestinal disease in humans and animals. Its pathogenicity is contributed to by the production of a variety of toxins. In addition, predisposing environmental factors are important for the induction of *C. perfringens*-associated enteritis as shown by infection models. Environmental contamination, gastric and intestinal pH, intestinal microflora, nutrition, concurrent infections, and medical interventions may influence the intestinal colonization, growth, and toxin production by *C. perfringens*. Prevention of *C. perfringens*-associated enteritis may be mediated by the use of feed additives like probiotics, prebiotics, organic acids, essential oils, bacteriophages, lysozymes, bacteriocins, and antimicrobial peptides. Here we summarize and discuss published data on the influence of different environmental predisposing factors and preventive measures. Further research should focus on feed composition and feed additives in order to prevent *C. perfringens*-associated enteritis.

Introduction

Clostridium perfringens, a gram-positive spore-forming anaerobic bacterium, has been implicated as one of the major pathogens in the development of humans and animal intestinal diseases and has been frequently diagnosed as the cause of human-foodborne disease (Songer, 1996; Songer, 2010). The bacterium lacks the ability to produce 13 of the 20 essential amino acids and obtains sufficient amounts of these amino acids by degrading host tissue via its toxins and degradative enzymes (Shimizu et al., 2002). *C. perfringens* is classified into five toxinotypes (A-E), based on the production of four major toxins: alpha, beta, epsilon, and iota toxin (Songer, 1996). Besides these four major toxins, the bacterium may produce additional toxins, such as enterotoxin, beta2 toxin, and NetB toxin (Gibert et al., 1997; Keyburn et al., 2008; Songer, 1996). Of the five toxin types, *C. perfringens* type A is the most common toxin type and is widespread in the environment and in the intestine of both healthy and diseased animals and humans (Petit et al., 1999; Songer, 1996). *C. perfringens* type A is part of the normal intestinal flora in the gastrointestinal tract which comprises a complex mixture of microbes including at least hundreds of bacterial species (Nicholson et al., 2005; Tancrède, 1992; Wise and Siragusa, 2007). *C. perfringens* types other than type A are less frequently cultured from the intestinal tract of animals and humans and can only occasionally be isolated from the environment in areas where clostridial disease is present (Songer, 1996). The heat resistant spores of *C. perfringens* can survive in the environment for years (Mueller-Spitz et al., 2010).

The pathogenesis of *C. perfringens*-associated enteritis is complex and still under investigation (Timbermont et al., 2011). The significance of some of the toxins produced by *C. perfringens* in the development of enteritis has been determined by deletion studies, e.g. the significance of NetB in the development of necrotic enteritis in broilers and the significance of enterotoxin in the development of food-borne enteritis in humans (Keyburn et al., 2008; Sarker et al., 1999). The role of some other toxins like iota toxin or beta2 toxin in the development of intestinal disease is still under debate. *C. perfringens*, beside the production of toxins, may suppress certain members of the normal intestinal flora which confer a health benefit to the host. Such a disbalance of the intestinal microflora may accelerate disease progression (Feng et al., 2010; Stanley et al., 2012). Furthermore, environmental circumstances may directly

or indirectly influence the normal intestinal flora, the colonization, growth, and toxin production of virulent *C. perfringens* strains, and subsequently the occurrence of intestinal disease (Schotte et al., 2004; Songer, 1996).

Different environmental predisposing factors may play a role in different animal species and humans and the significance of risk factors may variate among *C. perfringens* toxin types and subsequent diseases. However, many predisposing factors have been shown to play a role among diverse *C. perfringens* toxin types and in several animal species and humans. Therefore a comparative approach may increase the awareness of possible predisposing factors which is important in lowering the prevalence of outbreaks (Van Immerseel et al., 2004). In this review, possible predisposing factors which influence intestinal colonization, growth, toxin production of *C. perfringens*, and the development of *C. perfringens*-associated intestinal disease, and preventive measures are summarized and discussed.

Extracellular influence on survival, growth and toxin production

The transcription of a variety of genes of *C. perfringens* that encode for proteins required for intracellular metabolism, cell survival and multiplication, i.e. enzymes, transporters, and toxins, is regulated by the two-component VirR/VirS system (Ohtani et al., 2010). The proteins VirS and VirR act as a transmembrane protein and a response regulator protein regulating expression of the genes encoding alpha toxin, beta toxin, beta2 toxin, NetB toxin, and subsequently the production of these toxins (Ba-Thein et al., 1996; Cheung et al., 2010; Lyristis et al., 1994; Ohtani et al., 2003; Vidal et al., 2009). The regulation of epsilon toxin production and iota toxin production is still not clarified. Although enterotoxin production has been shown in non sporulating cultures in some studies, most studies show a strong relationship between sporulation and enterotoxin production (Czeczulin et al., 1996; Duncan, 1973; Melville et al., 1994). Both sporulation and the production of enterotoxin are regulated by sigma factors SigE, SigF, and SigK and occur under environmental conditions that provide insufficient quantity of nutrients for vegetative growth. The way in which environmental conditions may influence these sigma factors is still under investigation. Release of enterotoxin occurs upon cell lysis in

the intestinal lumen at completion of sporulation (Duncan et al., 1973; Harry et al., 2009; Labbe and Duncan, 1975; Li and McClane, 2010; Nakamura and Nishida, 1974; Sacks, 1983). The identification of the VirR/VirS system that regulates growth and alpha, beta, beta2, and NetB toxin production and the influence of environmental circumstances on sporulation and the production of enterotoxin show that extracellular signals might regulate survival rate, sporulation, growth, or toxin production by *C. perfringens*.

Predisposing factors

Environmental contamination

Although *C. perfringens* is a normal inhabitant of the intestine, virulent strains from the environment may displace resident *C. perfringens* strains in the gut (Barbara et al., 2008; Timbermont et al., 2009) or transfer plasmids that contain a toxin encoding gene to *C. perfringens* strains residing in the intestinal tract, converting these resident strains into potential enteropathogens (Brynestad and Granum, 2002; Kobayashi et al., 2009). Preventing oral uptake of virulent strains is thus considered to be a useful prophylactic (Barbara et al., 2008). Faeces of infected animals and humans are probably the most important source of infection. In hospitals, cross infections between patients have been identified by serotyping and genotyping of cultured strains from faecal samples from cases of diarrhoea. *C. perfringens* has also been isolated from dust, air, static surfaces, and other objects which can serve as fomites for cross contamination between patients (Borriello et al., 1985; Kobayashi et al., 2009; Schultz et al., 2003).

On farms, contamination has been demonstrated through the isolation of *C. perfringens* from fresh faecal samples, transport coops, bedding, drinking water, boots, fans, fly strips, and flies (Chalmers et al., 2008; Craven et al., 2001; Dhillon et al., 2004). Small numbers of *C. perfringens* in the faeces of sows are presumably able to overgrow the underdeveloped intestinal microflora of newborn piglets after ingestion (Songer and Uzal, 2005). Faecal shedding of *C. perfringens* by broodmares at the time of foaling increased the faecal shedding of *C. perfringens* by foals at 8-12 hours of age (Tillotson et al., 2002). Additional risk factors to equine neonates may include housing in a stall or drylot, foal born on dirt, sand or gravel surface, and other livestock on the premises in

the past, contributing to persistent faecal contamination of the environment (East et al., 2000). In broiler-breeder operations, environmental contamination includes transmission through the hatchery and grow-out operations, resulting in contamination of broiler carcasses as was determined by ribotyping of *C. perfringens* strains (Craven et al., 2003).

Raw protein foods of animal origin can frequently be contaminated during the slaughter process with *C. perfringens* originating from the intestinal tract or from faeces of animals. Improper cooling or inadequate cooking of contaminated meat or fish has been reported to cause foodborne *C. perfringens* infections in humans. Raw protein foods from which the bacterium has been cultured include beef, pork, poultry, and fish (Aschfalk and Müller, 2002; Craven et al., 2001; Craven et al., 2003; Hewitt et al., 1986; Kamber et al., 2007; Mataragas et al., 2008; Millar et al., 1985; Murrell et al., 1966; Nowell et al., 2010; Tavris et al., 1985). Reduction of faecal shedding of *C. perfringens* by infected animals and prevention of faecal contamination of the environment and raw protein foods may reduce the risk of infection and herewith intestinal disease.

Gastric and intestinal pH

Infection of the intestinal tract by *C. perfringens* via the oral route is lowered by a low gastric pH that is maintained by the excretion of hydrochloric acid by parietal cells. In hypochlorhydric mice the number of *C. perfringens* spores reaching the intestinal tract increased compared to control animals (Tennant et al., 2008). Intragastric gavage of *C. perfringens* type C strains resulted in less lethality in mice than did intraduodenal inoculation (Uzal et al., 2009). In a retrospective study it has been shown that antacids, which can increase gastric pH, may form a predisposing factor for the development of *C. perfringens*-associated diarrhoea (Asha et al., 2006).

An *in vitro* pH between 6.5 and 7.5 leads to optimal growth and optimal production of alpha, beta, beta2, and epsilon toxin by *C. perfringens* (Allaart et al., 2011; Fuchs and Bonde, 1957; Hauschild, 1966; Pivnick et al., 1964; Pivnick et al., 1965; Sakurai and Duncan, 1979). Therefore luminal pH may influence growth and toxin production by *C. perfringens* within the intestinal tract. The intestinal luminal pH is influenced by hydrochloric acid secretion from the stomach, intestinal epithelial bicarbonate secretion, alkaline pancreatic secretions, mucosal and bacterial lactate production, production of short chain fatty acids by bacterial fermentation, and absorption of short chain fatty acids by the colonic epithelium

(Nugent et al., 2001; Snoeck et al., 2004). Intestinal influences which increase gastric and intestinal pH may increase intestinal colonization, growth, and toxin production by *C. perfringens* and therefore may form predisposing factors for the development of *C. perfringens*-associated enteritis.

