NEW INSIGHTS INTO THE PATHOGENESIS OF GASTROINTESTINAL *CLOSTRIDIUM PERFRINGENS* INFECTIONS IN VEAL CALVES

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New insights into the pathogenesis of gastrointestinal *Clostridium perfringens* infections in veal calves

Nieuwe inzichten in de pathogenese van gastro-intestinale *Clostridium perfringens* infecties bij witvleeskalveren

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''*To study the phenomena of disease without books is to sail an uncharted sea, while to study books without patients is not to go to sea at all*.''

Sir William Osler (°1849)

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LIST OF ABBREVIATIONS

β2	beta2
BB	Belgian Blue
BBB	blood-brain barrier
BVD	bovine viral diarrhea
CPA	*Clostridium perfringens* type A
DNA	deoxyribonucleic acid
EC	European council
ELISA	enzyme-linked immunosorbent assay
HBS	haemorrhagic bowel syndrome
HE	haematoxylin and eosin
HF	Holstein Friesian
MR	milk replacer
OD	optical density
PCR	polymerase chain reaction
SF	solid feeds
STEC	shigatoxin-producing *Escherichia coli*
TMR	total mixed ration
VFA	volatile fatty acids

Enterotoxaemia is one of the most frustrating diseases known to cattle farmers and their veterinarians. The disease typically strikes the most vivid, fast growing and therefore most valuable calves. The rapid disease progress and inevitable death leave both farmer and veterinarian helpless. Although the disease is described worldwide and in all breeds, predominantly beef animals, such as Belgian Blues (BB), are affected.

Next to important losses in conventional suckler calves, enterotoxaemia is also responsible for up to 20% of the total mortality in BB veal calves (Pardon *et al.*, 2012). Because the disease especially occurs at the end of the production cycle, when the animals have a high value, for years enterotoxaemia has led to huge financial losses within the veal industry.

Notwithstanding its economic importance, the pathogenesis of enterotoxaemia in calves, both in suckler and in veal calves, is only marginally known. *Clostridium perfringens* is assumed to be the causative pathogen of enterotoxaemia in cattle. However, current hypotheses on the causative toxins (predominantly alpha toxin and β2-toxin) and their working mechanisms are not fully satisfactory, because evidence on their involvement in lesion development is lacking. Another issue which particularly hampers management of this disease in practice, is the lack of a reliable diagnostic test, in particular because the causative toxins are not identified. Since this pathogen is ubiquitous in both the environment and in the gastro-intestinal microbiota of cattle, in order to successfully prevent the disease in the future, identification of predisposing environmental factors, is equally essential as knowledge on the involved toxins.

In the first part of this thesis about the pathogenesis of fundic ulcerations, is elucidated. In the second part, the potential value of intestinal clostridial counts for diagnosis of enterotoxaemia is explored. Subsequently, the development of an intestinal loop model to induce and study enterotoxaemia-like lesions is described. In the final part, preliminary insights are acquired into the role of the feed regimen in the development of humoral immunity against the primary toxins involved in enterotoxaemia in calves.

CHAPTER 1

GENERAL INTRODUCTION

CHAPTER 1.1

STRUCTURE AND MANAGEMENT OF THE VEAL INDUSTRY IN BELGIUM

STRUCTURE AND MANAGEMENT OF THE BELGIAN CATTLE INDUSTRY

Historically, the cattle industry used local dual purpose breeds, such as the Belgian white and red breed of East Flanders and the Belgian red breed of West Flanders. In modern times, the traditional dual purpose farms are more and more directed towards exclusively beef- or dairy production.

Today, worldwide the dairy branch of the cattle industry uses predominantly HF cows, known for their supreme milk production. The beef industry is however more diverse and the used breeds and management structures are very region dependent.

The modern HF dairy cow has a very high milk yield and thus high energy and protein needs, especially in the first weeks of lactation. In practice, this is often achieved by balanced feeding of grass, corn and grains for energy provision and soy, alfalfa or even non-protein nitrogen for protein provision (Rankins *et al.,* 2002; Shen *et al.*, 2015; Liang and Cabrera, 2015). However, there is a large variation in quality and composition of the ration between farms.

Dairy calves are normally separated from their dams immediately after birth. Replacement heifers are raised either on cow's milk or milk replacer (MR) combined with concentrates and hay. The calves are weaned at an early age (between 6 and 12 weeks of age) for reasons of cost efficiency (Kahn *et al.*, 2011). Older calves are commonly fed hay and corn. Male calves, and less frequently surplus female calves, are destined for the veal industry and leave the dairy farm at approximately 2 weeks of age. On some farms, HF cows are inseminated with semen from beef breeds, in order to create heavier, more valuable crossbred calves for the veal industry (Domingo *et al.*, 2015).

In Belgium, predominantly BB cattle are raised for beef production. A minority of farms specializes in other breeds, such as (among others) Limousin, Blonde d'Aquitaine, Aberdeen Angus or Maine Anjou (Rapport annuel sur l'évolution de l'économie agricole et horticole de la Région wallonne, 2008; Landbouwrapport Vlaanderen, 2012). The BB beef industry can be divided into two major production systems, namely suckler calves (minority) and intensively reared calves (most conventional rearing system). The ration of BB cows can be very diverse, ranging from only pasture and roughage (for example hay or grass and corn silage), to a variety of products such as beets, beer draff or other waste products from the food industry. Depending on the season, suckler calves go outside with their dams, hereby gradually decreasing milk intake and increasing grass

intake. Alternatively, they are kept indoors, where they are fed concentrates and hay besides cow's milk. Weaning can be done abrupt, by taking the calf from the dam, or by gradually removing dams from a group, where more calves can drink from a decreasing number of remaining dams (Rasby, 2007; Lambertz *et al.*, 2015). Overall, suckler calves are often weaned at a later age compared to conventionally reared BB calves, and consume larger amounts of milk up to a later age. Conventionally reared calves are fed very intensively. Initially, the calves are fed MR (or in fewer cases with cow's milk), and supplemented with concentrates at a very young age. Milk provision is gradually decreased and often completely replaced by a concentrate ration by the age of 3 to 4 months. After weaning, calves are fed all-concentrate rations or a minimum of roughage. There is no real difference in the management of male and female calves until the age of 6 months. Heifers should have their first calf by the age of 24 months with a body weight of 600kg in a good body condition (not too fat). Therefore, insemination should be done at 15 months of age at a body weight of 375kg. To reach this objective, an average daily weight gain of 750g/day is optimal. This is possible by feeding good quality roughage and grass, which are cheaper than concentrates and more safe in terms of provision of sufficient structure. Bulls (and a minority of BB heifers) are fattened as fast as possible. Therefore, intensive feeding with a good feed conversion is pursued. The fattened animals are slaughtered at 650 to 800 kg live weight. Depending on genetics, housing, season and health issues, bulls reach this weight between 18 and 24 months of age (Technische brochure: Voeding van runderen van het Belgisch Wit-Blauwe ras, ILVO, 2013). Corn (maize) silage produces heavy weight yields per acre at a low cost and makes excellent roughage for beef-cattle finishing (De Campeneere *et al.*, 2002).

The intensive feed regimes in the specialized modern dairy and beef industry predispose for gastro-intestinal disorders, such as clostridial diseases. This is translated into a predisposition of high productive dairy cows fed protein- and energy-rich diets for haemorrhagic bowel disease (HBS), and in a predisposition for enterotoxaemia and overeating disease in intensively fed calves.

STRUCTURE AND MANAGEMENT OF THE BELGIAN VEAL INDUSTRY

Veal is an expensive and sumptuous quality product, which is appreciated in the western world for its high nutritional value, low fat content and tenderness. In the European Union (EU), veal is defined as meat from calves aged between 0 and 8 months of age. Since 2008, three effective 'veal' definitions are applicable in Europe (Regulation EC566/2008). White veal (milk-fed) is pale in color and the calves must be younger than 8 months at slaughter. White veal is the traditional form of veal production and still the most important segment of the veal industry. Rosé veal (grain-fed) also originates from calves younger than 8 months, but has a more red color due to the different diet. Meat from older animals (between 8 and 12 months) is locally marketed under different denominations, such as beef (United Kingdom) or older rosé veal (Ireland, the Netherlands) (Pardon *et al.,* 2014).

The veal industry has a great economic relevance for the dairy industry because of the valorisation of surplus male HF calves and because of the purchase of large amounts of milk byproducts. These byproducts, such as whey and whey protein concentrate, are of minor value to the food industry. Belgium produces almost exclusively white veal in three production types, in particular dairy calves (red Holstein and HF; 60%), purebred double muscled BB (15%) and crossbreds (mainly HF x BB; 25%). The BB segment is rather small, since these calves require a more specialized approach, have higher mortality and morbidity rates and an exclusively Belgian market (Pardon *et al.,* 2012a).

Unlike dairy herds or European beef herds, veal farms are typically highly integrated intensive livestock farming systems, with a complex network of tradesmen, milk powder plants, sorting centers, fattening herds, slaughterhouses and meat processing companies, owned by or in some way bound to large integrations that coordinate the sorting and distribution of the calves in a strict all-in/all-out production system (Pardon *et al.,* 2014). In Belgium, there are three main integrators that own milk powder plants and slaughterhouses, and that are complemented by five smaller integrations. Over 94% of the Belgian veal farms are situated in Flanders; more than 70% of those are in the province of Antwerp. The mean herd size is almost 600 calves per farm, which indicates an intensive production system (Pardon *et al.,* 2014). In the public opinion, veal farms are often criticized because the public still has the image of the small individual crates in

which veal calves were historically raised. Since 2007, however, group housing is obligatory in the European Union from the age of 8 weeks on (European Council, 1991, 1997; directives 91/629/EC and 97/2/EC). Only in the first 6 weeks of production, individual housing is allowed. Most commonly, calves are housed on slatted floors in small pens of 4-8 animals (figure 1.1). In the first 6 weeks of production, these pens are divided into individual baby boxes by fenced lateral partitions allowing social contact with neighboring calves (figure 1.2) (Pardon *et al.*, 2014). Other housing systems are used on a smaller scale: larger groups and automatic milk delivery systems are used in France, and the higher welfare standard systems as integrated in Peter's Farm in the Netherlands (large groups of 60 calves, nipple feeding, environmental enrichment) and Naturafarm in Switzerland (free access to outdoor pens, straw and water) (Bähler *et al.,* 2010; Brscic *et al.*, 2010).

Figure 1.1 Group housing of 26 week old HF veal calves in a one compartment stable.

Figure 1.2 Individual housing of 6 week old HF veal calves in baby boxes.

The production of white veal requires a very thorough follow-up of the meat color of the calves. On the one hand calves with too white meat color automatically also suffer from anemia, which diminishes appetite and growth performance. Furthermore, the European standards require that the haemoglobin content of calves at slaughter should minimally be 7.5 grams/L (European Council, 1991, 1997; directives 91/629/EC and 97/2/EC). On the other hand, calves with red meat lead to loss of valorization because the meat is not suitable for the exclusive market of white veal.

So, in order to comply with European standards and also to optimize the growth and meat value, a delicate iron balance must be maintained. In order to obtain white veal, the iron supply is held very limited in the ration. Iron measurements are carried out on full blood during the production period and extremely white meat color and anemia are counteracted by means of iron administration (Pardon *et al.*, 2014).

NUTRITIONAL MANAGEMENT

MILK REPLACER

Historically, the typical diet of veal calves is an all-liquid diet of milk powder. Two main products are used: MR based on variable percentages of skimmed milk powder, and MR which does not contain skimmed milk powder and therefore no casein (nill product). Skimmed milk powder is a high-quality, but more expensive, protein source. In cheaper MR's the casein-fraction is replaced by whey proteins. Whey is a side product from cheese production and is a cheap, but good quality, protein source. This whey powder is supplemented in variable degrees with lesser quality vegetable protein and energy sources as soy, pea and wheat to provide in the calves' needs. The milk powder composition can strongly vary in function of ingredient market prices and is adapted to the breed (Pardon *et al.,* 2014).

As described above, there are three predominant production types in Belgium, namely dairy calves (red Holstein and HF; 60%), purebred double muscled BB (15%) and crossbreds (mainly HF x BB; 25%). The different production types all have their specific feeding needs. In the start-up period, all calves start on a diet consisting of MR twice a day. The MR used in young calves (0-6 weeks) mostly consists, independent of the production type, of a mixture of a MR based on 50% skimmed milk powder, and nill product. In the group housing phase (after 6 weeks in production), the nutrition differs between management systems. In HF herds, the skimmed milk powder is gradually replaced by nill product, and the greatest part of the production cycle the calves are fed only nill product, supplemented with comparatively larger amounts of roughage than BB calves (figure 1.3; Pardon *et al.,* 2014). This is a cheap way of feeding, on which HF veal calves do reasonably well, obtaining a hot carcass weight of on average 152 kg after 22-26 weeks (Pardon *et al.,* 2013).