Nutrition

Dietary changes may cause an imbalance of the normal intestinal flora and may cause overgrowth by *C. perfringens* in the intestinal tract (Beaugerie and Petit, 2004; Van Immerseel et al., 2004). *C. perfringens* is one of the main proteolytic species in the intestinal tract and cannot grow in an environment where an amino acid supply is limited since it lacks the ability to produce certain essential amino acids (Roberfroid et al., 2010; Schimizu et al., 2002). *In vitro* the addition of these amino acids to the growth medium increased growth and toxin production by *C. perfringens*, while the addition of other amino acids did not increase *in vitro* growth rate or toxin production or even caused an antagonistic effect. The effect of addition of certain amino acids on the growth and toxin production by *C. perfringens* seems to depend on the strains examined (Fuchs and Bonde, 1957; Goldner et al., 1985; Jayko and Lichstein, 1959). *In vivo* studies on the effects of a protein-rich diet demonstrated that this resulted in a higher concentration of *C. perfringens* in the intestine of rats, cats, pigs, dogs, and chickens (Drew et al., 2004; Lubbs et al., 2009; Månsson et al., 1971; Takahashi et al., 1983; Zentek et al., 2003). The protein source seems to influence the effect of a higher dietary protein. In dogs, a protein-rich beef or poultry diet produced higher intestinal *C. perfringens* numbers than did a commercial standard diet (Zentek et al., 2004). In chickens, the feeding of fishmeal-based diets containing a high concentration of glycine and methionine increased the *C. perfringens* population in the intestine and could cause a higher mortality due to necrotic enteritis compared to the feeding of a soy-based diet containing a low concentration of these two amino acids (Drew et al., 2004; Prescott et al., 1978; Truscott and Al-Sheikly, 1977; Wu et al., 2010). Testing the two amino acids separately demonstrated that methionine decreased intestinal *C. perfringens* populations, while glycine increased the number of *C. perfringens* in the intestine and increased mortality rates (Dahiya et al., 2005; Dahiya et al., 2007a; Dahiya et al., 2007b). Further research on the effect of protein levels and sources on the development of *C. perfringens*-associated enteritis is important in the development of prevention strategies.

The *in vitro* growth of *C. perfringens* increased after adding carbohydrates to the growth medium but depended on the carbohydrate source. Addition of digestible carbohydrates like glucose, starch, lactose, ribose, trehalose, sucrose, maltose, triomaltose, dextrin, or fructose resulted in higher *in vitro* growth levels of *C. perfringens*, while the addition of indigestible carbohydrates like raffinose or cellobiose did not increase the growth rate (Fuchs and Bonde, 1957; Hauschild and Pivnick, 1965; Jayko and Lichstein, 1959; Labbe and Duncan, 1975; Labbe et al., 1976; Sakurai and Duncan, 1979). Digestible carbohydrates like glucose or dextrin resulted in an increased *in vitro* production of alpha, beta, and epsilon toxin by *C. perfringens* type C and D (Hauschild and Pivnick, 1965; Sakurai and Duncan, 1979). Injection of a suspension of starch into the abomasum of sheep and goats and administration of dextrin into the duodenum of cattle have all been used in an infection model for the reproduction of *C. perfringens* type D enterotoxemia (Layana et al., 2006; Niilo et al., 1963; Uzal and Kelly, 1998; Uzal et al., 2004). In cattle and sheep, overfeeding with carbohydrates resulted in higher numbers of *C. perfringens* in the rumen and caecum (Allison et al., 1975). Outbreaks of enteritis due to *C. perfringens* after feeding a diet containing large amounts of carbohydrates have been described in goats, sheep, cattle, and horses (Carroll et al., 1987; Griner and Bracken, 1953; McGowan et al., 1958; Ochoa and Velandia, 1978; Oliveira et al., 2010; Schofield, 1955; Stubbings, 1990; Waggett et al., 2010). The addition of fructose and sucrose, but not glucose, to chicken diets resulted in higher numbers of *C. perfringens* in the intestinal tract and a reduction in body weight gain. This is in contrast with the higher growth rate of *C. perfringens in vitro* after the addition of glucose to the growth medium (Riddell and Kong, 1992; Stutz and Lawton, 1984). The difference between the effects of the addition of fructose and glucose to chicken diets might be caused by a lower intestinal absorption capacity for fructose compared to glucose, which would result in a higher concentration of fructose in the intestinal tract that might be used by *C. perfringens* for growth and toxin production. In summary, high amounts of dietary digestible carbohydrates that exceed the digestion and absorption capacity of the intestinal mucosa seem to be utilized by *C. perfringens* for growth and toxin production and therefore may form a risk factor for the development of *C. perfringens*-associated enteritis.

Non-starch polysaccharides (NSPs), which can be divided into soluble and insoluble NSPs are other food components of interest. Soluble NSPs are fermented by the colonic microflora, while insoluble NSPs pass the intestinal

tract unaltered. Broilers fed diets based on high levels of soluble NSPs like wheat, rye, barley, and oat groats had increased levels of *C. perfringens* in their intestines and developed more necrotic lesions compared to broilers fed corn-based diets which contain low levels of soluble NSPs (Branton et al., 1987; Branton et al., 1997; Craven, 2000; Jia et al., 2009; Kaldhusdal and Hofshagen, 1992; Riddell and Kong, 1992; Takeda et al., 1995). An increased viscosity of the intestinal digesta induced by soluble NSPs may form an explanation for the higher susceptibility to the development of necrotis enteritis. A higher viscosity decreases the rate of diffusion of substrates and digestive enzymes and decreases their interaction at the mucosal surface providing more substrate for potential pathogens (Almirall et al., 1995; Annett et al., 2002; Huyghebaert et al., 2011; Kaldhusdal and Hofshagen, 1992; Wang et al., 1992). Viscosity has been shown to be reduced in the wheat-based diet by the addition of carbohydrase enzymes. However, growth, feed conversion, intestinal number of *C. perfringens*, intestinal lesion scores, and mortality among chickens after *C. perfringens* challenge were inconsistently influenced by the addition of carbohydrase enzymes (Engberg et al., 2004; Jackson et al., 2003; Jia et al., 2009; Riddell and Kong, 1992). Particle size reduction seems to be another method to increase the digestibility, to lower the viscosity of digesta, and to decrease the numbers of *C. perfringens* in the intestinal tract (Branton et al., 1987; Engberg et al., 2002). Whole wheat supplementation to wheat-based diets, which adds high levels of insoluble NSPs, also reduced the number of *C. perfringens*, and has been explained as an increased gizzard development that possibly caused a better regulation of the filling of the small intestinal tract. This would prevent an overload of digestible carbohydrates in the intestinal tract and subsequently lead to a decrease in substrate available for growth and toxin production by *C. perfringens* (Bjerrum et al, 2005; Engberg et al., 2004). Components in a corn-based diet that are activated by digestive enzymes and that suppress clostridial proliferation form another explanation for the lower susceptibility to the development of necrotic enteritis among broilers fed corn-based diets. This suggestion has been supported by *in vitro* studies that showed that *C. perfringens* proliferation was lower in digested corn-based diets compared to undigested corn-based diets and also compared to digested wheat and barley diets (Annett et al., 2002).

Total parenteral nutrition is used in the support of very-low-birth-weight infants. Compared to enteral feeding, parenteral feeding in neonatal piglets caused an increase in goblet cell numbers, an enrichment of

acidomucins in the intestine, and an increase in number of *C. perfringens*, one of the main mucolytic species in the intestinal tract (Collier et al., 2008; Conour et al., 2002; Deplancke et al., 2002; Roberfroid et al., 2010). In preterm piglets fed parenterally for a few days followed by enteral nutrition, the number of *C. perfringens* increased too compared to piglets fed enterally immediately after birth (Bjornvad et al., 2008).

Bismuth compound has been demonstrated to modify gastric mucin in humans which might reduce the availability of mucin as a substrate for *C. perfringens*. The feeding of bismuth compound to chickens reduced the colonization of *C. perfringens* and intestinal lesion scores in chickens after *C. perfringens* challenge (Stringfellow et al., 2009).

Trypsin is responsible for the cleavage of alpha, beta, and beta2 toxin resulting in complete loss of toxin activity (Baba et al., 1992; Gibert et al., 1997; Sakurai and Duncan, 1978). Trypsin activity in the intestinal tract is affected by dietary factors such as malnutrition in humans, which may cause a lack of intestinal proteases and persistence of clostridial toxins in the intestinal tract (Lawrence and Walker, 1976; Sato et al., 1978). Trypsin's ability to degrade alpha toxin is reduced by a high level of dietary zinc, which also increases the incidence of necrotic enteritis in chickens (Baba et al., 1992; Sato and Murata, 1973; Sato et al., 1978). Foods with high amounts of anti-trypsin factor or protease inhibitors, such as soybeans, sweet potatoes, and colostrum, have been shown to increase the incidence of hemorrhagic enterotoxaemia by *C. perfringens* in pigs, chickens, and humans (Hunter et al., 1993; Lawrence and Walker, 1976; Niilo, 1986; Palliyeguru et al., 2010; Palliyeguru et al., 2011; Shann et al., 1979). Although colostral anti-trypsin might form a predisposing factor for the development of disease, colostrum lowered the incidence of *C. perfringens*-associated intestinal disease compared to formula feeding in several animal species and humans, which was partly attributed to the presence of colostral antibodies against clostridial toxins (Bullen et al., 1976; Sangild et al., 2006; Songer, 1996; Songer and Uzal, 2005).

In summary, factors that can result in an overload of digestible carbohydrates, proteins and acidomucins in the intestinal tract and substances that counteract intestinal proteases may enhance the occurrence of *C. perfringens*-associated intestinal disease and should be avoided.

Concurrent infections

Concurrent infection with other pathogens can change intestinal circumstances that favour the proliferation and survival rate of *C. perfringens* in the intestinal tract. For example, a reduction of the digestion and absorption capacity of the intestinal mucosa after intestinal villous atrophy caused by intestinal viral infections can result in a higher amount of digestible carbohydrates and proteins in the intestinal tract providing a growth advantage for *C. perfringens* (Davidson et al., 1977; Guerrero et al., 2010; Katyal et al., 1999; Riddell and Kong, 1992; Roberfroid et al., 2010). This enhancing effect of viral infections on the proliferation of *C. perfringens* has been described in gnotobiotic mice infected with rotavirus and in dogs infected with parvovirus (Moreau et al., 1986; Turk et al., 1992). Viral infections also may cause a general immunosuppression as shown in chickens after the administration of a live commercial bursal disease vaccine at a level 10 times the dose recommended by the manufacturer. Viral infection of the bursa of Fabricius, the organ involved in the development of humoral immunity, probably causes an immunosuppression and increased intestinal lesion scores after *C. perfringens* challenge compared to the administration of *C. perfringens* alone (McReynolds et al., 2004).

Parasitic infections which damage the intestinal mucosa can also contribute to an increase in *C. perfringens* proliferation and toxin production. *Eimeria* spp. can alter the intestinal mucosa by multiplying in the cryptic glands followed by inhibiting epithelial regeneration and by destroying host tissue, in this way facilitating overgrowth of *C. perfringens*. Furthermore an increased mucogenesis induced by coccidial infections can enhance the growth of *C. perfringens* (Collier et al., 2008; Droual et al., 1994; Rosadio et al., 2010). Concurrent infection of *C. perfringens* with *Eimeria macusaniensis* has been described in alpacas dying from enterotoxaemia (Johnson et al., 2009; Rosadio et al., 2010). Severe coccidiosis was found in goats with enterotoxaemia caused by *C. perfringens* (Uzal et al., 1994). In poultry infection with *Eimeria* spp. has been associated with multiplication of *C. perfringens* in the intestinal tract and the development of necrotic enteritis (Al-Sheikhly and Al-Saieg, 1980; Baba et al., 1988; Baba et al., 1992; Baba et al., 1997; Bradley and Radhakrishnan, 1973; Droual et al., 1994; Droual et al., 1995; Hein and Timms, 1972). In an infection model it was impossible to produce necrotic enteritis by inoculation of *C. perfringens* solely, while administration of a combination of *C. perfringens* with *Eimeria maxima* or an overdose of a live coccidial vaccine led to grossly visible

intestinal necrotic lesions (Gholamiandehkordi et al., 2007; Pedersen et al., 2008). Application of coccidial vaccines before challenge with *Eimeria* spp. and *C. perfringens* or anti-coccidial treatment during challenge with *Eimeria* spp. and *C. perfringens* reduced coccidial lesions due to *Eimeria* spp. and necrotic enteritis lesions due to *C. perfringens*, emphasizing the role of coccidia in the development of *C. perfringens*-associated enteritis in chickens (Collier et al., 2008; Williams et al., 2003).

Trichuris spp., *Ascaris* spp., and *Ancylostoma* spp. are able to produce protease inhibitors to protect themselves against intestinal digestive enzymes (Delaney et al., 2005; Green, 1957; Hawley and Peanasky, 1992; Kageyama, 1998; Rhoads et al., 2000a; Rhoads et al., 2000b). Parasitic protease inhibitors have been suggested as a predisposing factor for the development of intestinal disease caused by trypsin-sensitive *C. perfringens* toxins. In a Hamadryas baboon a concurrent infection of *C. perfringens* with *Trichuris* worms was thought to be the cause of enteritis, with beta2 toxin demonstrated in the intestinal tract of the baboon by immunohistochemistry (Nikolaou et al., 2009). Ascaridiasis has been established as a concurrent intestinal infection found in turkeys suffering from necrotic enteritis caused by *C. perfringens* (Droual et al., 1995; Norton et al., 1992). *Ascaris* infection was apparently frequent among children suffering from necrotic enteritis in China (Shann et al., 1979). In an outbreak at an evacuation site in Thailand 95 % of faecal samples of children with necrotic enteritis due to *C. perfringens* type C contained intestinal parasites like *Stronglyloides stercoralis*, *Strongylida* spp*., Ascaris lumbricoides, Giardia lamblia, Hymenolepis nana*, and *Entamoeba histolytica*. This was significantly higher than among children without necrotic enteritis in the same camp. It was suggested that *S. stercoralis* might have reduced intestinal motility, obstructed intestine or penetrated the intestinal mucosa, which may have allowed a greater absorption of the beta toxin (Johnson et al., 1987). A concurrent infection of *Plasmodium relictum* with *C. perfringens* has been described in a captive king penguin. Anorexia due to avian malaria and moulting stress may have altered the intestinal environment and supported overgrowth by *C. perfringens* (Penrith et al., 1994).

In conclusion, viral and parasitic infections might increase the amount of substrate for growth and toxin production by *C. perfringens* or might enhance the persistence and absorption of clostridial toxins. Therefore, prevention and treatment of viral and parasitic infections is important in preventing *C. perfringens*-associated enteritis.

Antibiotics

Antibiotics that may be of value in the control of disease caused by pathogenic bacteria have antibacterial effects that extend beyond the target pathogen. Antibiotics administrated orally as well as parenterally followed by excretion in the intestinal tract can cause alterations of the composition of the indigenous flora (Goldstein, 2011; McFarland, 2000; Takesue et al., 2002). As a result the balance of the normal intestinal flora might get disturbed and non-sensitive micro organisms might overgrow the normal intestinal flora (Goldstein, 2011; McFarland, 2000). The effect of antimicrobial treatment on the colonization and toxin production by *C. perfringens* has been shown by multiple studies. *C. perfringens* has been isolated from stool of a dog with bloody diarrhoea after the use of cephalexin (Marjani et al., 2009). *C. perfringens*-associated colitis X has been described in horses after oral administration of tetracyclin and erythromycin (Gustafsson et al., 1997; Keir et al., 1999). The presence of beta2 toxin of *C. perfringens* in the intestinal tract was shown by immunohistochemistry after treatment with gentamycin causing severe typhlocolitis in horses. *In vitro* study of the *C. perfringens* strain cultured from the intestinal tract of these horses revealed an antibiotic-inducible ribosomal frameshifting of a cryptic beta2 toxin gene resulting in expression of the beta2 toxin. Abandoning the standard use of gentamycin during hospital care reduced the occurrence of colitis (Vilei et al., 2005). In humans suffering from antibiotic-associated diarrhoea, high counts of *C. perfringens* have been demonstrated (Abrahao et al., 2001; Ackermann et al., 2005; Asha and Wilcox, 2002; Borriello et al., 1984; Fisher et al., 2005; Modi and Wilcox, 2001; Schwartz et al., 1980; Vaishnavi et al., 2005). In conclusion, the use of antibiotics might result in overgrowth by *C. perfringens* in the intestinal tract and in an increased toxin production by *C. perfringens* and therefore might form a predisposing factor for the development of *C. perfringens*-associated enteritis.

Prevention

Probiotics

Bacterial species that may confer a health benefit to the host after oral administration e.g. by inhibition of harmful bacteria are called probiotics (Mikelsaar and Zilmer, 2009). The inhibition of harmful bacteria is mediated by

competition for nutrients, lowering of the pH, and the production of specific antibacterial substances (Crost et al., 2010; Du Toit et al., 1998; Gérard et al., 2008; Perelmuter et al., 2008; Romond et al., 1998). The *in vitro* activity against *C. perfringens* by potentially probiotic bacterial species is listed in Table 1.

Table 1 *In vitro* inhibitory effect of potential probiotic bacterial species against *C. perfringens* (CP)

Probiotic strain	Outcome	Reference
Bacillus cereus	+[a]	Bizani and Brandelli, 2002
Bacillus licheniformis	v[b]	Ducluzeau et al., 1976; Barbosa et al., 2005
Bacillus megaterium	-[c]	Barbosa et al., 2005
Bacillus pumilus	+	Barbosa et al., 2005
Bacillus subtilis	v	Barbosa et al., 2005; Klose et al., 2010; Teo and Tan, 2005; La Ragione and Woodward, 2003
Bifidobacterium infantis	+	Gibson and Wang, 1994
Bifidobacterium thermacidophilum	+	Klose et al., 2010; Kim et al., 2007
Enterococcus faecium	v	Klose et al., 2010; Chen et al., 2007; Shin et al., 2008
Lactobacillus acidophilus	v	Teo and Tan et al., Fukata et al., 1991
Lactobacillus amylovorus	v	Klose et al., 2010; Kim et al., 2007
Lactobacillus animalis	+	Biagi et al., 2007
Lactobacillus fermentum	-/+ [d]	Allaart et al., 2011; Teo and Tan, 2005
Lactobacillus mucosae	+	Klose et al., 2010
Lactobacillus murinus	+	Perelmuter et al., 2008
Lactobacillus pentosus	+	Weese et al., 2004
Lactobacillus reuteri	v	Klose et al., 2010; Kim et al., 2007; Hacin et al., 2008
Lactobacillus salivarius	+	Klose et al., 2010; Kim et al., 2007
Lactobacillus vaginalis	-	Hacin et al., 2008
Pediococcus acidilactici	-	Teo and Tan, 2005
Pediococcus pentosaceus	-	Teo and Tan, 2005; Shin et al., 2008
Streptococcus durans	+	Stark, 1960
Streptococcus faecalis	+	Stark, 1960; Bottone et al., 1974

[a] inhibition of growth CP
[b] inhibition of growth CP variable between strains or studies
[c] no inhibition of growth CP
[d] no inhibition of growth CP, inhibition of toxin production CP

Probiotic strains should be harmless to the host, resistant to acids and bile salts, and be able to persist and multiply in the intestinal tract (Biagi et al., 2007; Crost et al., 2010). Inhibitory capacity and the resistance to acids and bile salts differ among strains (Barbosa et al., 2005; Chen et al., 2007; Gibson and Wang, 1994; Klose et al., 2010; Teo and Tan, 2005; Weese et al,. 2004).

As *in vivo* studies in mice, pigs, and chickens have shown, several probiotic strains are able to colonize the intestinal tract after oral inoculation and reduce the colonization and persistence of *C. perfringens* and mortality after oral challenge with this bacterium (Table 2).

Table 2 *In vivo* effect of potential probiotic bacterial species against *C. perfringens* (CP)

Probiotic strain	Species	Outcome	Reference
Bacillus licheniformis	Mice Chickens	RC[a] RNLac [b]	Ducluzeau et al., 1976 Knap et al., 2010
Bacillus subtilis	Chickens	RC	Teo and Tan, 2005; La Ragione and Woodward, 2003
Mixture of *Bifidobacterium animalis*, *Lactobacillus acidophilus*, *L. casei*, *L. pentosus*, and *L. plantarum*	Pigs	RC / RNL[c]	Siggers et al., 2008
Bifidobacterium infantis	Quails	RC	Butel et al., 1998
Clostridium butyricum	Mice	RC	Kong et al., 2011
Lactobacillus acidophilus	Chickens	RC	Fukata et al., 1991
Lactobacillus johnsonii	Chickens	RC	La Ragione et al., 2004
Lactobacillus plantarum	Mice	RC / RNLac	Xia et al., 2011
Lactobacillus salivarius	Mice	RC	O'Mahony et al., 2001
Lactobacillus sp. No. I-2673	Chickens	RC	Gérard et al., 2008
Ruminococcus gnavus	Mice	RC	Crost et al., 2010
Streptococcus faecalis	Chickens	RNLac	Fukata et al., 1991

[a] reduction of intestinal colonization CP
[b] reduction of necrotic lesions after challenge with CP
[c] reduction of necrotic lesions

As demonstrated *in vitro*, the transcription of *cpb2*, the gene encoding the beta2 toxin and the production of beta2 toxin by *C. perfringens* were reduced after co-culturing of *C. perfringens* with *Lactobacillus fermentum*, which lowered the environmental pH without influencing bacterial viability (Allaart et al., 2011). Considering the fact that alpha toxin, beta toxin, beta2 toxin, and NetB toxin are all regulated by the VirR/VirS system, a decrease in the production of all these toxins due to the presence of *L. fermentum* could be hypothesized.