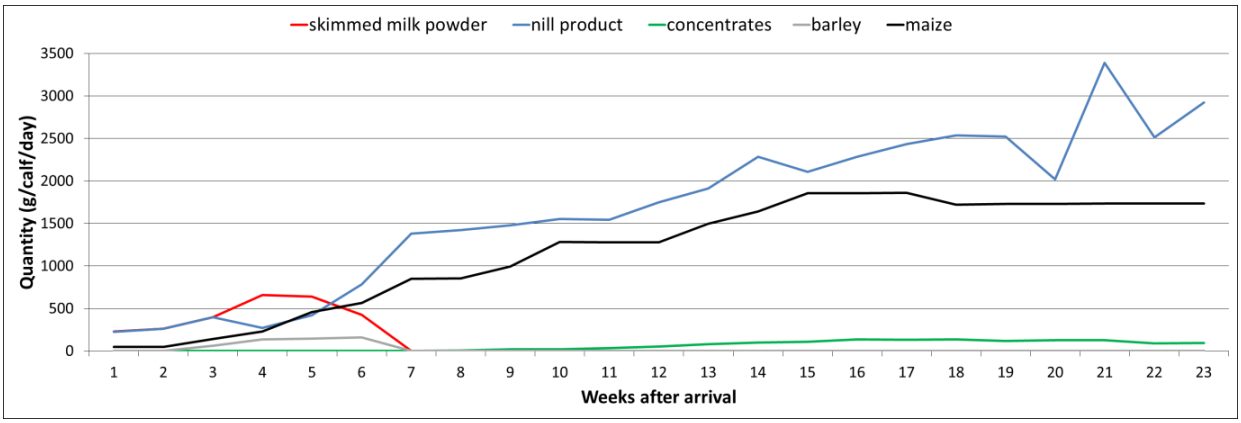

Figure 1.3: Example of a typical feed regimen on a HF veal farm

BB veal calves are continuously fed a diet consisting of MR based on skimmed milk powder, combined with small amounts of chopped wheat straw or another roughage source, as is obligated by European Welfare standards (European Council Directive 2008/119/EC, European Council, 2008). The quantity and concentration of the MR is gradually increased, up to more than 10 liters per feeding, and highly concentrated (Figure 1.4; Pardon *et al.*, 2010). Although MR based on skimmed milk powder is more expensive than nill product, the use of skimmed milk powder is profitable since BB calves do very well on this system as they have an excellent feed conversion, slaughter efficiency, meat quality and conformation (Pardon *et al.,* 2014). BB calves reach up to 200 kg carcass weight between 28 and 32 weeks after arrival (Pardon *et al.,* 2013).

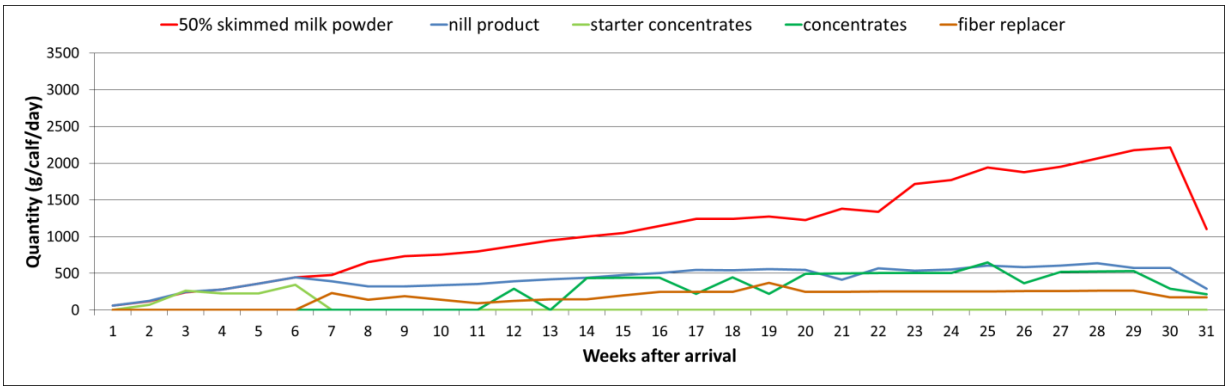

Figure 1.4: Example of a typical feed regimen on a BB veal farm.

ROUGHAGE PROVISION

European legislation demands a minimum amount of fibrous feed, ranging from 50 g/day at the age of 2 weeks (start of the production cycle), up to 250 g/day at the age of 20 weeks, in addition to the MR diet (European Council Directive 2008/119/EC, European Council, 2008). However, there are no specifications on the type of solid feed (SF) provided. In the first years following implementation of this legislation, most farmers opted for cereal grains, in order to restrict the iron supply, and maintain the white meat color. Additionally, grain provides easily fermentable sugars, contributing to body weight gain. However, the welfare effect of the provision of grains is limited, since it does not promote chewing, nor ruminating, does not prevent oral stereotyping, and even predisposes for hyperkeratosis and plaques as a reaction on the increased volatile fatty acids (VFA) concentration in the rumen (Brscic *et al.,* 2011; Cozzi *et al.*, 2002; Prevedello *et al.,* 2012). Therefore, there is a tendency towards other SF sources. Chopped wheat straw is a popular alternative, since it does not affect meat color (due to the low bioavailability of iron), and it provides enough structure to fulfill chewing and ruminating needs, leading to less abnormal behavior (Cozzi *et al.,* 2002; Mattiello *et al.*, 2012). Unfortunately, chopped wheat straw increases the number of abomasal lesions, and its low energy content does not contribute to an increased growth (Prevedello *et al.,* 2012; Brscic *et al.*, 2011). Other roughage sources, such as maize and dried beet pulp, evoke too red carcasses (Cozzi *et al.,* 2002).

Currently, in most herds, SF mixtures are used to optimize benefits of the different roughage sources, while limiting the health risks. A popular mixture is corn silage, barley or wheat straw, and grains. The fluctuating price of milk derived products, and the increasing ability to develop more adapted SF mixtures, has led to a drastic decrease in MR use in predominantly HF veal calves, and replacement by SF feeding, easily doubling the European recommendations. SF mixtures of corn grain and straw enriched with extruded pea or urea have been shown to be able to reduce the amount of MR consumption, and thus lower the total feeding cost without compromising on meat color, growth performance, behavior or carcass quality (Brscic *et al.,* 2014).

This shift away from MR towards more SF has important implications on calf management, welfare and health. The increased roughage provision influences gastrointestinal development, nutrient utilization and gastro-intestinal microbiota composition. In calves with better rumen development, ruminal microbes can lead to

more ammonia-N recycling, optimizing protein utilization for growth (Berends *et al.,* 2015). Since relatively less water can be retained from MR feeding, there might be an increased need for water provision when SF provision is increased further (Berends *et al.,* 2012). In the current proportions, not providing drinking water does not lead to dehydration (Gottardo *et al.,* 2002). The provision of water however does reduce milk refusals and, in calves fed large amounts of SF, it positively affects the development of rumen mucosa, positively influencing production characteristics. Moreover, providing water acts as an environmental enrichment (Gottardo *et al.,* 2002).

In addition to an effect on the production characteristics, the adapted gastro-intestinal microbiota can influence the gastro-intestinal health. Several intestinal bacteria are known to have a bacteriostatic effect on *C. perfringens* or other pathogenic bacteria (Schoster *et al.,* 2013), and dietary changes can thus have an important effect on the occurrence of gastro-intestinal problems. Empirically, it is well known that nutritional management greatly influences the incidence of enterotoxaemia and abomasal ulcers in veal calves. However, at present it is unknown what dietary factors exactly provoke these diseases. Subsequently a detailed overview of current knowledge on the pathogenesis and diagnosis of enterotoxaemia and abomasal ulcers in calves is provided, with a focus on the specific risk factors in veal calves.

CHAPTER 1.2

DISEASES ASSOCIATED WITH *CLOSTRIDIUM PERFRINGENS* IN CATTLE

DISEASES ASSOCIATED WITH *CLOSTRIDIUM PERFRINGENS* IN CATTLE

CLOSTRIDIUM PERFRINGENS

MICROBIOLOGICAL CHARACTERISTICS

C. perfringens is a large gram-positive rod (figure 1.5). The opportunistic pathogen is non-motile, mesophile (with an optimum growth temperature of 37°C), and produces endospores (Hatheway, 1990). It is an anaerobic bacterium, which produces energy using nitrate as its final electron acceptor. When grown in the presence of nitrate, the bacterial growth rate increases, as this inorganic electron acceptor leads to an increase in energy production (Hasal and Hall, 1975). Apart from the anaerobic phosphorylation reactions, *C. perfringens* can also undergo anaerobic fermentation to produce carbon dioxide and other gases and create an anaerobic environment in host tissues (Shimizu *et al.*, 2002). Although the growth is strictly anaerobic, *C. perfringens* has an extremely good oxygen tolerance (Hasal and Hall, 1975). In other anaerobic bacteria, the superoxide dismutase activity and oxygen reduction rates are known predictors for aerobic survival. However, these factors do not seem to influence the environmental survival of *C. perfringens*, since oxygen tolerance is very high despite low superoxide dismutase activity and oxygen reduction abilities (Rolf *et al.*, 1978). The environmental survival contributes to the buildup of a high infection pressure in environments with faecal contamination, such as cowsheds, stables and corrals.

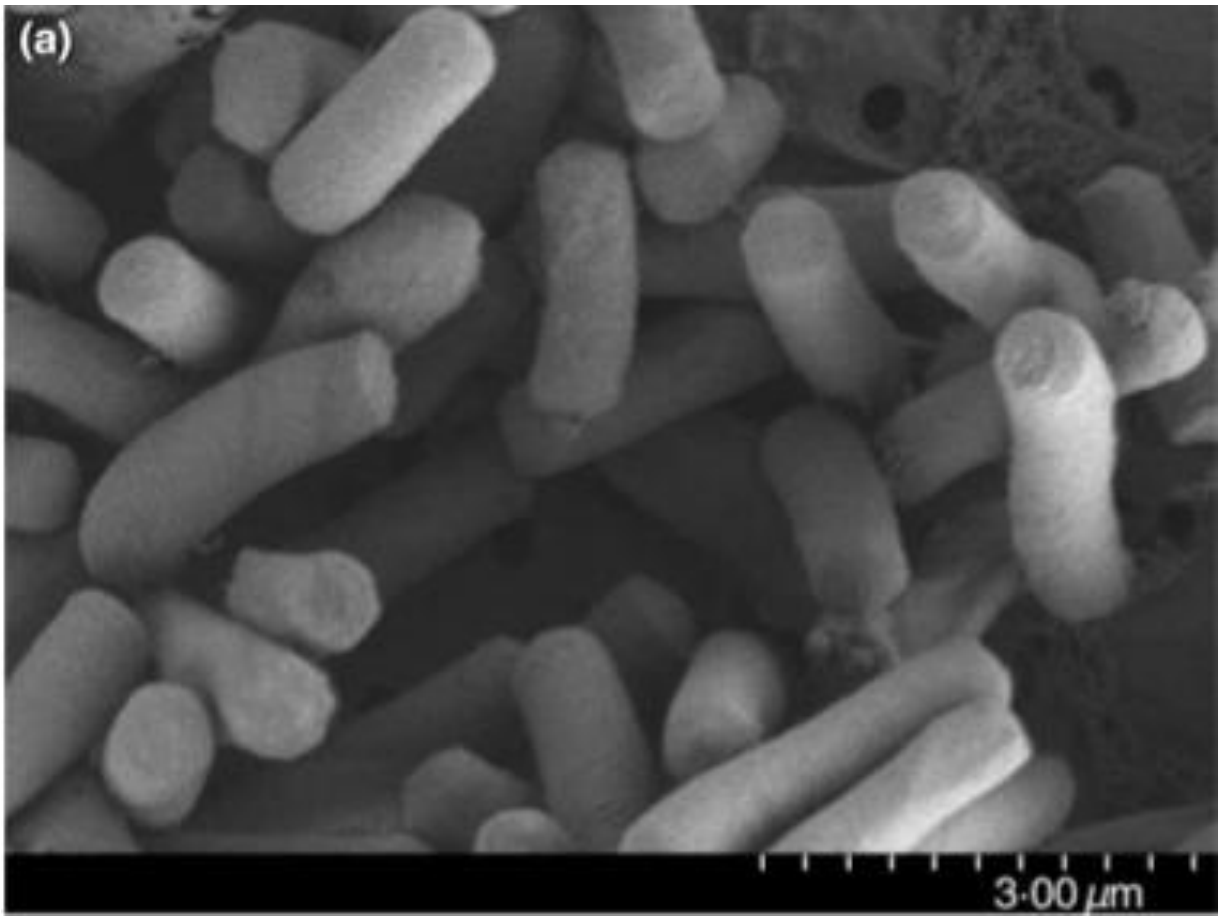

Figure 1.5: Scanning electron micrographs of *Clostridium perfringens* (Zhang *et al.*, 2006).