All studies reviewed here indicated that probiotics may diminish growth, colonization, and toxin production by *C. perfringens* in the intestinal tract and herewith the occurrence of *C. perfringens*-associated intestinal disease. The mechanisms behind these effects are not yet clear for all cases.

Prebiotics

Stimulation of the growth of intestinal bacteria which diminish the growth and toxin production by *C. perfringens* like lactobacillae and

bifidobacteria might be another way to prevent overgrowth by *C. perfringens* in the intestinal tract. Prebiotics are non-digestible food ingredients, mainly non-digestible carbohydrates, which positively affect the host by selectively stimulating the growth and activity of harmless bacteria in the colon, favourably altering the colonic microflora and herewith increase general gut health. Non-digestible carbohydrates are fermented by bifidobacteria and lactobacilli into short-chain fatty acids which lower the intestinal pH and amongst others suppress the growth of pathogens (Biggs et al., 2007; Pan et al., 2009; Roberfroid et al., 2010; Swanson et al., 2002). *In vivo* studies on the effect of prebiotics on the intestinal presence of *C. perfringens* are summarized in Table 3.

Fructo-oligosaccharides and inulin, a polymer of fructo-oligosaccharides, occur in feed ingredients such as wheat and rye (Bornet et al., 2002). Pectin and arabinogalactans are polysaccharides present in feed ingredients such as apples and sugar beets. Inulin, pectin and arabinogalactans are fermented by bifdobacteriae and lactobacillae *in vitro*, but not by *C. perfringens* (Fuchs and Bonde, 1957; Roberfroid et al., 2010; Shinohara et al., 2010). Feeding of natural occurring prebiotic carbohydrates showed a reduction of the number of *C. perfringens* in some *in vivo* studies (Al-Tamimi et al., 2006; Biggs et al., 2007; Catala et al., 1999; Flickinger et al., 2003; Gómez-Conde et al., 2007; Kleessen et al., 2003; Shinohara et al., 2010). In other studies no effect on the intestinal number of *C. perfringens* was determined (Barry et al., 2010; Grieshop et al., 2002; Menne et al., 2000; Sparkes et al., 1998a; Sparkes et al., 1998b; Swanson et al., 2002; Yang et al., 2008a; Zentek et al., 2003). Possibly the level of prebiotic carbohydrates in these studies has been too low to reduce the intestinal number of *C. perfringens*. Increasing the level of dietary fructo-oligosaccharides seemed to increase the effect on the composition of the intestinal microflora (Kolida et al., 2007; Yap et al., 2008). However, very high levels of fructo-oligosaccharides might cause anti-nutritive side-effects or diarrhoea contributed to the laxative effect of fructo-oligosaccharides (Biggs et al., 2007; Sairanen et al., 2007).

Lactose is a disaccharide that occurs naturally in mammalian milk. The disaccharide is digested into glucose and galactose by the enzyme lactase. Lactose can be used as a prebiotic in non-mammalian animals that do not possess the enzyme lactase like chickens. Fermentation of lactose by the caecal microflora increases fatty acid levels in the caecum and lowers intestinal pH (Corrier et al., 1990; Van der Wielen et al., 2002). Lactose has been shown by two studies to reduce

intestinal numbers of *C. perfringens* and *C. perfringens*-associated disease amongst chickens, indicating the possibility of using lactose as a potential alternative to antibiotics in poultry (McReynolds et al., 2007; Takeda et al., 1995).

Mannan-oligosaccharides (MOS), present in the outer cell wall of yeast, might have a prebiotic effect and enhance the production of immunoglobulin A, which could help to control pathogens (Biggs et al., 2007; Chee et al., 2010; Sims et al., 2004). However, the feeding of yeast to pigs resulted in a reduction of feed intake and daily weight gain while immunoglobulin A and intestinal numbers of *C. perfringens* remained unaffected (White et al., 2002).

Table 3 Influence of prebiotic feed-additives on *in vivo* activity of *C. perfringens* (CP)

Prebiotic feed-additive	Species	Test	Result	Reference
FOS/Inulin	Dogs	RC[a]	v -	Zentek et al., 2003; Swanson et al., 2002; Flickinger et al., 2003
	Cats	RC	v +	Barry et al., 2020; Sparkes et al., 1998a; Sparkes et al., 1998b
	Chickens	RC	-	Biggs et al., 2007; Kleessen et al., 2003; Yang et al., 2008a
	Quails	RC		Catala et al., 1999
	Humans	RC		Menne et al., 2000
Arabinan	Dogs	RC	-	Grieshop et al., 2002
	Humans	RC	+	Al-Tamimi et al., 2006
Arabinan + Pectin (beet-apple pulp)	Rabbits	RC	+	Gómez-Conde et al., 2007
Pectin	Cats	RC	-	Barry et al., 2010
Pectin (apple)	Humans	RC	+	Shinohara et al., 2010
Lactose	Chickens	RC	+	Takeda et al., 1995
		RNL/Mac[b]	+	McReynolds et al., 2007
MOS	Pigs	RC	-	White et al., 2002
	Chickens	RC	v	Biggs et al., 2007; Chee et al., 2010; Kim et al., 2011; Yang et al., 2008a; Yang et al., 2008b
	Chickens	RNL/Mac	-	Thanissery et al., 2010
	Turkey	RC	+	Sims et al., 2004
Lactulose	Pigs	RC	+	Krueger et al., 2002
	Pigs	RC clostridia[c]	+	Kien et al., 2007
	Humans	RC LP-clostridia[d]	+	Terada et al., 1992
Lactosucrose	Cats	RC LP-clostridia	+	Terada et al., 1993
	Chickens	RC LP-clostridia	+	Terada et al., 1994
GOS	Humans	RC clostridia	+	Costalos et al., 2008

[a] reduction of intestinal colonization CP
[b] reduction of necrotic lesions and mortality after challenge with CP
[c] reduction of intestinal colonization clostridia
[d] reduction of intestinal colonization lecithinase-positive clostridia

In poultry, the effect of MOS on intestinal numbers of *C. perfringens* was inconsistent between studies (Biggs et al., 2007; Chee et al., 2010; Kim et al., 2011; Sims et al., 2004; Thanissery et al., 2010; Yang et al., 2008a; Yang et al., 2008b) and seemed to be both dose- and diet-dependent (Biggs et al., 2007; Kim et al., 2011; Yang et al., 2008b). Addition of MOS to the diet of chickens for ten days could not reduce intestinal colonization by *C. perfringens* and intestinal necrotic lesion scores after challenge with *C. perfringens.* As the authors of that study suggested, supplementation of chicken diets with MOS for a longer period might have reduced intestinal necrotic lesions scores (Thanissery et al., 2010).

Lactulose, lactosucrose, and galacto-oligosaccharides (GOS) are synthetic prebiotic carbohydrates which escape digestion in the upper intestinal tract and become fermented by lactobacillae and bifidobacteriae in the colon. In most studies on the administration of these synthetic prebiotics the focus is on the effect on clostridia in general, without a differentiation between *C. perfringens* and other Clostridial species (Costalos et al., 2008; Kein et al., 2007; Terada et al., 1992; Terada et al., 1993; Terada et al., 1994). A reduction of faecal numbers of *C. perfringens* has been shown only in sows after oral administration of lactulose, with a possible reduction of neonatal infections in piglets after birth (Krueger et al., 2002).

In summary, it is difficult to draw conclusions from published studies due to large differences between studies in subjects, age, diet, outcome parameters, substances tested, dose, and duration of the experiments. Most substances have only been tested once in a given animal species, while those that have been studied several times show mainly inconsistent results. The lack of consistent results may be due to the fact that studies focus not only on the prevention of colonization of *C. perfringens* but on many intestinal health and growth parameters. More specific research on the effect of prebiotics on the colonization of *C. perfringens* might lead to optimization of choice of substances and dosages which might result in better strategies for the prevention of *C. perfringens*-associated intestinal disease.

Plant extracts and essential oils

Many extracts and essential oils derived from plants have been shown to exhibit *in vitro* growth-inhibiting activity towards *C. perfringens* (Candan et al., 2003; Gadhi et al., 1999; García et al., 2002; Johnston et al., 2001; Kim and

Lee, 2009; Lee and Ahn, 1998; Lee et al., 2001; Magwa et al., 2006; Nevas et al., 2004; Shanmugavelu et al., 2006; Si et al., 2009; Sokmen et al., 1999; Sökmen et al., 2003; Unlü et al., 2002; Wannissorn et al,. 2005; Wilkinson et al., 2003). Growth-inhibiting activity of plant extracts and essential oils may vary within the same plant species due to differences in genotype, geographical location, growth circumstances, growth stage, and drying methods (Nevas et al., 2004; Wilkinson et al., 2003). Activity is also influenced by the distillation or extraction method, which may influence the level of growth-inhibition after preparation (García et al., 2002; Sokmen et al., 1999; Wilkinson et al., 2003). This variation in growth-inhibiting activity between extracts and essential oils of the same plant species may hinder their application for the control of *C. perfringens*-associated enteritis. Additional features needed for their *in vivo* usage are resistance to acids, food substances, and the intestinal microbiota, and a lack of inhibition towards the intestinal level of lactobacillae and bifidobacteriae (Nevas et al., 2004; Si et al., 2009). Only a few essential oils have been tested *in vivo* for their ability to reduce intestinal proliferation of *C. perfringens* and to reduce intestinal lesion scores. In chickens, thymol and thymol mixed with carvacrol showed an inhibitory effect on the intestinal colonization and proliferation of *C. perfringens* (Mitsch et al., 2004). The addition of thymol to broiler feed in another study did not significantly reduce the numbers of *C. perfringens* in the intestinal tract, but increased body weight gain (Cross et al., 2007). In an infection model a mixture of thymol, cinnamaldehyde, and eucalyptus reduced necrotic lesions after challenge with *C. perfringens* (Timbermont et al., 2010). The effect of thymol looks promising, while the effect of other essential oils will need further investigation.

Organic acids

The growth of bacteria is inhibited by organic acids; organic compounds with weak acidic properties. This inhibitory effect has been through a lower environmental pH caused by its weak acidic properties, a prebiotic effect on intestinal microflora, or a direct effect on the bacterial cell integrity. Organic acids may cross the bacterial cell membrane and dissociate in the higher intracellular pH inside the bacteria, resulting in the release of charged anions and protons that cannot cross the cell membrane. This decrease in bacterial intracellular pH and inhibition of essential metabolic reactions reduces bacterial growth (Biggs and Parsons, 2008; Brul and Coote, 1999). Caprylic acid,

capric acid, lauric acid, myristic acid, and oleic acid all reduced the growth of *C. perfringens in vitro* (Skrivanová et al., 2005; Timbermont et al., 2010). In *in vivo* studies, organic acids were shown to reduce the number of *C. perfringens*, but in these studies the number of lactobacillae and bifidobacteria in the intestinal tract decreased too (Biggs and Parsons, 2008; Garrido et al., 2004; Geier et al., 2020). Severity of intestinal lesions and mortality due to necrotic enteritis did not decrease after feeding a diet with organic acids (Geier et al., 2010; Lensing et al., 2010), except for one study in which intestinal lesion scores were reduced by feeding a diet supplemented with lauric acid (Timbermont et al., 2010). The effect of organic acids on growth parameters of chickens was inconsistent (Biggs and Parsons, 2008; Garrido et al., 2004; Geier et al., 2010; Lensing et al., 2010). It can be concluded from studies that organic acids have a bactericidal effect on *C. perfringens in vitro* and that the administration of organic acids to chickens might reduce the intestinal colonization of *C. perfringens in vivo* (Biggs and Parsons, 2008; Garrido et al., 2004; Geier et al., 2010; Skrivanová et al., 2005; Timbermont et al., 2010). However, this does not consistently improve simultaneously intestinal health and growth performance of young chickens after challenge with *C. perfringens* ((Biggs and Parsons, 2008; Garrido et al., 2004; Geier et al., 2010; Lensing et al., 2010; Timbermont et al., 2010). Of the acids studied, sodium lauroyl lactylate, an ester of lauric acid and fatty acids is a hundred to thousand-fold more effective inhibitor of *C. perfringens* growth activities than fatty acids alone (Lensing et al., 2010). A reduction in necrotic lesions and in mortality after challenge with *C. perfringens* was shown after the administration of a high dosage of sodium lauroyl lactylate to chickens. This nominates it as a potential feed additive for the reduction of necrotic enteritis in chickens due to *C. perfringens* (Lensing et al., 2010).

Bacteriophages, lysozymes, and antimicrobial peptides

Bacteriophages are viruses that infect and multiply in bacteria. The production of lytic enzymes by bacteriophages results in lysis of the host bacterium and release of progeny viruses in the environment. Bacteriophages as well as phage lytic enzymes are of interest for the treatment of bacterial infections due to their ability to kill pathogenic bacteria and for their highly discriminatory nature leaving other bacterial species unaffected (Joerger, 2003). *C. perfringens* bacteriophages and there lytic enzymes have recently been determined and molecular characterized (Kim et al., 2012; Morales et al.,

2012; Nariya et al., 2011; Oakley 2011; Schmitz et al., 2011; Seal et al., 2011; Seal, 2013; Simmons et al., 2010; Volozhantsev et al., 2011; Volozhantsev et al., 2012). However, there are few available data on *in vivo* studies determining the effect of phage therapy against *C. perfringens*-associated disease. Only one study shows the effect of bacteriophage therapy on the depression of broiler performance after infection with *E. maxima* and *C. perfringens*. Weight gain was significantly higher while feed conversion ratios and mortality rates were reduced after oral administration of bacteriophages (Miller et al., 2010). Administration of bacteriophages by water or feed was as effective as administration by oral gavage providing a potential prevention strategy for the control of *C. perfringens*-associated disease in poultry.

Lysozymes are single peptides which cleave glycosidic linkages in the peptidoglycan layer of bacterial cell walls (Zhang et al., 2010). Lysozymes preventing bacterial growth are abundantly present in animal products as milk and hen eggs (Hughey and Johnson, 1987). Egg white lysozyme did not show *in vitro* activity against two *C. perfringens* strains derived from a human food poisoning outbreak (Hughey and Johnson, 1987), however, the growth of three *C. perfringens* strains derived from broilers with necrotic enteritis was inhibited (Zhang et al., 2006). Egg white lysozyme has been shown to increase weight gain and decrease feed conversion ratio, necrotic lesions, and mortality after oral challenge with *C. perfringens* combined with either *E. maxima* or a glycin-rich diet (Liu et al., 2010; Zhang et al., 2010) providing a potential feed additive for the control of *C. perfringens*-associated disease in poultry.

Bacteriocins are proteinaceous compounds that are antagonistic against bacteria other than the producing strain with in general a rather narrow killing spectrum in comparison to antibiotics (Joerger, 2003). Bacteriocins antagonistic against *C. perfringens* are produced by *Carnobacterium divergens* (Holck et al., 1996), *Lactococcus lactis* (Guerlava et al., 1998; Udompijitkul et al., 2012), *Bacillus* spp (Niu and Neu, 1991), *Enterococcus faecalis* (Sparo et al., 2009), *Pediococcus acidilactici* (Elegado et al., 1997), *Pediococcus pentosaceus* (Grilli et al., 2009), and *Ruminococcus gnavus* (Crost et al., 2011; Dabard et al., 2001; Ramare et al., 1993) as shown by *in vitro* studies. Divercin AS7 produced by *C. divergens* and Pediocin A produced by *P. pentosaceus* have been studied as a potential feed additive for performance improvement in broilers after oral challenge with *C. perfringens*. *C. perfringens* challenge combined with a diet rich in wheat, barley, and fishmeal increased the feed conversion ratio in broilers,

while feed conversion ratio did not increase after the same treatment combined with divercin AS7 feed supplementation (Józefiak et al., 2012). Average daily weight gain increased after pediocin A feed supplementation, but this effect seemed to be independent of *C. perfringens* challenge. Administration of pediocin A did not decrease *C. perfringens* counts or intestinal lesion scores and did not improve growth parameters after challenge with *C. perfringens* combined with an overdose of a live coccidial vaccine. However, pediocin A inhibited the growth of *C. perfringens in vitro*. The authors suggest that the large amount of gram-positive bacteria in the intestinal tract may have had a competitive diluting effect on pediocin A and might have decreased the inhibiting effect of pediocin A on the intestinal level of *C. perfringens* (Grilli et al., 2009).

Antimicrobial peptides *in vitro* antagonistic against *C. perfringens* other than bacteriocins have been produced by *Fusarium proliferatum* and *Fusarium tricinctum* (Meca et al., 2010; Meca et al., 2011). A low but inhibitory toxic concentration of Enniatin B produced by *F. tricinctum* was not toxic to Caco-2 cells making Enniatin B in a low concentration a potential substance for further *in vivo* investigation (Meca et al., 2011). It can be concluded that bacteriocins produced by several bacterial species and antimicrobial peptides produced by *Fusarium* spp may have a role in the control of *C. perfringens*-associated intestinal disease due to their antagonistic activity against *C. perfringens*, but more *in vivo* studies are needed.

Summarizing conclusion

C. perfringens is ubiquitous in the environment and in the intestinal tract of animals and humans and is frequently involved in the development of enteritis. Faeces of infected animals and contaminated meat are the most common sources of infection. Environmental factors are thought to influence the colonization, intestinal growth, and toxin production by *C. perfringens* and might play a key-role in the development of disease.

Colonization, growth, and toxin production are increased by high levels of dietary protein and dietary digestible carbohydrates which exceed the digestion and absorption capacity of the intestinal tract. These effects are most likely enhanced by factors that negatively influence the digestion and absorption capacity of the intestinal tract.

Probiotics, bacteriophages, and lysozyme have been shown to be

useful in the prevention, although more *in vivo* studies in different animal species are needed. The effect of prebiotics on the development of *C. perfringens*-associated enteritis remains unclear. Such a possible relationship should be the focus of *in vivo* studies, in order to optimize choice of substances and dosages for the prevention of enteritis associated with *C. perfringens*. Several organic acids, plant extracts, plant essential oils, bacteriocins, and antimicrobial peptides have been shown to exhibit an inhibitory effect on *C. perfringens in vitro* which makes them possible alternatives for the use of antibiotics. However, an inhibitory effect on the development of *C. perfringens*-associated enteritis of these substances has not been proven yet, due to a lack of sufficient *in vivo* studies.

Further research should focus on the composition and digestibility of diets and the administration of feed additives in order to reduce the intestinal growth and toxin production of virulent *C. perfringens* strains and to support intestinal health.

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Chapter 8

Summarizing discussion

Clostridium perfringens is one of the most important causes of intestinal disease in animals and humans (Songer, 1996). Its virulence is attributed to the several toxins it can produce, including the beta2 toxin encoded by *cpb2* (Gibert et al., 1997). In this thesis we studied the role of the beta2 toxin produced by *C. perfringens* in the development of intestinal disease. We especially addressed the development of reliable diagnostic test methods; the relationship between *cpb2*-harbouring *C. perfringens* and intestinal disease; the pathogenesis of *cpb2*-harbouring *C. perfringens*-associated intestinal disease, and the involvement of the normal intestinal flora and predisposing factors for the development of *cpb2*-harbouring *C. perfringens*-associated intestinal disease.

Diagnostic methods for detection of *cpb2* and *in vitro* and *in vivo* beta2 toxin production

The isolation of *C. perfringens* from the intestinal tract followed by the demonstration of *cpb2* in the genome of isolates forms a vital part of research on the role of the beta2 toxin in the pathogenesis of intestinal disease associated with *cpb2*-harbouring *C. perfringens*. Since the identification of two different allelic subpopulations of *cpb2,* the consensus allele and the atypical allele, two different primer sets are used (Jost et al., 2005). To increase test efficacy a new primer set was developed which recognizes both alleles of *cpb2* (**chapter 2**). Digestion with MboI of the PCR product enabled a distinction between the consensus and the atypical allele. A third restriction pattern was exclusively found in porcine isolates, which suggests a separate subpopulation of *cpb2*-harbouring *C. perfringens* among piglets which might not be pathogenic to other animal species.

Application of the PCR on faecal samples showed that both *cpb2*-positive and *cpb2*-negative isolates and different *cpb2* allelic subpopulations could be present within the same faecal sample. This result demonstrated the shortage of testing only one or a few isolates per clinical sample. A comparison of three different sampling methods showed that pooling of ten *C. perfringens* isolates followed by the newly developed primer set appeared to be the most sensitive, time-efficient and cost-effective way of testing for the presence of *cpb2* in faecal samples.