The bacterium has a single circular chromosome, with a relatively low guanine-cytosine content compared to other Gram-positive bacteria, suggesting better tolerance against high temperatures (Shimizu *et al.*, 2002; Hurst and Merchant, 2001). Like other Gram-positive bacteria, it has a thick protective cell wall made up of peptidoglycan, surrounding an inner membrane. The thickness of this peptidoglycan wall is associated with the heat resistance of both spores and vegetative germs. Normal heat resistance D-values (the time required at a certain temperature to kill 90% of the organisms being studied) for *C. perfringens* spores varies between 1 and 124 minutes at 100°C (Orsburn *et al.,* 2008). So, the spores are very heat-resistant and can survive boiling temperatures (Doyle, 2002). Furthermore, spores are also very resistant to ultraviolet (UV)-radiation and disinfectants, complicating the disinfection of materials (Paredes-Sabja *et al.*, 2008).

C. perfringens spores are thought to be the important infectious cell morphotype, and after inoculation into a suitable host, these spores must germinate and return to active

growth to cause GI disease (Hatheway, 1990). While bacterial spores can remain dormant for many years, they can return to life in as little as 20 min via spore germination and outgrowth if nutrients are added (Paredes-Sabja *et al.*, 2008).

Although the pathogen has all enzymes necessary for glycolysis and glycogen metabolism, it does not contain all enzymes for amino-acid biosynthesis (Shimizu *et al.,* 2002). *C. perfringens* has exacting growth requirements. The list of essential amino-acids is long and exists of arginine, aspartic acid, cystine, glutamic acid, histidine, leucine, phenylalanine, threonine, tryptophan, tyrosine and valine. In addition, adenine, calcium pentothenate, pyridoxine and biotine are essential vitamins (Fuchs and Bonde, 1957). Consequently, the growth rate, and possible intestinal overgrowth, of *C. perfringens* is rather determined by the availability of essential amino-acids and growth factors, than by the abundance of sugars or starch.

Based on the produced extracellular toxins, alpha-toxin, beta-toxin, epsilon-toxin and iota-toxin, *C. perfringens* is categorized into 5 serotypes (A, B, C, D and E) (table 1.1).

Table 1.1. *Clostridium perfringens* toxinotypes (Niilo, 1980)

Type	α	β	E	I	Enterotoxin	β2
A	++				(x)	(x)
B	+	++	+			(x)
C	+	++				(x)
D	+		++			(x)
E	+			++	(x)	(x)

+ Classic

(x) Potential

Historically, typing was done based on the neutralization of the major toxins with type-specific antisera using rodents as test animals (mouse lethality tests) (Petit *et al.*, 1999). However, today, for animal welfare and practical reasons, this technique is abandoned and replaced by detection of toxins by enzyme-linked immunosorbent assay (ELISA) or genotyping by multiplex PCR (Naylor *et al.*, 1997; Baums *et al.*, 2004). For ELISA and for the rodent-test with antisera the antigenicity of the toxin is essential. The PCR-technique

detects the presence of the toxin genes (namely *cpa*, *cpb*, *etx* and *iap*, for the major toxins; often complemented with *cpe* for enterotoxin and *cpb*2 for β2-toxin).

Apart from the described major toxins, a whole series of minor toxins are produced by *C. perfringens.* The differentiation between major and minor toxins is outdated, and was founded upon the believe that especially the major toxins were important in the development of disease. Enterotoxin was one of the first and most important minor toxins known, for its important role in human food poisoning. Today, the role of several minor toxins and 'new' toxins as the β2 toxin has been elucidated and has been suggested to be important in different animal diseases, including in gastro-intestinal disease in cattle (Gibert *et al.*, 1997; Muylaert *et al.*, 2010).

SPECIFIC DISEASES ASSOCIATED WITH *CLOSTRIDIUM PERFRINGENS* IN CATTLE

All 5 toxinotypes have been isolated from, and associated with, disease in bovines. The most important disease presentations associated with *C. perfringens* in both adult cows and calves are enterotoxaemia, HBS, neonatal haemorrhagic abomasitis and necrotic enteritis, overeating disease and abomasal ulcers. Enterotoxaemia and abomasal ulcers are seen in conventionally reared calves as well as in veal calves, but due to their high prevalence and economic importance in the veal industry, these diseases are discussed under 'diseases associated with CPA in veal calves'.

HAEMORRHAGIC BOWEL DISEASE

HBS is an emerging syndrome of adult cattle, for which no specific cause has been elucidated. The disease is characterized by acute, progressive segmental necro-haemorrhagic enteritis of predominantly the jejunum, with intraluminal blood clots (figure 1.6; Abutarbush *et al.,* 2004). These clots lead to obstructive ileus. Clinical signs include depression and ileus, with abdominal distention, melena and colic, often accompanied by anorexia, lethargy, dehydration, shock, milk drop and death. In some cases sudden death is observed, without any previous symptoms (Abutarbush and Radostitis, 2005; Ceci *et al.*, 2006). The mortality varies between 85-100% (Kirkpatrick *et al.*, 2001).

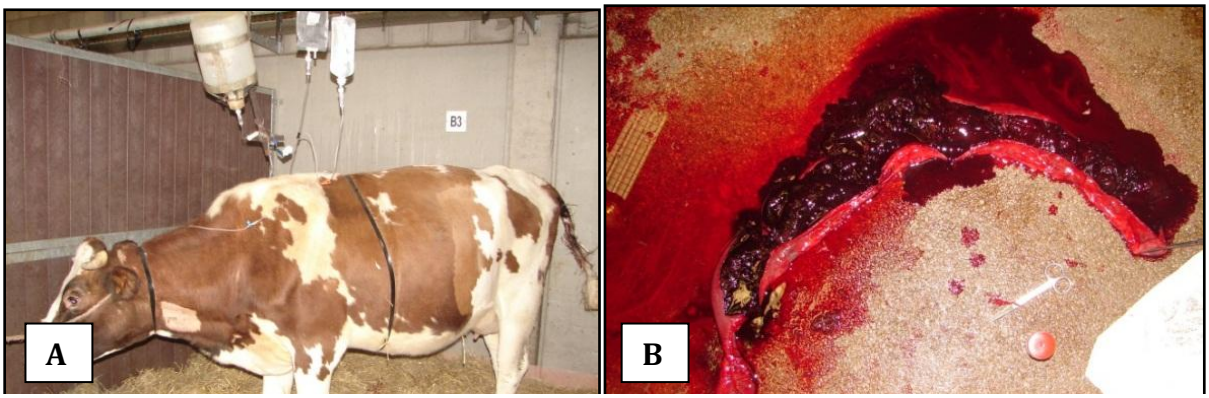

Figure 1.6: A: Cow with HBS after resection of the affected intestinal segment, treated with perfusion, neomycine, penicillin, lidocaine, flunixine, and erythromycin. Despite therapy, the cow died 2 days after surgery. On pathology a diffuse necro-haemorhagic enteritis was observed. B: Opened resected intestinal segment (proximal jejunum), filled with blood clots.

Similarly to other Clostridium-linked diseases, HBS is believed to be multifactorial, and in literature several predisposing factors have been suggested. HBS is predominantly seen in highly productive dairy cows, but with a special predisposition in Brown Swiss. Prevalence seems to be higher in autumn and winter, and in older cows, often in early lactation, when high energetic and high protein diets are fed with low structure (Godden *et al.*, 2001, Berghaus *et al.*, 2005). The disease is most seen in farms with a Total Mixed Ration (TMR). HBS has been associated with CPA, but also with other pathogens, such as *Aspergillus fumigatus*, mycotoxins, and shigatoxin-producing *Escherichia coli*'s (STEC's) (Forsberg, 2003; Socket, 2004; Baines *et al.,* 2011; Elhanafy *et al.*, 2013).

The most common hypothesis is that the pathogenesis of HBS is similar to this of enterotoxaemia in other animals. There are in fact a lot of similarities between HBS and enterotoxaemia, such as risk factors (intensive feed regimens), acute evolution of the disease, and the presence of necro-haemorrhagic enteritis (figure 1.7). This enforces the hypothesis that CPA and its toxins make an important contribution to the development of HBS. Additionally, CPA was isolated from the intestine of diseased animals in several case reports. CPA β2-toxin positive strains are frequently isolated from the lesions (Dennison *et al.*, 2002; Ceci *et al.*, 2006; Savic *et al.*, 2012). The contribution of both alpha toxin and β2 toxin to the development of the observed lesions is as yet unclear. Because of the risk factors, bacterial overgrowth due to overfeeding with protein is

suspected, followed by intraluminal toxin production. Consequently alpha toxin might lead to damage to the cell membranes of the enterocytes, leading in turn to the production of inflammatory mediators, such as thromboxane, leukotriene and prostaglandins. This cascade triggers a local inflammatory reaction, damaging the intestinal microvascularization and resulting in haemorrhages in the intestinal lumen. Due to the affected integrity of the epithelium, the facilitated penetration of bacteria and toxins can lead to septicaemia and toxaemia (Elhanafy *et al.,* 2013).

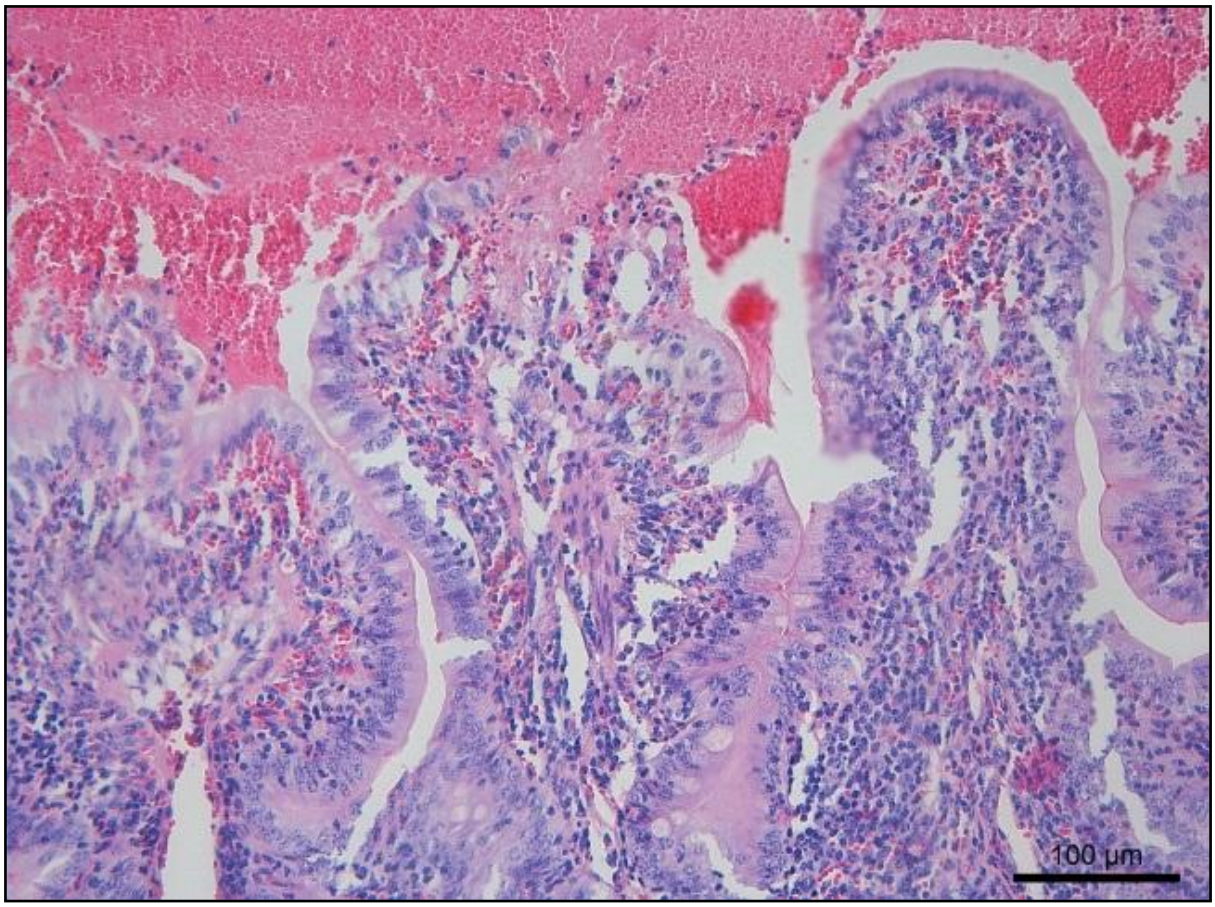

Figure 1.7: Jejunum: necrotic tip of the villi with intraluminal haemorrhages (HE-staining, bar: 100 µm).

Given the lack of knowledge about causative virulence factors, confirmation of the clinical diagnosis is difficult and often based on clinical findings, autopsy and exclusion of other causes of intraluminal bleedings, such as *Salmonela* spp., BVD-virus, and coccidia. Treatment can be surgical, by removal of the affected intestinal segment, or conservative with antibiotics and prokinetics. Treatment is seldom succesful, and the mortality varies between 85 and 100% (Kirkpatrick *et al.,* 2001). Preventive measures

should be directed towards the feeding management. Vaccination with currently existing clostridial vaccines, or with autolog bacterin-toxoid vaccines from affected farms does not seem to prevent HBS (Elhafany *et al.*, 2013).

NECROTIC ENTERITIS IN NEWBORN CALVES

Necrotic enteritis in newborn calves has a very acute evolution, with sudden onset, and is characterized by either sudden death or acute (often haemorrhagic) diarrhea. The disease is most commonly seen in beef cattle, of between 0 to 7 days of age. Clinical signs may include weakness, depression, abdominal distention and abdominal pain (Niilo *et al.*, 1974; Garcia *et al.,* 2013). On autopsy, the small intestines show diffuse congestion and haemorrhage (figure 1.8). Microscopically, there is severe villus blunting, with diffuse haemorrhage and severe infiltration of leukocytes (figure 1.9). Microscopic changes can also be found outside the small intestine. Additionally, segmental neutrophilic necrotizing vasculitis and fibrin thrombi of the mucosal blood vessels can be observed. Frequently, haemorrhages are detected in the rumen, reticulum, abomasum, cecum and colon. This disease is associated with *C. perfringens* type C, with β-toxin as the most important virulence factor. This toxin forms membrane pores in enterocytes, and leads to intestinal necrosis (Garcia *et al.,* 2013). Particularly neonatal calves are highly susceptible to the disease, since β-toxin is inactivated by the action of trypsin. Neonatal calves have insufficient levels of intestinal trypsin activity, due to the trypsin inhibitor effect of colostrum (Garcia *et al.*, 2012). Diagnosis is made by confirming the presence of β-toxin in the intestinal contents of diseased animals. Neonates are believed to acquire type C bacteria from an environment contaminated by sick animals or by asymptomatic carriers (Garcia *et al.,* 2013).

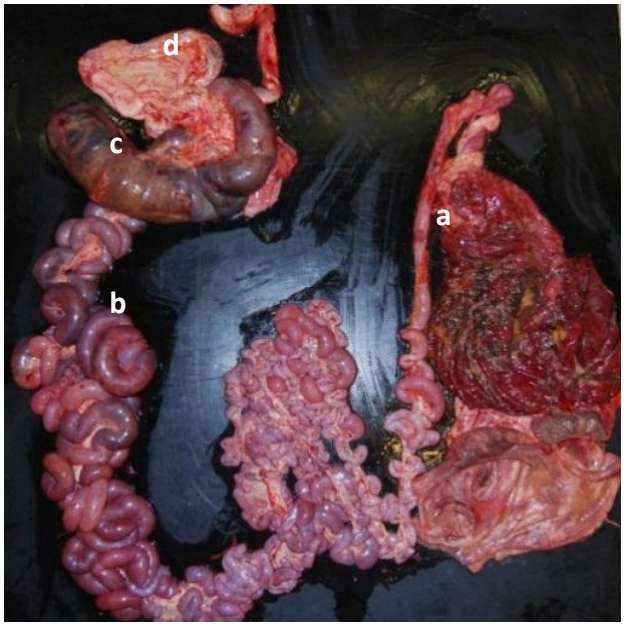

Figure 1.8: Gastro-intestinal tract of a newborn calf with haemorrhagic abomasitis (a) and necrotic entero (b) – typhlo (c) – colitis (d)

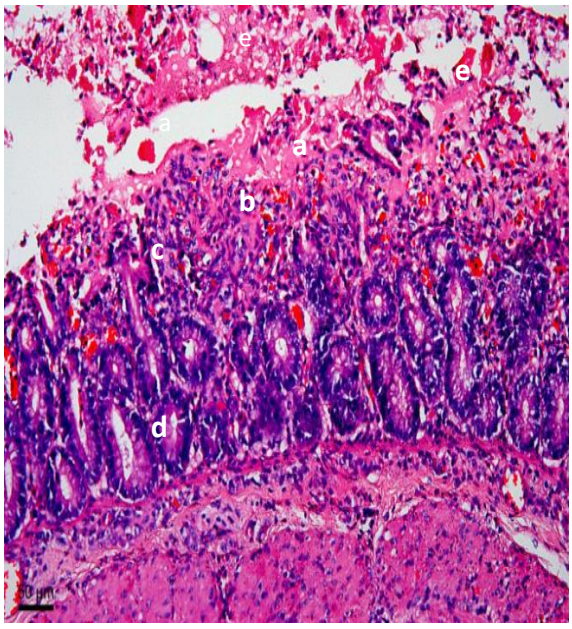

Figure 1.9: HE stain of haemorrhagic small intestine with necrosis of the epithelium (a), neutrophilic infiltration (b), hyperemia of the lamina propria (c) and submucosa (d) and haemorrhages (e) (bar: 50 µm)

ABOMASITIS IN NEWBORN CALVES

Haemorrhagic abomasitis is quite commonly observed in neonatal calves, with or without necrotic enteritis. This abomasitis has similarities with braxy in sheep. However, braxy is often related to the consumption of frozen food, and is characterized by acute haemorrhagic abomasitis with wall oedema, and *Clostridium septicum* can be found in the abomasal wall. In neonatal calves however, *C. septicum* cannot be isolated from the lesions, but instead a pure growth of non-enterotoxic CPA was obtained by several authors (Manteca *et al.,* 2001; Songer and Miskimins, 2005; Van Kruiningen, 2009). This is in contrast with neonatal necrotic enteritis, where typically type C is isolated. In 1998, Roeder *et al.* experimentally induced abomasitis in calves inoculated with CPA. The clinical signs were sudden death or a short period of anorexia, abdominal distention, regurgitation, nervous signs and shock. Typical lesions on necropsy were bloody contents of the abomasum, and a necrotizing haemorrhagic inflammation of the mucosa, often with emphysema (Songer and Miskimins, 2005). Although no publications are available to confirm this, similar risk factors as for necrotic enteritis are believed to

be important in haemorrhagic abomasitis in neonatal calves, such as the lack of competitive flora and the inadequate presence of protective enzymes. Additionally, the delivery of large amounts of colostrum can lead to abomasal distention, which might slow the drop of pH, the rennet formation and the emptying of the abomasum and hereby allowing for better survival of bacteria.

OVEREATING DISEASE

There is a thin line between what is considered 'overeating disease' and 'enterotoxaemia', and the majority of authors use both terms. However, in analogy with the situation in sheep, 'overeating disease' mostly refers to type D enterotoxaemia. Epsilon-toxin has been isolated from both adult cows and calves, and both in beef cattle and in dairy cattle (Keast and McBarron, 1954; Aichelman, 1956; Griesemer and Krill, 1962; Atkinson, 1998). Most typical for type D enterotoxaemia or 'overeating disease' in comparison with the traditional enterotoxaemia, is the presence of neurological symptoms and a focal symmetrical encephalomalacia. This could recently be experimentally induced by inoculating either bacteria or supernatant in the duodenum of healthy 9-month old Holstein calves, fulfilling Koch's postulates for this disease (Filho *et al.,* 2009). The produced epsilon toxin is absorbed into the blood stream, and induces lesions in the brain and lungs, characterized by perivascular proteinaceaous edema, predominantly in the brain and lung (figure 1.10). Often the neurologic and respiratory symptoms are far more prominent than the gastro-intestinal complaints, and acute loss of consciousness, hyperesthesia, intermittent tonic and clonic convulsions, recumbency and dyspnea have been reported (Niilo *et al.,* 1963).

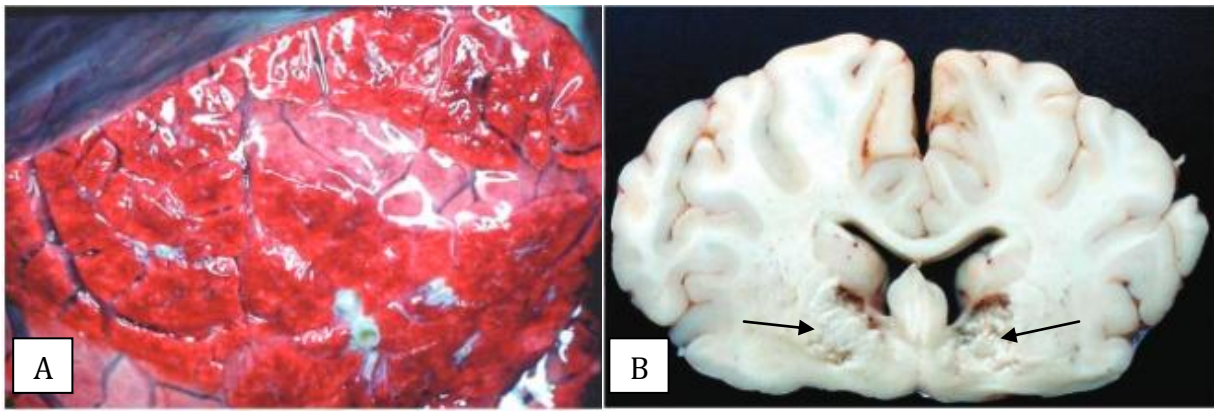

Figure 1.10: Lesions of calves experimentally infected with type D enterotoxaemia. A: Lung with acute pulmonary edema B: Corpus striatum with focal symmetrical encephalomalacia. The arrows point to the necrotic foci in the brain (Filho *et al.,* 2009).

The content of the duodenum seems to facilitate the proliferation and toxin production by the bacteria. Therefore, it is probable that a starch overload in the duodenum provides a substrate for clostridial overgrowth and abundant toxin production (Filho *et al.*, 2009). However, this seems to be a risk factor for all clostridial diseases, and is probably not specific for type D enterotoxaemia. More likely it is the presence of Type D *C. perfringens* in the intestinal microbiota of the animals that predisposes to type D enterotoxaemia with ensuing nervous symptoms, rather than the more traditional type A enterotoxaemia, where the gastro-intestinal signs are more striking (Niilo *et al.*, 1963).

CLOSTRIDIUM PERFRINGENS TYPE A PATHOLOGY IN THE VEAL INDUSTRY

In this chapter a detailed overview of abomasal ulcerations and enterotoxaemia is given as the most important clostridial diseases in the veal industry.

ABOMASAL ULCERATIONS

PATHOGENESIS: INFECTIOUS AGENTS

A general consensus is that abomasal ulcerations concern a multifactorial disorder on which infectious agents as well as environmental factors have an important influence (Jelinski *et al.,* 1995; Braun *et al.,* 1997; Dirksen *et al.*, 1997; Bähler *et al.*, 2010).
Although CPA has been cultured from affected abomasa, it is also isolated from abomasal content from abomasa without lesions at necropsy (Roeder *et al.,* 1987), and not all authors report the presence of CPA in gastric lesions (Mills *et al.*, 1990). In a case control study, CPA was isolated in about 75% of the abomasa of animals both with or without ulcers (Jelinski *et al.*, 1995). Therefore, the presence of this bacterium in the abomasum does not prove a causative role in the pathogenesis of abomasal ulcerations, and could be due to post-mortem multiplication or simply due to passage through the gastro-intestinal tract. However, experimental inoculation with CPA was able to reproduce abomasal ulcerations in neonatal calves (Roeder *et al.*, 1998) and in two case reports, huge numbers of *C. perfringens* were isolated from a case of abomasitis, in which Gram-positive rods were associated with necrotic lesions in the gut tissue (Songer *et al.,* 2004; Van Immerseel *et al.*, 2010). In one case, a clonal population was isolated from the abomasum and small intestine (Van Immerseel *et al.,* 2010). These reports clearly suggest a role for CPA in the pathogenesis of abomasal lesions in at least part of the cases. CPA could however only play a secondary role in these cases, and there are no studies on the reproduction of abomasal ulcerations in older animals.
'Candidatus Helicobacter bovis' has been described in the abomasum of cattle (De Groote *et al.,* 1999), although no information is available on its possible role in the

formation of lesions, in contrast to several other pathogenic gastric *Helicobacter spp.*, among others in humans (Alfizah *et al.,* 2012), pigs (Hellemans *et al.*, 2012) and mice (Flahou *et al.,* 2010). A variety of other bacteria, including *Campylobacter spp.* and *Streptococcus spp.*, and fungi have also been linked to abomasal lesion formation (Mills *et al.,* 1990).