The presence of *cpb2* in the genome of isolates does not implicate that the beta2 toxin is produced by *C. perfringens*. Several studies showed differences in the ability to produce the beta2 toxin and the level of beta2 toxin

produced *in vitro* by different *cpb2*-positive *C. perfringens* strains (Bueschel et al., 2003; Harrison et al., 2005; Jost et al., 2005; Waters et al., 2005). Therefore, in **chapter 3**, the level of *in vitro* beta2 toxin production by *C. perfingens* strains isolated from roe deer with intestinal disease was investigated. None of the isolated strains from roe deer with intestinal disease and only two isolates from a healthy roe deer produced beta2 toxin *in vitro* confirming the shortage of testing only for the presence of *cpb2* in the genome of isolates.

In vitro beta2 toxin production may not be related to *in vivo* beta2 toxin production. Therefore, immunohistochemistry on intestinal tissue samples from all roe deer was accomplished to verify the presence of beta2 toxin in the intestinal tract. A clear relationship has been shown between the presence of the beta2 toxin in intestinal tissue by immunohistochemistry and the presence of *cpb2*-positive *C. perfringens* (Bacciarini et al., 2003). However, as shown in **chapter 3**, all *C. perfringens* isolates from roe deer with signs of intestinal disease harboured *cpb2,* while no beta2 toxin was demonstrated in intestinal tissue by immunohistochemistry. In conclusion, these data emphasize the shortcomings of only *in vitro* investigation and the need for determining the presence of beta2 toxin *in vivo* in epidemiological studies.

The relationship between *cpb2*-harbouring C. *perfringens*, beta2 toxin, and enteritis

Studies on the relationship between *cpb2*-harbouring *C. perfringens*, beta2 toxin, and intestinal disease are summarized in Table 1.

The current studies on the relationship between *cpb2* and intestinal disease show inconsistency regarding study design and outcome. At first the definition of *cpb2*-harbouring *C. perfringens*-associated disease is not consistent between studies. Some studies describe lesions in the intestinal tract, while most studies just mention diarrhoea or intestinal disease. Secondly, not all studies include a negative control group (Fisher et al., 2005; Klaasen et al., 1999; Thiede et al., 2001). Also, some studies compare the presence of *cpb2* between diseased or healthy animals, testing one strain per animal, while other studies investigate the presence of *cpb2* among different strains per animal (Bueschel et al., 2003; Lebrun et al., 2007). If more strains per animal are tested, this random effect is not always included in statistical analysis (Lebrun et al., 2007). Furthermore, only few studies investigate the ability of strains to produce the beta2 toxin *in vitro* or, if possible, the presence of the beta2 toxin in

the intestinal tract. One study demonstrated low transcription levels of *cpb2* by Northern blot analysis, however, detectable levels of beta2 toxin could not be demonstrated by Western blot analysis (Waters et al., 2005). Finally, none of the studies describes clearly defined lesions or symptoms related to the presence of *cpb2*-harbouring *C. perfringens* as well as the ability of these strains to produce the beta2 toxin *in vitro* and *in vivo*. This suggests that solely the presence of *cpb2*-harbouring *C. perfringens* in the intestinal tract, does not cause intestinal disease. Possibly, *cpb2*-harbouring *C. perfringens* is just a member of the normal intestinal flora, of which the growth is facilitated by circumstances which also facilitate the growth of other pathogens. These pathogens may form a major cause in the development of intestinal disease instead of *cpb2*-harbouring *C. perfringens*. Another option is that other members of the intestinal flora or intestinal circumstances which *e.g.* influence growth or toxin production by *C. perfringens* may play a role in the development of *cpb2*-harbouring *C. perfringens*-associated intestinal disease.

Table 1: Relationship between *cpb2*, *in vitro* beta2 toxin production, *in vivo* presence of beta2 toxin, and disease

Species	Disease	Relationship *cpb2*-disease	Relationship *in vitro* beta2 toxin- disease	Relationship *In vivo* beta2 toxin-disease	Reference
Piglets	Diarrhoea	+[a]	nd[b]	nd	Garmory et al., 2000
	Enteritis or diarrhoea	+	+	nd	Bueschel et al., 2003
	Diarrhoea	+	+	nd	Waters et al., 2003
	Diarrhoea	-[c]	nd	-	Farzan et al., 2013
Cows/ calves	Enteritis, enterotoxaemia, sudden death	+	nd	nd	Bueschel et al., 2003
Calves	Diarrhoea	-	nd	nd	Garmory et al., 2000
	Diarrhoea	-	nd	nd	Manteca et al., 2002
	Diarrhoea	+	+	nd	Lebrun et al., 2007
Lambs	Diarrhoea	-	nd	nd	Garmory et al., 2000
Roe deer	Intestinal disease	+	-	-	**Chapter 3**
Birds	Necrotic enteritis	-	-	nd	Crespo et al., 2007
Layers	Subclinical necrotic enteritis	-	-	nd	**Chapter 4**
Foals	Diarrhoea	-	nd	nd	Garmory et al., 2000
Horses	Typhlocolitits / intestinal disease	+	-	nd	Herholz et al., 1999 / Waters et al., 2005

[a] Positive relationship
[b] Not determined
[c] No relationship

The pathogenesis of *cpb2*-harbouring C. *perfringens*-associated enteritis

The mode of action of the beta2 toxin on intestinal cells is still under investigation. Earlier studies established a cytotoxic effect of beta2 toxin on human intestinal cell lines (Fisher et al., 2005; Gibert et al., 1997). However, the significance of the beta2 toxin in the development of *cpb2*-harbouring *C. perfringens*-associated intestinal disease in animals and humans has never been examined by genetic or protein deletion studies and *cpb2*-associated intestinal disease has never been reproduced by infection models.

In **chapter 5** the significance of porcine and human beta2 toxin in the development of cell cytotoxicity caused by *cpb2*-harbouring *C. perfringens* was investigated by an *in vitro* protein deletion study. Although supernatants of porcine *cpb2*-harbouring *C. perfringens* cultures and part of the supernatants of human *cpb2*-harbouring *C. perfringens* cultures were cytotoxic to both porcine and human intestinal cells, a significant role for the beta2 toxin in the development of this cell cytotoxicity could not be determined. These data suggest the absence of a potential role for beta2 toxin in the induction of intestinal cell cytotoxicity and subsequent diarrhoea. *In vivo* studies are necessary to determine or exclude a role of beta2 toxin in the development of intestinal disease.

A potential role for the intestinal microflora

The intestinal microflora comprises a complex mixture of at least several hundred's of bacterial species and is recognized as being beneficial to its host. Bacteria which are beneficial to the host diminish the colonization and growth of pathogenic bacteria by colonization resistance, competition for nutrients, and lowering the intestinal pH by the production of acid. Furthermore intestinal bacteria can influence each other by the production of signal molecules and metabolic products which regulate each other's growth and gene expression (Nicholson et al., 2005; Tancrède, 1992; Wise et al., 2007).

In **chapter 6** the effect of co-culturing *Lactobacillus fermentum*, a member of the normal intestinal flora, with *C. perfringens* on beta2 toxin production by *C. perfringens* was studied. A lower production of beta2 toxin was demonstrated with the presence of *L. fermentum* while the viability of *C. perfringens* was not affected. Incubating the supernatant of a beta2 toxin producing *C. perfringens* culture with *L. fermentum* did not result in degradation of the beta2 toxin. It was hypothesized that the reduction of

beta2 toxin production after co-culturing with *L. fermentum* might result from acid production by *L. fermentum*. Therefore the effect of pH on beta2 toxin production by *C. perfringens* was determined. A decrease in beta2 toxin production was shown when *C. perfringens* was grown at a lower pH, while the viability of the bacterial strains and the stability of the beta2 toxin were not affected. Q-PCR's performed on mRNA isolated from *C. perfringens* cultures grown at various pH's showed a lower transcription level of *cpb2* at a lower pH indicating that the mechanism by which environmental pH influences beta2 toxin production is via effects on gene transcription although other effects can not be excluded.

The transcription of *cpb2* is regulated by the two-component VirR/VirS system (Ohtani et al., 2003). The VirS protein shows similarity with sensor histidine kinase proteins of other bacteria, suggesting that *virS* encodes a transmembrane protein which acts as a receptor for external regulation. The VirR protein shows similarity with response regulator proteins from other bacteria that regulate gene expression (Lyrisitis et al., 1994). The identification of the VirR/VirS system indicates that possibly also other external circumstances than pH may upregulate or downregulate the gene expression of *cpb2*. Such an upregulation of *cpb2* gene expression has been shown by intestinal Caco-2 cells (Vidal et al., 2009). An upregulated expression of the gene may lead to a cytotoxic beta2 toxin concentration in the intestinal tract and subsequently disease.

Intestinal bacteria are able to produce bacteriocins, proteinaceous compounds that are antagonistic against bacteria other than the producing strain. Several members of the normal intestinal flora produce bacteriocins against *C. perfringens* (**chapter 7**). However, bacteriocins are also produced by *C. perfringens* itself (Barbara et al., 2008; Garnier and Cole, 1986, Mahony, 1974; Mahony and Li, 1978; Mahony and Swantee, 1978) which may increase their pathogenicity by inhibition of other *C. perfringens* strains in the intestinal tract (Barbara et al., 2008; Nauerby et al., 2003; Timbermont et al., 2009). Besides intra-species growth inhibition, inhibition of the normal intestinal flora by *C. perfringens* bacteriocins has been shown (Barbara et al., 2008; Feng et al., 2010; Stanley et al., 2012). Possibly, beta2 toxin may act as a bacteriocin and may play a role in the inhibition of other bacteria in the intestinal tract instead of exhibiting a direct effect on intestinal cells. However, a *cpb2*-negative strain inhibited the normal intestinal flora, while a *cpb2*-positive strain did not

suppress other bacteria (Barbara et al., 2008). Also in **chapter 6** the level of *L. fermentum* did not decrease after incubating *L. fermentum* with supernatant of *cpb2*-harbouring *C. perfringens.*

Another hypothesis may be that beta2 toxin only plays a role in the development of intestinal disease in combination with other virulence factors. *Cpb2*-harbouring *C. perfringens* strains may transfer their plasmid containing *cpb2* to other *C. perfringens* strains in the intestinal tract which may produce additional virulence factors, converting these strains into potential enteropathogens. Such a spread of plasmids containing virulence factors between *C. perfringens* strains has been described before (Brynestad and Granum, 2002; Kobayashi et al., 2009; Li et al., 2007). Additional virulence factors may include adhesion characteristics, rapid multiplication, the production of bacteriocins, and the production of other toxins (Barbara et al., 2008).