PATHOGENESIS: ENVIRONMENTAL RISK FACTORS

Suspected risk factors among environmental factors are the availability of roughage, changing climate, physical and environmental stress, overfeeding, presence of hairballs, no access to water and large animal density (Wensing *et al.,* 1986; Mills *et al.,* 1990; Jelinski *et al.,* 1995; Bähler *et al.,* 2010). Several studies have observed an increase in abomasal lesions when feeding larger amounts of wheat straw mixtures (Welchman and Baust, 1987; Mattiello *et al.*, 2002; Brscic *et al.,* 2011). Presumably, the mechanical irritation of straw-fibres causes microlesions, leading to a disruption of the mucosal barrier. Similarly, long periods of an empty abomasum predispose for abomasal lesions by permitting prolonged exposure of the mucosa to stomach acid (Matiello *et al.*, 2002). Also the use of acidifying products (ascorbid acid, cider vinegar, betaine hydrochloride) or products decreasing the production of protective mucus (anti-inflammatory drugs) can contribute to the development of gastric lesions (Gisbert and Abreira, 2006).

It is interesting that fundic lesions are more likely associated with environmental risk factors, while pyloric lesions are associated with infectious causes (Breukink *et al.*, 1989; Bähler *et al.,* 2010). It is clear that the pathogenesis of abomasal ulceration has not yet been clarified. The existence of different grading systems makes it difficult to compare studies concerning the prevalence and severity of abomasal ulcerations in different populations. Often, the location of the lesions (fundic or pyloric region) is not reported, which complicates the interpretation of possible risk factors in the context of the hypothesis raised by Bähler *et al.* that the pathogenesis differs between fundic and pyloric ulcerations. It is clear that the pathogenesis of both lesions is incompletely known, and more research is needed in order to develop an efficient prophylactic protocol.

ABOMASAL ULCERATIONS IN THE VEAL INDUSTRY: PREVALENCE

Historically, abomasal ulcers are a very common problem in veal calves. Older studies reported a prevalence of non-perforating abomasal lesions in veal calves at slaughter of between 52% and 95% (Groth and Berner, 1971; Wiepkema *et al.,* 1987; Welchman and Baust, 1987; Breukink *et al.,* 1989). These data date back to before the European legislation of veal calf welfare, and may not be applicable to modern populations (European Council Directive 2008/119/EC, European Council, 2008).

More recent data are available for Switzerland (Bähler *et al.,* 2010), which has a veal system claiming improved animal welfare, and for Italy, the Netherlands and France (Brscic *et al.,* 2011), which have a more mainstream veal production system comparable to the production system in Belgium. In Switzerland conventional farms were compared to welfare-labeled farms ('Naturafarms') and a prevalence of 83 % in conventional farms and 79% on 'Naturafarms' was detected. The Swiss conventional production system differs from the Flemish production system in a way that the Swiss system is not integrated to such a high degree as the Flemish industry, and as a result there is significantly more variation in the management. The 'Naturafarm' system has even less similarity to the Flemish system, seen that it gives *ad libitum* access to hay and water, as well as access to outdoor pens. Bähler *et al.* (2010) relied for their conclusions on a total of only 125 abomasa selected at random in one large abattoir. Brscic *et al.* (2011) have recently published a study including more than 10,000 abomasa spread over 170 veal farms in three major producing countries (99 in the Netherlands, 47 in France, and 24 in Italy). The Dutch and Italian production systems approximate the Flemish production system, and data collected in these circumstances are most suitable for comparison with the Flemish population. Brscic *et al.* (2011) only studied the pylorus-region, where other studies have taken the entire abomasum into account. The overall prevalence of pyloric lesions in this study is 74.1%.

Bähler *et al.* (2010) have shown that in Swiss veal calves, pyloric lesions occur more often than fundic lesions. On conventional farms, 13% of abomasa had fundic lesions, while 60% had pyloric lesions, and 10% had lesions in both pylorus and fundus. This is, however, the only study that reported on the prevalence of fundic and pyloric ulcerations separately. Brscic *et al.* have concluded that 74.1% of the investigated abomasa had pyloric lesions, but no information was gathered about fundic lesions.

Nevertheless, a high ratio of pyloric ulcerations to fundic ulcerations can be expected in Belgian veal calves.

Recent research in Flemish veal calves has shown an average overall mortality of 5.2 %. Abomasal ulceration accounts for 3% of this mortality, varying between 6.4% in HF and 1.15% in BB calves (Pardon *et al.,* 2010). These fatal ulcers are only the tip of the iceberg, and high mortality rates reveal a larger underlying problem of (a)symptomatic non-fatal ulcers, which are a welfare issue and might influence productivity.

The extent of ulceration in the abomasum can vary significantly between affected abomasa. Different grading systems have been proposed by several authors, taking into account a series of parameters (Whitlock *et al.,* 1980; Wiepkema *et al.,* 1987; Marshal *et al.,* 2009; Bähler *et al.,* 2010; Van Immerseel *et al.*, 2010; Brscic *et al.*, 2011). As a consequence of differences in used grading systems, comparisons between studies are difficult. The system described by Whitlock is acknowledged through-out the world (Type 1: erosion or non-perforation ulcer with no apparent blood loss; Type 2: Non-perforating ulcer with blood-loss; Type 3: Perforating ulcer with local peritonitis; Type 4: Perforating ulcer with generalized peritonitis). Ulcers of type 2 to 4 are potentially fatal, in contrast to ulcers of type 1, which mostly do not induce any clinical signs. However, the occurrence of type 1 lesions cannot be neglected, since it leads to higher feed conversions and lower growth rates (Dirksen *et al.,* 1997).

ABOMASAL ULCERATIONS IN THE VEAL INDUSTRY: RISK FACTORS

The recent dietary changes towards higher amounts of roughage in the veal diet have improved production results, and decreased abnormal oral stereotyped behavior and rumen disorders (Mattiello *et al.,* 2002; Suarez *et al.*, 2006). However, several studies have observed an increase in abomasal lesions when feeding larger amounts of wheat straw mixtures (Welchman and Baust, 1987; Mattiello *et al.,* 2002; Brscic *et al.,* 2011). The effect of a replacement of MR by SF on abomasal lesions is two-sided. Historically, the veal calf received only 2 daily meals of MR, predisposing for abomasal ulceration due to overdistention of the stomach wall, and long periods of an empty abomasum (Matiello *et al.,* 2002). Increased SF feeding decreases the quantitity of MR fed per feeding, and can thus benificially influence the prevalence of abomasal lesions. However, SF are often fed immediately after MR feeding, contributing to the abomasal overload, and can

additionally have an abrasive effect on the solid particles on the abomasal wall, explaining the observed predisposition in several studies (Prevedello *et al.,* 2012).

CONCLUSIONS

Pyloric ulcerations are highly prevalent in European veal calves, and also a high prevalence of fundic ulceration is expected based on a limited study on Swiss veal calves. The pathogenesis for this condition is largely unknown, although the feed regimen (large amounts of MR in only two feedings and the use of chopped straw) in veal calves is believed to predispose for this disease. A role for CPA is suggested based on case reports and experimental inoculation in neonatal calves, but could not yet be demonstrated for ulcerations in older calves.

ENTEROTOXAEMIA

EPIDEMIOLOGY

Enterotoxaemia is a worldwide, fatal disease of young cattle (Muylaert *et al.*, 2010). Although the disease is widely spread, there are differences in incidence of enterotoxaemia between different breeds and production systems. Historically, the disease has mainly been reported in beef suckler calves (Griner and Bracken, 1953, Niilo *et al.*, 1974). Enterotoxaemia can occur in calves of all ages, but the disease is mostly seen in calves up to 10 weeks of age (Troxel *et al.*, 1997). In contrast to enterotoxaemia outbreaks in small ruminants, calf enterotoxaemia is a sporadic disease, often only affecting a single animal or a very limited number of cases in the same herd (Lebrun *et al.*, 2010).

Specifically in veal calves, compared to HF veal calves, BB calves are predisposed for enterotoxaemia. Up to 20% of the total mortality, especially in the last weeks of production, can be attributed to this disease, making it the second most important cause of mortality in BB veal calves after pneumonia (Manteca *et al.*, 2001; Pardon *et al.*, 2012a). In contrast to beef calves, in veal calves a peak is seen in enterotoxaemia at 22 weeks in production (Troxel *et al.*, 1997; Pardon *et al.,* 2012a). It is important to realize that although enterotoxaemia is a 'rare event disease', with a low morbidity (for example in BB veal calves morbidity for enterotoxaemia is 1,3% versus 56% for pneumonia), the economic losses are very large. This is because the mortality in diseased animals is close to 100% (in comparison, pneumonia has 4.8% mortality risk), and the disease affects the most valuable, heaviest, fastest growing animals, while most other mortality occurs in the beginning of the production round (Pardon *et al.*, 2012a).

DISEASE PRESENTATION

Clinically, enterotoxaemia is a peracute disease, in which death occurs within a few minutes to a few hours (Barker *et al.*, 1993). In only a minority of the affected calves, signs prior to death are noticed by the farmer. In those cases, abdominal pain, nervous symptoms, and signs of agony (respiratory distress, lateral recumbency and convulsions, followed by coma) can be noted (Lebrun *et al.*, 2010; Muylaert *et al.*, 2010). *In vivo* signs of diarrhea and meteorism are rather rare (6% and 2% respectively) (Manteca *et al.*, 2000).

Given the scale in which veal calves are housed, individual observation is limited, and the disease almost always presents itself as sudden death. Presumably, enterotoxaemia is overdiagnosed in the absence of a necropsy. Acute pneumonia is highly prevalent in veal calves, and may present itself as sudden death since in the large scale structure of the veal herds, farmers easily miss the preliminary signs of respiratory disease (Pardon *et al.,* 2012a). Other causes of sudden death in veal calves include cardiac problems, perforated abomasal ulcers and mineral disturbances (hypomagnesemia, iron intoxication) (Pardon *et al.,* 2012a; Glock and DeGroot, 1998).

Post-mortem, a remarkable and rapid meteorism of the abdomen, and rapid putrefaction with foul smell is typical for clostridial infections (figure 1.11). On necropsy, the disease is characterized by diffuse or localized small intestinal haemorrhage with bloody intestinal contents and, most often, the absence of other clinical signs (figure 1.12) (Lebrun *et al.,* 2010; Manteca *et al.,* 2002). The extension of the lesions in the gastro-intestinal tract of the calves is variable, and affected loops from only 10 cm to the entire length of the small intestine are found, as well as lesions to other gastro-intestinal structures as the caecum, colon or abomasum. In the majority of the cases, also the mesenteric lymph nodes are affected (Glock and DeGroot, 1998; Lebrun *et al.*, 2010). Other internal organs might show signs of enterotoxaemia, namely petechiae or congestion (Lebrun *et al.,* 2010).

Figure 1.11: Acute death in a Belgian Blue calf with a distended abdomen and a marked meteorism

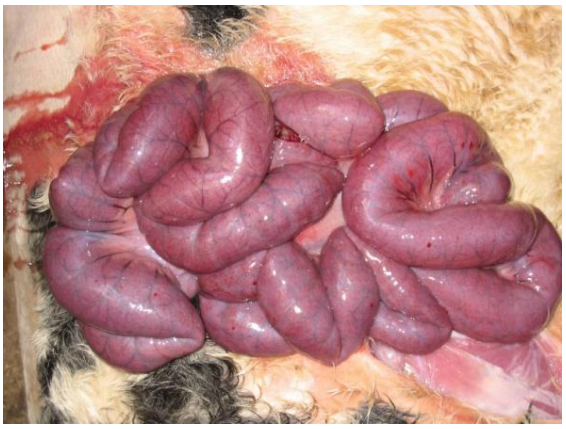

Figure 1.12: Diffuse haemorrhagic enteritis in an enterotoxaemia-case

Microscopic examination often reveals an extensive necro-haemorrhagic enteritis, with erosion of the villi, cell necrosis reaching from the tip of the villi to the base of the crypts,

and the infiltration of neutrophils and lymphocytes (Worrall *et al.*, 1987, Manteca *et al.*, 2002, Lebrun *et al.*, 2010). Manteca *et al.* (2004) specifically described a hydropic degeneration of the ortho- and parasympathetic ganglia of the muscular and mucosal layers. In the intestinal lumen, groups of bacteria similar to *C. perfringens* vegetative stages can be found in the necrotic areas clutching to the necrotic cells. They are, however, not found in the mucosa of the intestinal wall (Manteca *et al.*, 2001).

PATHOGENESIS

The cause of enterotoxaemia remains controversial. Epidemiological studies showed a strong association between *C. perfringens* and enterotoxaemia (Manteca *et al.*, 2001). Altough all toxinotypes have been isolated from enterotoxaemiacases, in most cases, type A is isolated (Daube *et al.,* 1996; Petit *et al.*, 1999; Manteca *et al.*, 2001; Lebrun *et al.*, 2010). The quantification of CPA strains in affected tissues is significantly higher in calves with enterotoxaemia than in control cases (Manteca *et al.*, 2001). However, Koch's postulates could not yet be fully fulfilled for enterotoxaemia (Songer, 1996; Manteca *et al.,* 2001).

The most commonly accepted hypothesis on the pathogenesis of enterotoxaemia is a local overgrowth of *C. perfringens* (of the whole clostridial population, rather than specific strains), associated with overproduction of alpha toxin and its systemic absorption. Other authors hypothesize an overgrowth of specific clones, which are presumed to produce higher amounts of this toxin, or a more stable variant of the toxin (Bullifent *et al.*, 1996; Ginter *et al.*, 1996). Another explanation is that not the virulence of the strain matters, but that host factors and nutrition contribute to a higher sensitivity (Petit *et al.*, 1999).

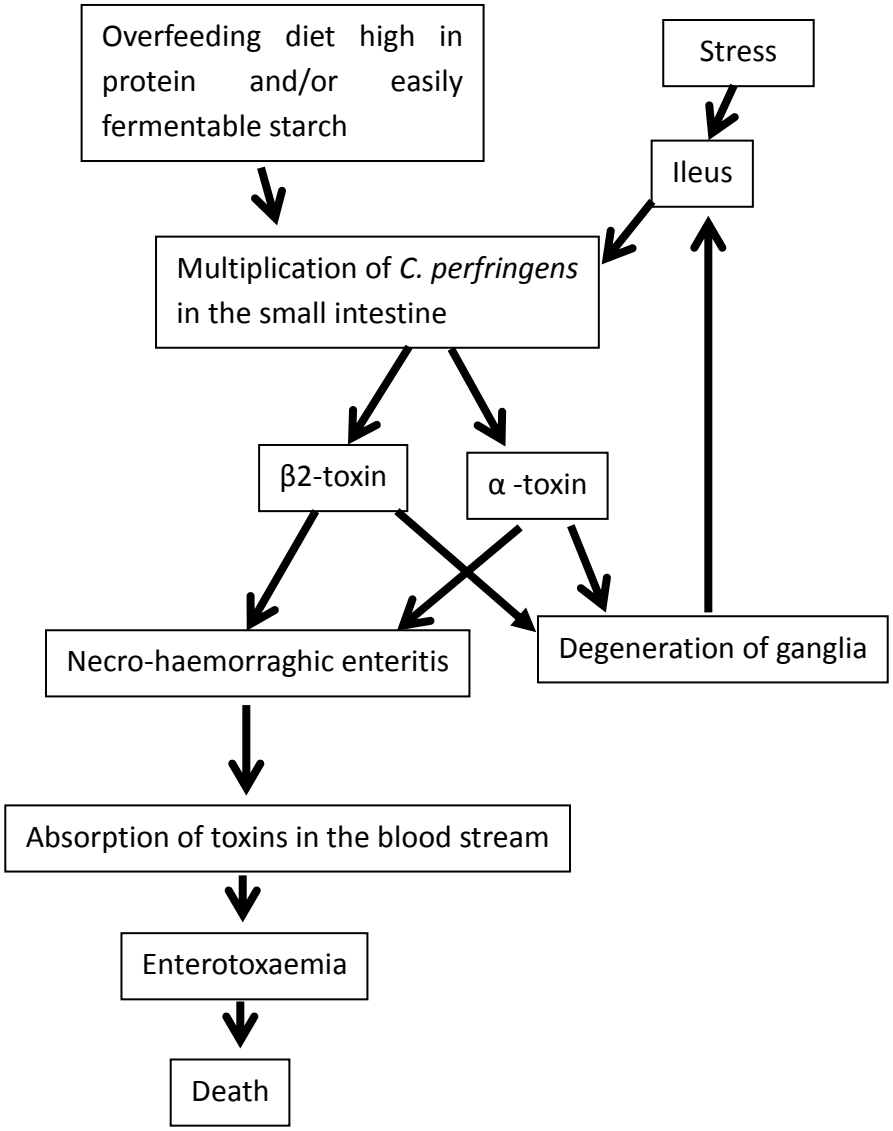

Figure 1.13: Schematic overview of the most commonly accepted hypothesis on the pathogenesis of *C. perfringens* enterotoxaemia

In addition to alpha toxin, β2-toxin has been linked to enterotoxaemia by different authors. Gibert *et al.* (1997) and Smedley *et al.* (2004) suspected a role of β2-toxin based on the *in vitro* potential to cause cell lesion in bovine cell lines. The *in vivo* importance of these findings is, however, controversial. Garmory *et al.* (2000) could not detect a difference in prevalence of β2-producing strains between control calves and bovine enterotoxaemia cases, and could only isolate these strains in about 60% of the cases. However, Lebrun *et al.* (2007) confirmed a marginally significant difference between

enterotoxaemia and control groups for the presence of the *cpb2*-gene, encoding for the β2-toxin. Among isolates from calves, an atypical allele was found at the cpb2 locus, corresponding to poor expression of β2-toxin. However, Lebrun *et al.* (2007) revealed the exclusive presence of 'consensus' cpb2 in enterotoxaemia cases, compared with control cases, with high expression rates in isolates from enterotoxaemic calves (44%) and low expression rates in isolates from control calves (7%). Moreover, *cpb2* positive isolates induced higher lesions scores (necrohaemorrhagic enteritis) in an intestinal loop compared to *cpb2* negative isolates (Manteca *et al.,* 2001). These data suggest that the typical variant of the β2-toxin may play a role in the pathogenesis of calf enterotoxaemia.

Apart from the causative toxins and their effect on host cells, the most important remaining question in the pathogenesis of enterotoxaemia is the identification of the trigger causing clostridial overgrowth and toxin production. In literature, stress and drastic feeding changes are the most commonly mentioned risk factors. Stressful environmental influences, such as regrouping, transporting, handling and medical treatments have been mentioned as risk factors for enterotoxaemia (Lebrun *et al.*, 2010). In addition, severely diseased animals often develop enterotoxaemia, presumably as a consequence of very important stress. Worrall *et al.* (1987) already suggested a post-stress modification of the intestinal microbiota by induction of a paralytic ileus. In addition to this stress, feeding changes can lead to inadequate digestive processes, and consequently, a higher availability of the nutrients for bacterial growth (Niilo, 1986). The finding of Manteca *et al.* (2004) that ganglia are involved, suggests a direct effect on intestinal motility, additional to the general paralytic effect of enteritis, leading to a vicious circle. The consequent intestinal stasis diminishes flushing of bacteria and toxins, possibly triggering the development of enterotoxaemia.

Also other forms of feed adaptations are often observed to induce enterotoxaemia. Most common examples are suckler calves that develop enterotoxaemia after a cow in heat abandons her calf for 12 to 24 hours, whereafter the calf drinks a large quantity of milk when allowed to suckle again. Likewise, the absence of drinking water for a period of time, followed by esurient intake of large amount of drinking water in a few minutes, can also be followed by enterotoxaemia.

RISK FACTORS IN THE VEAL INDUSTRY

BB veal calves are fed intensively with very rich diets. The oversupply of both protein and easily fermentable carbohydrates favors enterotoxaemia by triggering clostridial overgrowth and toxin production (Annett *et al.,* 2002), presumably explaining the predisposition of BB veal calves compared to HF veal calves or BB beef calves.

Increasing the MR ration should always be done gradually over the course of the production cycle. Empirically, some farmers report more enterotoxaemia-problems after feeding from a freshly filled silo. Presumably, differences in composition of the MR (often fluctuating in function of the costs of ingredients) might attribute to this phenomenon. Therefore, the advice to the integrations that own the MR-producing plants and steer the production processes should be to avoid fluctuations in the composition of MR for BB veal calves as much as possible. Moreover, soy is often used as an alternative protein source in MRs. However, raw soybeans contain anti-trypsin agent. Since alpha toxin is sensible to proteases that are potentially inhibited by soy products, large amounts of soy in the MR might predispose for enterotoxaemia. As mentioned above, the dietary management of enterotoxaemia in veal calves is based on empiric observations, and studies evidencing the benefit of a certain dietary fraction are lacking.

In addition to dietary factors, other factors influenced by management might play a role, in particular stress. Veal calves are regrouped routinely in order to obtain a uniform growth in the cohort. This regrouping is necessary to optimize production results, but can lead to a higher incidence of enterotoxaemia losses due to the induction of stress (Manteca *et al.,* 2000). Therefore, regrouping has to be limited to the bare necessity, done in a calm manner, and the feed management has to be kept very stable during this period.

DIAGNOSIS

Although the clinical presentation and necropsy findings of enterotoxaemia are typical (sudden death with haemorrhagic intestines, and the absence of other clinical signs), they are far from pathognomonic and the confirmation of an enterotoxaemia-suspicion is difficult (Manteca *et al.,* 2001; Philippeau *et al.,* 2004). Therefore, a number of ancillary laboratory techniques are described, which are used to confirm a clinical

diagnosis of enterotoxaemia. These are clostridial quantification, toxinotyping of strains and toxin detection.

CLOSTRIDIAL QUANTIFICATION

Bacterial analysis is the most frequently used laboratory test to diagnose enterotoxaemia. *C. perfringens* is a normal inhabitant of the gastro-intestinal tract, with counts between 10^2 and 10^5 colony-forming units (CFU) per ml of intestinal content. To confirm enterotoxaemia, in suckler calves, a cut-off value between 10^6 and 10^7 cfu/ml intestinal content has been recommended, dependent on the time between death and sampling (Popoff 1989, Manteca *et al.,* 2001, Philippeau *et al.,* 2003). Samples should be taken as soon as possible after death. This means that sampling directly after finding the dead calf on farm is to be preferred over sampling hours later when the calf has arrived at a pathology institution (Lebrun *et al.*, 2010). An affected intestinal loop should be ligated and kept under 4°C to prevent bacterial growth after sampling (Songer and Miskimmins, 2004). Lebrun *et al*. (2010) advised transport under anaerobic conditions in an anaerobic jar, to avoid reduction of the clostridial count during transport. However, *C. perfringens* is rather tolerant to oxygen, and the ligation of the loops should be sufficient for practical reasons (Rolf *et al.*, 1978; Bergey *et al.*, 1939). In order to quantify *C. perfringens*, 10-fold dilutions are made, plated on blood agar, and grown overnight. The colonies are identified based on their typical form surrounded by a double β-haemolysis zone (Manteca *et al.,* 2001).

C. perfringens multiplies in high numbers in the intestine as part of the post-mortem putrefaction process and can rapidly invade the internal tissues. Therefore, the time period between death and sampling is important for quantitative bacterial analysis (Philippeau *et al.,* 2003, Lebrun *et al.,* 2010). Philippeau *et al.* (2003) determined that 3 hours after death, the clostridial count in healthy euthanized animals approached the counts in enterotoxaemia-cases (table 1.2). This leads to serious difficulties, primarily because most cases are 'sudden death', and the exact time of death is often unknown, and secondly, because samples have to be obtained as soon as possible after death, preferably by a trained veterinarian, which is not always possible under field conditions. The previously described studies, conducted to achieve cut-off values, were performed on suckler calves (Popoff *et al.,* 1989; Manteca *et al.,* 2001; Phillipeau *et al.,* 2003). Feed

has an important influence on the intestinal microbiota, and as such on the basal clostridial counts in healthy calves. Therefore, described cut-off values in suckler calves can possibly not be extrapolated towards veal calves, due to the important dietary differences.

Table 1.2: Decision tree for diagnosis of enterotoxaemia in suckler calves based on clostridial quantifications in intestinal content (Philippeau *et al.,* 2003).

Quantification (CFU/ml content)	**Time between death and sampling (hr)**		
	0-3	**3-6**	**6-15**
$<10^5$	No enterotoxaemia	No enterotoxaemia	No enterotoxaemia
10^5-10^6	Indecisive	Indecisive	Indecisive
10^6-10^7	Enterotoxaemia	Indecisive	Indecisive
10^7-10^8	Enterotoxaemia	Enterotoxaemia	Indecisive
$>10^8$	Enterotoxaemia	Enterotoxaemia	Enterotoxaemia

TOXIN TYPING

In order to do toxin typing, strains have to be cultivated and purified first, which is time-consuming. Post-mortem, an overgrowth of all clostridial strains present in the intestinal lumen occurs, leading to practical problems to set cut-off values in function of the time and conditions between death and sampling. In order to have a representative result, several colonies have to be tested, both for major toxins, and for β2-toxin (Lebrun *et al.,* 2010). Therefore, toxin typing of isolated strains as a diagnostic technique for enterotoxaemia is circuitous and time consuming, limiting its practical value.