In conclusion, more research on the interaction between intestinal bacteria and the exchange of additional virulence factors between *C. perfringens* strains may increase the knowledge on the pathogenesis of *cpb2*-harbouring *C. perfringens*-associated intestinal disease.

Intestinal circumstances which may influence *cpb2*-harbouring *C. perfringens*

In **chapter 7** intestinal conditions are described which can influence the balance between probiotic bacteria and pathogens in the intestinal tract and which can induce growth and toxin production by *C. perfringens*. Since the growth of *C. perfringens* and the production of alpha, beta, netB, and beta2 toxin are all regulated by the VirR/VirS system, environmental circumstances influencing growth of *C. perfringens* and the production of alpha, beta, or netB toxin may influence the growth of *cpb2*-positive *C. perfringens* and the production of beta2 toxin too.

High levels of digestible carbohydrates or proteins favour the growth and toxin production by *C. perfringens in vitro* and *in vivo*. An overload of digestible carbohydrates or proteins in the intestinal tract may be obtained by a nutritional overload or by a decreased digestion and absorption rate. In **chapter 4**, a new syndrome is described among laying hens with focal necrosis in the intestinal tract. The pathological lesions in these laying hens resembled the pathologic lesions found in broilers with subclinical necrotic enteritis (SNE), which has been related to the presence of *C. perfringens* in the intestinal tract in combination with intestinal predisposing factors which increase substrate for *C.*

perfringens (Gholamiandehkordi et al., 2007; Kaldhusdal and Hofshagen, 1992). Both healthy and diseased laying hens carried *cpb2*-positive *C. perfringens* in the intestinal tract which were able to produce the beta2 toxin *in vitro*. A relationship between beta2 toxin producing *C. perfringens* and intestinal disease could not be determined. However, subclinical necrotic enteritis was only established in laying hens with an increased amount of intestinal watery content. Although the watery content may be a result of *C. perfringens* infection itself, it could be speculated that the watery content has resulted from other factors which caused villus atrophy and villus fusion as observed by histology. This villus atrophy and villus fusion may have caused a decreased digestion and absorption rate of intestinal digesta and herewith an increase of watery content and substrate for beta2 toxin production by *C. perfringens* resulting in the development of SNE.

Anti-trypsin in sow colostrum has been determined as a key-factor in the development of diarrhoea in neonatal piglets associated with *C. perfringens* producing beta toxin, another trypsin-sensitive toxin (Songer and Uzal, 2005). Therefore, anti-trypsin factor in sow colostrum may cause an accumulation of trypsin-sensitive beta2 toxin too, which may lead to cytotoxic concentrations of beta2 toxin (**chapter 5**) and subsequent development of diarrhoea. Protease-inhibitors may also be produced by intestinal parasites like *Trichuris* spp., which might have resulted in enteritis in a Hamadryas baboon after concurrent infection with *Trichuris* worms and *cpb2*-harbouring *C. perfringens*. The presence of beta2 toxin in the intestinal tract of the baboon was demonstrated by immunohistochemistry (Nikolaou et al., 2009).

Finally, antibiotics may cause alterations of the composition of the normal intestinal microflora (**chapter 7**) which may influence growth and beta2 toxin production by *cpb2*-harbouring *C. perfringens* (**chapter 6**) and subsequently the development of *cpb2*-harbouring *C. perfringens*-associated intestinal disease.

Conclusion

Results described in this thesis demonstrate that a possible role for *cpb2*-harbouring *C. perfringens* in the development of intestinal disease is still disputable. The development of a new primer set which recognizes both alleles

of *cpb2* and the development of a new test strategy for application of the newly developed PCR on fecal samples improved test sensitivity for the presence of *cpb2*. However, a relationship between *cpb2*-harbouring *C. perfringens*, beta2 toxin, and intestinal disease could not be proven in chickens and roe deer. Studies on the relationship between *cpb2*, beta2 toxin, and intestinal disease in other animal species show inconsistent results. Moreover a significant role for the beta2 toxin in the development of intestinal disease could not be established in an *in vitro* model and reproduction of the disease by other infection models has not been achieved yet. A proven role for the beta2 toxin in the development of intestinal disease by appropriate *in vivo* gene or protein deletion studies or infection models has not been established in any animal species. Therefore the role of the beta2 toxin in the development of intestinal disease remains at least questionable. Other virulence factors, the intestinal microbiota, and environmental circumstances might influence the significance of the intestinal presence of *cpb2*-harbouring *C. perfringens* in the development of intestinal disease. This hypothesis was supported by the finding of a reducing effect of *L. fermentum* on beta2 toxin production by *cpb2*-harbouring *C. perfringens*. In this way, environmental influences might increase the role of *cpb2*-harbouring *C. perfringens* in the development of intestinal disease too. However, this hypothesis needs further *in vivo* investigation.

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Samenvatting in het Nederlands

Clostridium perfringens

Het darmkanaal van gezonde mensen en dieren zit vol met bacteriën, eencellige micro-organismen met een afmeting van niet meer dan enkele micrometers. In totaal zijn er tien keer zo veel bacteriële cellen in het darmkanaal van dieren en mensen dan dat er lichaamseigen cellen zijn. De darmbacteriën spelen een belangrijke rol in de vertering van voedsel, het handhaven van de juiste zuurgraad in de darmen en het onderdrukken van andere bacteriesoorten die mogelijk schadelijk kunnen zijn. *Clostridium perfringens* is een darmbacterie die voorkomt in de darmen van gezonde dieren en mensen. Onder voor *C. perfringens* gunstige omstandigheden gaat de bacterie zich vermenigvuldigen en toxines (gifstoffen) produceren die de cellen van de darmwand beschadigen (necrose) en een ontstekingsreactie (enteritis) veroorzaken. De eiwitten die vrij komen door de celschade gebruikt *C. perfringens* als voedingsstoffen om zich weer verder te vermenigvuldigen en weer meer toxines te produceren. Doordat er zo steeds meer celschade aan de darmcellen ontstaat, worden voedingsstoffen en vocht niet meer goed opgenomen uit de darm en ontstaat er diarree.

Het beta2 toxine

Er zijn meer dan 15 verschillende toxines die door *C. perfringens* geproduceerd kunnen worden waaronder het alpha en het beta2 toxine. Het stukje DNA (gen) wat codeert voor het alpha toxine, *cpa,* komt in alle *C. perfringens* stammen voor en kan dus gebruikt worden om *C. perfringens* te identificeren. Het beta2 toxine is een van de vele *C. perfringens* toxines waarvan nog onbekend is in hoeverre het bijdraagt aan het ontstaan van celschade en enteritis. Het stukje DNA wat codeert voor het beta2 toxine, *cpb2*, komt onder *C. perfringens* stammen wijdverbreid voor. Het aantonen van *cpb2* vormt een belangrijk middel in het onderzoek naar de rol van beta2 toxine in het ontstaan van ziekte en gebeurt met behulp van een polymerase chain reaction (PCR). Daarbij wordt (een stukje van) het gen net zo vaak vermenigvuldigd totdat er genoeg van is om het met behulp van een kleuring voor het blote oog zichtbaar te maken. Het is de kunst om een PCR zo te ontwikkelen dat de test specificiteit hoog is, dat wil zeggen dat het alleen dat ene gen aantoont wat je wilt aantonen en dus niet ook andere genen. Daarnaast wordt een hoge

test sensitiviteit nagestreefd, dat wil zeggen dat de test alle *cpb2* varianten die voorkomen aan kan tonen, ook in zeer lage concentraties.

In **hoofdstuk 2** is door middel van een internet database gezocht naar een stukje van het *cpb2* gen wat in alle *cpb2* varianten voorkomt en niet in andere genen en is een nieuwe PCR voor de detectie van *cpb2* ontwikkeld. Vervolgens is gekeken hoe deze PCR het best toegepast kan worden om *cpb2* aan te tonen in mestmonsters. Een vergelijking is gemaakt tussen het toepassen van de PCR op geïsoleerde darmbacteriën die gekweekt waren uit mestmonsters en het toepassen van de PCR op DNA wat rechtstreeks geïsoleerd was uit dezelfde mestmonsters. Bij het toepassen van de PCR op darmbacteriën bleek *cpb2* in meer mestmonsters gevonden te worden dan bij het toepassen van de PCR op DNA rechtstreeks geïsoleerd uit de mestmonsters. Zowel *cpb2*-positieve als *cpb2*-negatieve *C. perfringens* isolaten werden aangetoond in hetzelfde mestmonster. Het testen van een mengsel van het DNA van tien isolaten leverde uiteindelijk de meest sensitieve test methode op.

De aanwezigheid van *cpb2*-positieve *C. perfringens* betekent niet dat het beta2 toxine ook altijd geproduceerd wordt. Er kan een mutatie in het gen zitten, waardoor het beta2 toxine niet geproduceerd kan worden. Soms is het gen volledig intact maar wordt het beta2 toxine toch niet geproduceerd door *C. perfringens* onder laboratoriumomstandigheden of het toxine wordt wel geproduceerd onder laboratoriumomstandigheden, maar niet in de darm. Dit laatste kan onderzocht worden door een speciale kleuring (immunohistochemie) die het beta2 toxine aankleurt in de darm. Welke factoren de productie van het beta2 toxine door *C. perfringens* beïnvloeden is nog onduidelijk.

De rol van *cpb2*-positieve *C. perfringens* in verschillende diersoorten

In varkens, koeien, paarden en mensen is een mogelijke relatie gevonden tussen de aanwezigheid van *cpb2*-positieve *C. perfringens* in de darm en enteritis. In de meeste studies is echter niet onderzocht in hoeverre het beta2 toxine ook echt in de darm aanwezig was.