The actual typing can be done using mouse seroneutralisation assays, which is considered the gold standard (Hatheway, 1990). This technique has important ethical implications. In these tests, mice are injected with culture supernatants and seroprotected with seroneutralising antibodies against the representative *C. perfringens* toxinotypes (Hatheway, 1990). For animal welfare reasons, this test should be avoided. In addition to the mouse test, other phenotypic tests such as intradermal injections in guinea pigs were developed and abandoned for the same reasons (Petit *et al.,* 1999). ELISA's have been developed and historically used to detect the production of toxins (Hatheway, 1990). Nowadays, most laboratories routinely conduct PCR-tests to detect

toxin genes, because PCR can detect any combination of toxin-encoding genes present in an isolate. Moreover, some PCR methods have been described that do not require prior purification of the strains, and can directly be done on samples of faeces or intestinal content (Persson and Olson, 2005; Zhang *et al.*, 2006). However, since CPA, the most prominent toxinetype in the pathogenesis of enterotoxaemia, is a natural part of the intestinal microbiota, the cpa-gene can also be detected in the intestinal contents of healthy calves and is therefore of very limited importance in the diagnosis of enterotoxaemia. Knowledge on the causative toxins could lead to more reliable gene detection methods. However, Koch's postulates could not yet be fulfilled for neither toxin, and therefore the lack of knowledge in the pathogenesis prohibits the use of gene detection as a reliable technique in the diagnosis of enterotoxaemia in calves.

TOXIN DETECTION

In contrast to toxin typing, toxin detection tests are commercially available directly for intestinal content or other biological samples. Toxin detection is done with ELISA or slide latex agglutination kits (McClane and Snyder, 1987; Ginter *et al.,* 1994). Commercial, easy to use, on-site tests for practitioners are available for alpha toxin, beta toxin and epsilon toxin, but not for iota and β2-toxin (Lebrun *et al.,* 2010). The detection of alpha toxin above the detection limit is meaningful, and is a reliable way to confirm a clinical diagnosis of enterotoxaemia. However, since alpha toxin is very sensitive to degradation by intestinal proteases, samples must be taken rapidly after death, and a negative result does not exclude the diagnosis of enterotoxaemia (Popoff, 1989). A variation on these toxin detection tests is immunohistochemistry, as is done for other clostridial diseases (for example epsilon toxin in small ruminants) (Uzal and Songer, 1996). However, alpha toxin is very unstable, and fixation in formaldehyde could easily affect the tertiary structure of the protein. As for PCR techniques, the lack of knowledge on causative toxins complicates the use of toxin detection as a reliable diagnostic technique.

Overall, the interpretation of the laboratory tests and thus the confirmation of a clinical suspicion of enterotoxaemia often remains difficult, and in atypical clinical presentations the diagnosis is often questionable. Especially the time between death and sampling has

a major influence on the test results. Therefore, samples should be taken from the cadaver within the first hours, transported and stored anaerobically and chilled, in order to optimize chances on a correct diagnosis. At present, in the field, most people rely on isolation of *C. perfringens*, with or without quantification.

PREVENTIVE STRATEGIES

Because the evolution of the disease is very acute, curative treatment comes too late once the animals show clinical symptoms. In the next paragraphs an overview of possible preventive measures is provided.

MANAGEMENT

Primarily, the focus of the prophylaxis is to be on good management practices. Since sudden feed changes or protein- and soluble carbohydrate-rich diets (Niilo, 1986) are believed to predispose for enterotoxaemia, these should be avoided. In order to avoid clostridial overgrowth, a constant feed ration and feed rate should be maintained, with sufficient amounts of fibre to obtain a stable intestinal microbiota (Lebrun *et al.,* 2010).

VACCINATION

Vaccination is not common in the veal industry, especially due to the costs. In beef cattle, vaccination is more routinely done in herds with a high incidence of enterotoxaemia, but results are conflicting, and clinical disease and fatalities despite vaccination are common (Glock and Degroot, 1998). Immunization of the calves is obtained by vaccination of the mother, 8 and 2 weeks before parturition. Active immunity is induced by vaccinating twice with a 4-6 week interval and repeating every 6 to 12 months. Calves from unvaccinated mothers can be vaccinated, starting from the age of 2 weeks (Troxel *et al.*, 1997). Manteca *et al.* (2004) demonstrated the presence of naturally acquired alpha-toxin antibodies in the serum of healthy cows, suggesting that part of the toxin produced by the normal microbiota in healthy intestine is absorbed in the blood and processed by the immune system without any clinical symptoms of enterotoxaemia. This leads to the presence of maternal antibodies in the sera of the calves.

Most available commercial vaccines are combination vaccines against several clostridial species, often including multiple toxinotypes of *C. perfringens* (Lebrun *et al.,* 2010). All available vaccines are toxoid vaccines, for systemic use. These vaccines only induce systemic antibodies. Moreover, no vaccines contain the β2-toxin. Due to the lack of knowledge on the causative toxins, no targeted vaccines against enterotoxaemia in calves were developed. Therefore, current vaccines are raw culture supernatants, inactivated for example by formalin, and filtrated to remove bacterial bodies. These cultures contain a large number of different proteins, such as minor toxins and metabolic waste. These elements may disturb the immune response against the toxins of interest (Lebrun *et al.*, 2010). Moreover, inactivation, especially formalin inactivation, can induce morphological changes in the tertiary protein structures of relevant toxins, influencing the immunogenicity, and possibly negatively influencing the immune response. However, similar vaccines are used in other animal species (predominantly sheep), where they do induce efficient protection against clostridial diseases (Songer, 1996). Possibly, the causative toxins in cattle enterotoxaemia are more sensitive to coagulation by formalin than those in small ruminant enterotoxaemia.

Since interference with maternal antibodies is to be expected when vaccinating young calves, and since in the veal sector, no information is known on the vaccination status of the mother, it is difficult to recommend correct vaccination protocols to the veal industry.

ANTIBIOTICS

Antibiotics are sometimes used metaphylactically on farms in the event of an enterotoxaemia outbreak (multiple cases in a short period of time) (Daube *et al.*, 1992, Popoff, 1989; Pardon *et al.,* 2012b). Since antibiotic use is commonly high on veal farms, the presence of multiresistant *C. perfringens* is not inconceivable (Pardon *et al.,* 2012b). The metaphylactic use of antibiotics will increase the selection pressure of all present pathogens, and should therefore be discouraged.

Generally, β-lactam antibiotics as penicillin or amoxicillin are used based on their efficiency against *Clostridia* (Lebrun *et al.,* 2010). However, the effects of such

metaphylactic use are difficult to assess, since enterotoxaemia is often limited to a few cases on a farm, even without the use of antibiotics. In the context of responsible use of antibiotics, these practices should be discouraged. Commonly, *C. perfringens* has little resistance to antibiotics, but it can acquire multiple resistance mechanisms in the case of frequent antibiotic use on the farm. The curative use of oral penicillin and serotherapy (serum from vaccinated cows) is used in practice. However, no published data are available on success rates, and according to feedback from veterinarians, the therapy is often not successful.

PRE- AND PRO-BIOTICS

In enzootic-like presentations of the disease, pre- en probiotics are administered to prevent clostridial overgrowth and to promote a stable intestinal microbiological environment (Lebrun *et al.,* 2010). Prebiotics are organic molecules which are indigestible by the host, but serve as a substrate for a certain subpopulation of intestinal bacteria. The term probiotics refers to live bacteria or yeasts which are administered to the host in order to improve the intestinal microbial balance (Callaway *et al.,* 2008). For example, *Bifidobacter lactis* and *Lactobacillus rhamnosus* are known to synergistically reduce the adherence of *Salmonella*, *E. coli* and *Clostridum spp*. to the intestinal mucosa in swine (Collado *et al.,* 2007). In broiler chickens, *Bacillus* isolates, amongst others, are known to prevent necrotic enteritis (Jimoh *et al.*, 2013; Lee *et al.,* 2013). However, no studies have explored the effect of pre- or probiotics on enterotoxaemia in cattle.

In BB veal calves, enterotoxaemia has an enzootic-like presentation in a number of herds. Therefore, many products are employed in order to decrease the enterotoxaemia incidence in BB veal calves (for example based on *Saccharomyces cerevisiae,* butyric acid, garlic,…), with variable, but often disappointing, results. To date, there are no reliable studies on the effect of these pre- and probiotics on the incidence of enterotoxaemia in veal calves.

CONCLUSIONS

Enterotoxaemia continues to be an economically important problem in veal calves. Although sudden death in combination with haemorrhagic intestines is typical for this disease, it is not pathognomonic. Confirmation of the diagnosis is difficult, since available diagnostic methods are strongly affected by the time between death and sampling. Despite abundant empiric communication on management of enterotoxaemia, the pathogenesis of the disease is actually still largely unclear. Presumably, intensive feeding regimes predispose for this disease. A better understanding of the pathogenicity of *C. perfringens* and the provoking risk factors is needed in order to make progress in preventive medicine (vaccination and nutritional management) and reliable diagnostic methods to confirm the disease.

CHAPTER 2

SCIENTIFIC AIMS

SCIENTIFIC AIMS

In veal calves, *C. perfringens* has been associated with enterotoxaemia and abomasal ulceration, two diseases causing important losses and jeopardizing animal welfare. The pathogenesis of *C. perfringens* associated diseases in calves is still largely unclarified, and causative toxins are unknown. As a consequence, diagnostic tools are limited to clostridial isolation and quantification of intestinal content, but no evidence exists on its reliability in veal calves. Despite a lot of empiric experience with the prevention of enterotoxaemia outbreaks in cattle, no evidence is available on what factors elicit the disease.

Therefore, the overall objective of the present doctoral thesis was to gain new insights in the role of *C. perfringens* in haemorrhagic enteritis (enterotoxaemia) and abomasal ulcerations in veal calves and in the pathogenesis of enterotoxaemia. Four specific objectives are formulated below.

1) *C. perfringens* type A has been isolated from abomasal ulcerations, and ulcerations could be induced after inoculation of the bacteria in neonatal calves. However, there are no studies available on the role of *C. perfringens* in the pathogenesis of fundic ulcerations in veal calves. Therefore the first objective was to determine the association of *C. perfringens* with fundic ulcerations in veal calves (chapter 3).

2) *C. perfringens* is known to be associated with enterotoxaemia. In the field, clostridial quantification of intestinal content is the most commonly used diagnostic technique for enterotoxaemia. However, the technique is very sensible to post-mortem clostridial overgrowth. Moreover, feed can influence the basal clostridial counts, potentially biasing the value of clostridial counts as a diagnostic technique in veal calves fed very specific diets (large amounts of MR). Therefore, the second objective of this thesis was to determine the value of *C. perfringens* counts for the diagnosis of enterotoxaemia in veal calves (chapter 4).

3) In order to get a better understanding of enterotoxaemia, and so better preventive tools, it is essential to determine the causative virulence factors of *C. perfringens* in the pathogenesis of enterotoxaemia. In pursuance of identifying these causative toxins, a reproducible and practically achievable model is needed. Consequently, the third aim was to develop an intestinal loop model and to study the pathogenicity of different strains and conditions in the development of necro-haemorrhagic lesions (chapter 5).

4) With alpha toxin and perfringolysin identified as the most important virulence factors in enterotoxaemia, the road is open for the development of better vaccines against this disease. In order to develop good vaccination schemes, it is important to determine the natural occurrence of antibodies against these toxins, and especially the duration of maternal immunity. Since the feed management can influence the contact of the calves with *C. perfringens* and its toxins, the natural occurrence of antibodies might differ between different production types and breeds. This information might be useful in the development of other preventive measures against enterotoxaemia, next to vaccination. Therefore, the last objective of this thesis was to determine the onset of humoral immunity against the most important *C. perfringens* toxins, and the influence of production type, breed and SF provision on the natural occurrence of antibodies against alpha toxin and perfringolysin (chapter 6).

CHAPTER 3

PREVALENCE AND BACTERIAL COLONIZATION OF FUNDIC ULCERATIONS IN VEAL CALVES

The aim of this work is to detect a potential link between CPA and abomasal ulceration. CPA has been isolated from abomasal ulcerations, and ulcerations could be induced after inoculation of CPA in neonatal calves. However, there are currently no studies available on the role of C. perfringens in the pathogenesis of fundic ulcerations in veal calves.