In **hoofdstuk 3** wordt een nieuw ziektebeeld beschreven bij leghennen. In de darm van een deel van de leghennen van acht verschillende pluimveebedrijven werden kleine necrosehaardjes gevonden. Op zeven van de acht bedrijven kon *C. perfringens* geïsoleerd worden uit een deel van de leghennen. Alle *C. perfringens* bacteriën droegen *cpb2* bij zich en in elk

genomen monster waar *C. perfringens* isolaten in zaten, zaten een aantal isolaten die het beta2 toxine onder laboratoriumomstandigheden konden produceren. *Cpb2*-positieve C. *perfringens* werd ook geïsoleerd uit leghennen die geen necrose haardjes in de darmen hadden. Dit kan betekenen dat het voorkomen van *cpb2*-positieve C. *perfringens* onvoldoende is om de kleine necrosehaardjes te laten ontstaan en dat waarschijnlijk risicofactoren een rol spelen bij het aanslaan van de infectie. Deze hypothese wordt ondersteund door het feit dat necrosehaardjes alleen gevonden werden in combinatie met een hoger vochtgehalte van de darmen. Uit histopathologisch onderzoek (het bekijken van weefsel met een microscoop) bleek dat de darmvlokken verkort waren waardoor het verterings- en absorptie-oppervlak van de darm verkleind was. Dit kan leiden tot een verminderde verterings- en opnamecapaciteit van de darmwand en dus een verhoogd gehalte aan vocht en voedingsstoffen in de darm die weer als substraat kunnen dienen voor beta2 toxine productie door *C. perfringens.* Een onderliggende oorzaak van het hogere vochtgehalte in de darmen kon niet gevonden worden. Helaas kon het beta2 toxine in de darm niet worden aangekleurd met behulp van immunohistochemie doordat ook de darminhoud zelf mee kleurde, wat leidde tot onbetrouwbare resultaten. Daarom kan geen conclusie getrokken worden over het verband tussen het produceren van beta2 toxine in de darm en het ontstaan van de focale necrosehaardjes in de darmen van de leghennen.

In **hoofdstuk 4** is gekeken naar de aanwezigheid van *cpb2*-positieve *C. perfringens* in de darm bij reeën. De mogelijkheid van geïsoleerde stammen om het beta2 toxine te produceren en de aanwezigheid van het beta2 toxine in de darmen van dieren die cpb2-positieve *C. perfringens* bij zich droegen werden tevens onderzocht. *Cpb2*-positieve *C. perfringens* werd gevonden in de darm van alle reeën met darmafwijkingen en in de darm van een deel van de gezonde reeën. Slechts twee *cpb2*-positieve *C. perfringens* stammen uit de darm van een gezonde ree produceerden het beta2 toxine onder laboratoriumomstandigheden. Het beta2 toxine kon in geen van de reeën in de darm door aankleuring worden aangetoond. De studie laat zien dat het belangrijk is om niet alleen naar de aanwezigheid van de bacterie te kijken maar ook om na te gaan of het beta2 toxine in de darm wordt geproduceerd.

In **hoofdstuk 5** is een *C. perfringens* stam die veel beta2 toxine produceert opgegroeid in groeimedium. De bacterie is vervolgens uit het groeimedium verwijderd en het supernatant (groeimedium zonder bacteriën) met daarin het

beta2 toxine is toegevoegd aan Caco-2 cellen (darmcellen afkomstig uit de darm van mensen). Een groot deel van de Caco-2 cellen raakte door het groemedium met daarin het beta2 toxine beschadigd. Om te meten in hoeverre dit veroorzaakt werd door het beta2 toxine is het beta2 toxine uit supernatant verwijderd en is het supernatant vervolgens aan Caco-2 cellen toegevoegd. Opnieuw raakte een groot deel van de Caco-2 cellen beschadigd. Waarschijnlijk wordt de schade dus niet veroorzaakt door het beta2 toxine, maar door andere stoffen zoals andere toxines die ook in het supernatant aanwezig zijn. De *C. perfringens* stam waarmee getest werd was afkomstig uit de darm van een varken. Mogelijk is beta2 toxine wat geproduceerd wordt door *C. perfringens* stammen geïsoleerd uit de darm van een varken niet toxisch voor darmcellen van mensen. Daarom werd het experiment herhaald met IPI-21 cellen (darmcellen afkomstig van varkens). Weer werd geen verschil gezien tussen celschade veroorzaakt door supernatant met en het supernatant zonder beta2 toxine. Dit maakt het onwaarschijnlijk dat beta2 toxine een rol speelt in het ontstaan van darmonsteking bij biggen. Mogelijk zouden bepaalde omstandigheden in de darm ervoor kunnen zorgen dat hogere concentraties beta2 toxine kunnen ontstaan die wel toxisch zijn voor darmcellen. Dit dient verder onderzocht te worden.

De invloed van andere darmbacteriën op beta2 toxineproductie door *C. perfringens*

Hoofdstuk 6 laat zien dat een *cpb2*-positieve *C. perfringens* stam geïsoleerd uit de darm van een varken onder laboratoriumomstandigheden grote hoeveelheden beta2 toxine kan produceren. Maar onder invloed van de darmbacterie *Lactobacillus (L.) fermentum* bleek de beta2 toxine productie door *C. perfringens* sterk af te nemen. De afname van de beta2 toxine productie heeft mogelijk te maken met het zuurder maken van het groeimedium door *L. fermentum.* Daarom is er ook getest of de productie van beta2 toxine door *C. perfringens* afneemt bij een lagere zuurgraad wat inderdaad zo bleek te zijn. Het aantal *C. perfringens* bacteriën en de levensvatbaarheid van deze *C. perfringens* bacteriën nam niet af door de aanwezigheid van *L. fermentum* en het gevormde beta2 toxine werd niet afgebroken onder invloed van *L. fermentum.* Nadere analyse liet zien dat het DNA dat codeert voor het beta2 toxine in mindere mate afgelezen wordt bij een lagere pH en er dus inderdaad minder beta2 toxine wordt gevormd.

Voedingsfactoren die de groei en toxineproductie door *C. perfringens* beïnvloeden

Het is tot nog toe nooit gelukt om enteritis op te wekken door dieren te infecteren met *cpb2*-positieve *C. perfringens*. In infectieproeven met *cpb2*-negatieve *C. perfringens* was het meestal onmogelijk om enteritis te veroorzaken door het infecteren met de bacterie alleen. Risicofactoren als een hoog gehalte aan eiwit in de voeding of gelijktijdig optredende infecties waren essentieel voor het opwekken van enteritis. Daarom is het denkbaar dat risicofactoren tevens een rol spelen bij het ontstaan van (mogelijk) door *cpb2*-positieve *C. perfringens* veroorzaakte enteritis.

Hoofdstuk 7 geeft een overzicht van alle omgevingsfactoren die de groei en toxine productie door *C. perfringens* beïnvloeden. Onder laboratoriumomstandigheden is aangetoond dat *C. perfringens* zich sneller vermenigvuldigt en sneller toxines produceert wanneer de juiste voedingsstoffen aanwezig zijn. Verteerbare koolhydraten en eiwitten vormen een belangrijke voedingsbron voor *C. perfringens.* Het gehalte aan deze voedingsstoffen in het maagdarmkanaal kan verhoogd zijn door een te hoog gehalte van deze voedingsstoffen in de voeding waardoor de verterings- en opnamecapaciteit overschreden wordt of doordat de verterings- en opnamecapaciteit van de darm verlaagd is. Dit laatste gebeurt in het geval van een virusinfectie of wanneer de viscositeit (= stroperigheid) van de darminhoud toeneemt door de opname van voedsel met een hoog gehalte aan niet-verteerbare koolhydraten.

De toxines die door *C. perfringens* geproduceerd worden zijn eiwitten die onder normale omstandigheden afgebroken worden door het verteringsenzym trypsine. Wanneer dit enzym in onvoldoende mate aanwezig is, kunnen de toxines ophopen in het darmkanaal en zo voor schade zorgen. Een gebrek aan trypsine ontstaat bijvoorbeeld bij langdurige ondervoeding. Maar ook kunnen er in de voeding stoffen zitten die de afbraak van toxines door trypsine afremmen zoals in zoete aardappelen of sojabonen. Parasieten in het maagdarmkanaal kunnen anti-trypsine produceren om zichzelf te beschermen en hiermee dus ook de toxines van *C. perfringens*. Ook in biest, de eerste moedermelk, zitten anti-trypsine factoren die ervoor zorgen dat antilichamen die via de biest aan de nakomeling worden overgedragen niet worden afgebroken. Bijkomend effect is dat eventueel in de darm van de zuigeling aanwezige toxines van *C. perfringens* niet afgebroken worden

en er darmschade ontstaat. Wanneer antilichamen tegen de toxines van *C. perfringens* aanwezig zijn in de biest wordt dit effect weer teniet gedaan.

Conclusie

De rol van het beta2 toxine in het ontstaan van enteritis is nog onduidelijk. Een relatie tussen de aanwezigheid van beta2 toxine in de darm en het ontstaan van darmontsteking kon niet worden aangetoond in de uitgevoerde studies. Ook had het beta2 toxine geen effect op verschillende cellijnen. Dat maakt het onwaarschijnlijk dat alleen de aanwezigheid van *cpb2*-positieve *C. perfringens* in de darm leidt tot darmontsteking. Andere toxines, andere darmbacteriën of voedingsfactoren zouden ervoor kunnen zorgen dat *cpb2*-positieve *C. perfringens* wel een significante rol speelt in het ontstaan van darmontsteking. Om deze hypothese te bevestigen is verder onderzoek nodig.

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Curriculum Vitae

Janneke Allaart werd op 6 april 1979 geboren te Amstelveen. Na het behalen van haar VWO diploma in 1997 aan het Hermann Wesselink College in Amstelveen begon zij aan de studie diergeneeskunde in Utrecht.

Als nieuwsgierig kind van een gedreven natuurwetenschapper was de interesse voor wetenschappelijk onderzoek al vroeg gewekt en de studie werd zo veel mogelijk benut om onderzoekservaring op te doen. De keuze voor een monotraject varken zorgde ervoor dat zij tot twee maal toe de kans kreeg om een onderzoeksstage te doen aan Wageningen University and Research Centre naar gedrag & welzijn van zeugen in groepshuisvesting en preventie van speendiarree in de biologische varkenshouderij.

In 2004, na haar studie diergeneeskunde, werd zij aangesteld als junior docent bij de vakgroep varkensgezondheidszorg van de hoofdafdeling gezondheidszorg landbouwhuisdieren aan de faculteit diergeneeskunde te Utrecht. Naast onderwijs, tutoraat en patiëntenzorg werd wederom zo veel mogelijk tijd besteed aan het leveren van een bijdrage in onderzoek naar voor de varkenshouderij relevante onderwerpen. Stond tijdens de studie de bevlogenheid voor welzijnsonderzoek in de varkenssector nog voorop, nu begon het enthousiasme voor het onderzoek naar de pathologie van infectieziekten het toch echt te winnen.

In 2007 begon Janneke daarom aan haar promotieonderzoek bij de hoofdafdeling pathobiologie aan de faculteit diergeneeskunde te Utrecht, met dit proefschrift als resultaat.

In 2012 werkte Janneke een jaar als varkensdierenarts bij diergeneeskundig centrum de Overlaet in Oss. Sinds juni 2013 is zij als onderzoeker werkzaam bij het Nutreco Poultry Research Centre in Casarrubios del Monte (Toledo, Spanje).

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