PREVALENCE AND BACTERIAL COLONIZATION OF FUNDIC ULCERATIONS IN VEAL CALVES

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Bonnie Valgaeren, Bart Pardon, Bram Flahou, Stefanie Verherstraeten, Evy Goossens, Leen Timbermont, Freddy Haesebrouck, Richard Ducatelle, Filip Van Immerseel and Piet Deprez (2013). Prevalence and bacterial colonization of fundic ulcerations in veal calves. VETERINARY RECORD. 172(10)

ABSTRACT

The veal industry remains concerned with the welfare of veal calves. Several management improvements, such as group housing and access to roughage, have already been implemented. Detection of fundic ulcerations at slaughter has been suggested as a possible welfare indicator in several species, including calves, although the pathogenesis behind fundic ulcerations is still largely unknown. Several studies demonstrated *C. perfringens* in fundic ulcerations in cattle and recently, a clonal strain of *C. perfringens* has been isolated out of a fundic ulceration from a BB calf which died of enterotoxaemia. The present study aims at estimating the prevalence of fundic ulcerations in Flemish veal calves from dairy and beef origin. In addition, the prevalence of *C. perfringens* in abomasums with or without fundic ulcerations was estimated. In a large integrated slaughterhouse 604 calves from 10 Flemish veal herds were sampled (5 dairy origin and 5 beef origin; 60 ± 5 calves, selected according to slaughter order). The abomasal fundus of each calf was opened, and the presence and intensity of each ulceration (score I to IV) was noted. A total of 334 swabs were taken for the isolation of *C. perfringens*, both from healthy fundi (n= 158) and from fundic ulcerations (n= 176). The overall prevalence of fundic ulceration was 16.7%. Beef calves showed significantly more lesions (23.3%) than dairy calves (11.5%) ($P<0.01$). 99% of the ulcerations (205/207) were type 1 (non-perforating, non-bleeding ulcerations); only two ulcerations were type 2 (bleeding ulcerations). *C. perfringens* could be isolated from 26.6% of the swabs taken out of healthy fundi, whereas 21% of swabs taken out of fundic ulcerations were positive ($P>0.05$). In conclusion, the present study did not demonstrate any difference in the prevalence of *C. perfringens* in fundic ulcerations compared to normal abomasa. However, the significantly higher prevalence of fundic ulcerations in BB veal calves compared to HF veal calves implies that other factors than *C. perfringens* colonization need to explored.

INTRODUCTION

The European veal industry is specialized in raising surplus dairy calves on a low iron milk diet to obtain white meat. Next to HF dairy calves, also beef calves of local breeds, such as the BB breed in Belgium, are raised as white veal calves. Marked differences in milk diet exist between these breeds, especially considering the quality of the protein (Pardon *et al.,* 2012). In a recent survey on mortality in veal calves in Belgium, perforating abomasal ulcerations, either fundic or pyloric, accounted for 3% of mortality, with marked differences between HF (6.4%) and BB (1.2%) (Pardon *et al.,* 2012). Fatal ulcerations are only the tip of the iceberg, revealing a larger underlying health and welfare issue of non-fatal ulcerations. A recent study determined the within herd prevalence of pyloric ulcerations in The Netherlands, France and Italy at 74.1% on average, ranging from 31.7% to 100% (Brsic *et al.,* 2011), but no information on fundic ulcerations was mentioned. The only study on the prevalence of fundic ulcerations in the European veal production system is a Swiss study in a veal production system with exceptionally high welfare standards, resulting in a total of 23% of the abomasa with fundic lesions in the standard production system and 8% in the naturafarm production system (Bähler *et al.,* 2010). In addition to stress and feeding regime, *C. perfringens* has often been suggested to be related with bovine abomasal ulceration, but so far no studies could confirm this relationship (Roeder *et al.,* 1988, Jelinski *et al.,* 1995, Van Immerseel *et al.,* 2010). 'Candidatus Helicobacter bovis' has been described in the abomasum of cattle (De Groote *et al.,* 1999, Haesebrouck *et al.,* 2009), although no information is available on its possible role in the formation of ulcerations, in contrast to several other pathogenic gastric Helicobacter spp., amongst others in humans (Alfizah *et al.,* 2012), pigs (Hellemans *et al.,* 2012) and mice (Flahou *et al.,* 2010). This study aimed at estimating the prevalence of fundic ulceration in veal calves of different breeds in Belgium and the possible association of *C. perfringens* and *Helicobacter spp.* with fundic ulcerations.

METHODS

Between November 2011 and April 2012, abomasa from 604 calves from 10 randomly selected veal herds (5 HF and 5 BB) within the same integration were examined at slaughter. Within each batch 60 ± 5 (mean ± standard deviation) calves were sampled. Abomasa were opened with a sterile scalpel from omasum to duodenum following the curvatura major. Lesions on the mucosa of the fundic region were graded according to Whitlock (1980). Swabs for isolation of *C. perfringens* were taken from every lesion, and additionally from a random surface of about 4 cm^2 in 132 fundi without lesions. Swabs were transported at 5°C and were streaked (within 4 hours of sampling) on Columbia agar (Oxoid, Basingstoke, UK) with 5 % defibrinated sheep blood containing 12 mg kanamycin sulphate and 30,000 U/l polymyxin B sulphate. Plates were incubated for 24 hours at 37°C under anaerobic conditions. *C. perfringens* colonies were identified by characteristic colony morphology and dual haemolysis. Forty-four full-thickness tissue samples were removed from different lesion sites in order to determine the presence of *Helicobacter spp*. A 'Candidatus Helicobacter bovis' species-specific PCR, as well as 2 Helicobacter genus-specific PCR primer sets were applied, as described previously (Moyaert *et al.*, 2008). Following agarose (1.5%) gel electrophoresis, PCR amplicons of interest were sequenced using the BigDye Terminator sequencing kit (Applied Biosystems, Foster City, Ca) and sequences were determined on an automatic DNA sequencer (ABI PrismTM 3100 Genetic Analyzer, Applied Biosystems). The electropherograms were exported and converted to Kodon software (Applied Maths, Sint-Martens-Latem, Belgium).

RESULTS

The mean within herd prevalence of fundic ulceration was 16.7% ± 10.6 (mean ± standard deviation), ranging from 1.5% to 40%. As illustrated in figure 3.1, there was a trend towards a higher prevalence in beef calves (22.3% ± 10.3) as compared to dairy calves (11.0% ± 8.1) (P= 0.089, independent samples t-test). Using the grading system of Whitlock (1980) almost all lesions (99%) belonged to type 1 (erosion or non-perforating ulceration with no apparent blood-loss). The two remaining lesions belonged to type 2 (non-perforating ulceration with blood-loss). In total 26 percent of the swabs (59/225 swabs) taken from lesions were positive for *C. perfringens* after anaerobic incubation. This is similar to the 27 percent positive swabs (36/132 swabs) taken from healthy abomasa. There was no significant difference between the two breeds (figure 3.2). No Helicobacter DNA was amplified.

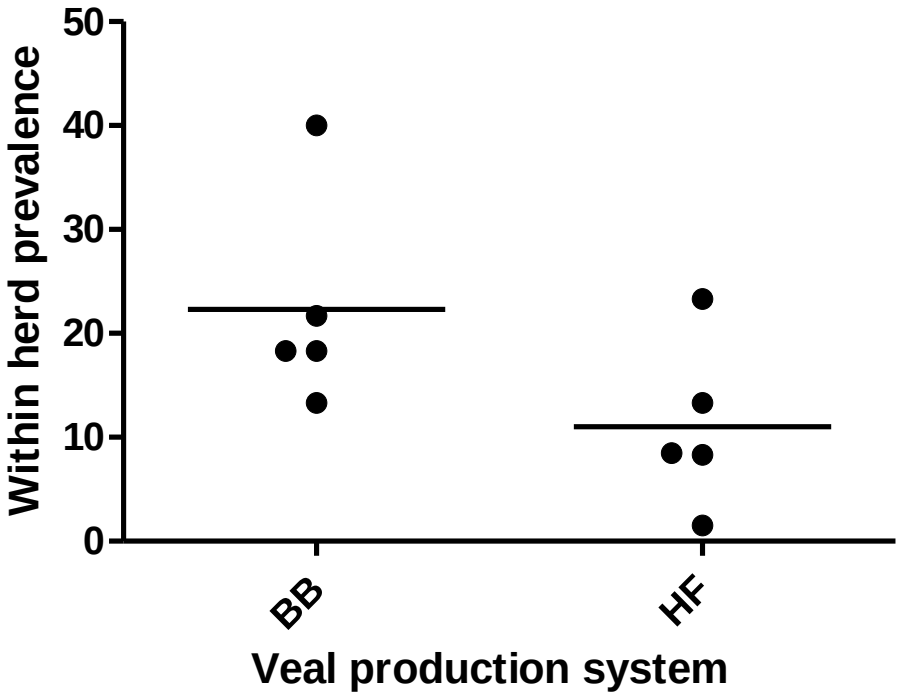

Figure 3.1: Mean within herd prevalence of fundic ulcerations depending on breed. Abbreviations: BB= Belgian Blue, HF= Holstein Friesian

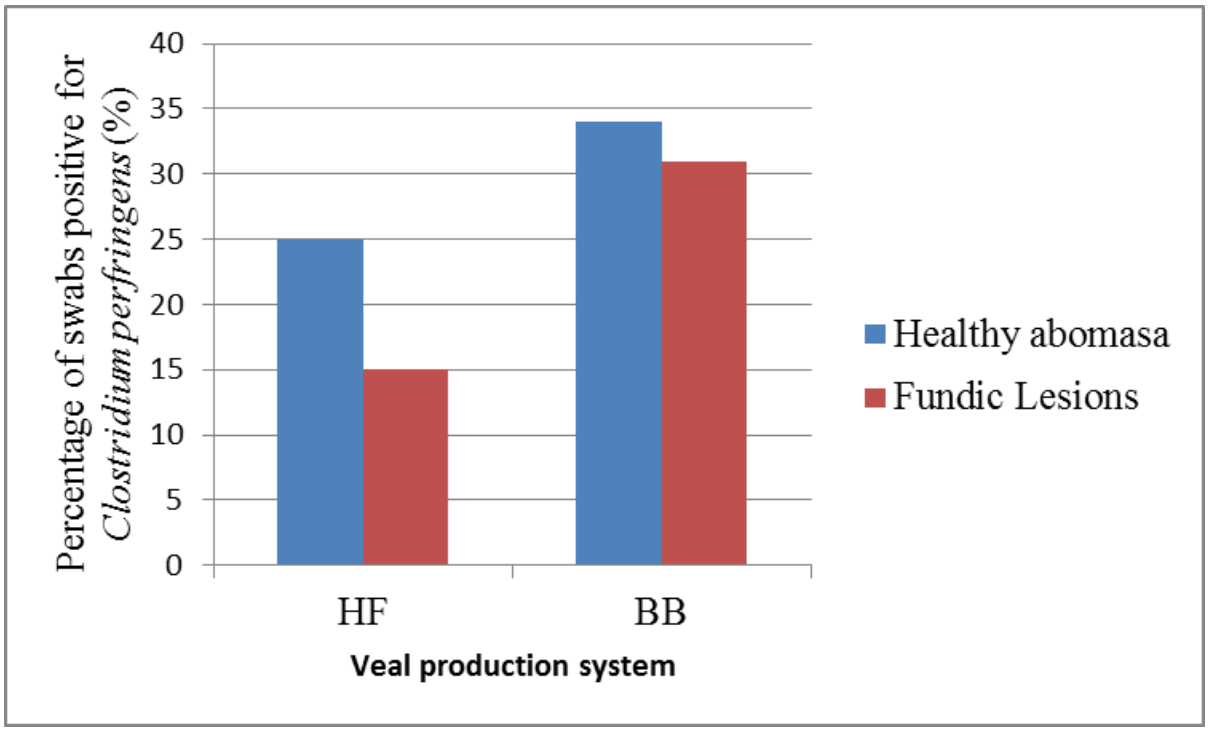

Figure 3.2: Percentage of swabs positive for *Clostridium perfringens* after cultivation of swabs taken from healthy abomasa and from lesions. In the HF group 9/62 swabs from lesions were positive for *C. perfringens*, while 24/97 swabs from healthy abomasa were positive. In comparison, in the BB group 50/163 swabs from lesions were positive, versus 12/35 swabs from healthy abomasa. Abbreviations: HF= Holstein Friesian, BB= Belgian Blue

DISCUSSION

In the present study, the prevalence of fundic ulcerations in the most common veal production system in Europe, based on the Belgian situation, was estimated at 11% in dairy veal. This is lower than what has been reported in standard Swiss veal production but higher than in the Swiss high welfare system (naturafarm). However, a possible bias of the integration cannot be excluded, due to specific dietary differences.

Most of the ulcerations belonged to type 1, which in general do not induce detectable clinical signs, but do lead to a higher feed conversion and lower growth rate (Dirksen, 1997). No information was available on the occurrence of fatal ulcerations (type 3 or 4) in the examined cohorts of calves. BB veal calves trended towards a higher prevalence of fundic ulcerations compared to dairy veal, most likely due to dietary differences (higher animal protein/vegetable protein ratio in MR for BB calves), although a breed effect (e.g. susceptibility to stress) cannot be excluded. BB calves are also slightly older than HF calves at slaughter age (28-32 weeks vs 22-26 weeks), potentially contributing to the higher ulcer incidence. This apparent paradox of lower prevalence of fundic ulcerations but higher mortality due to perforating ulcerations in HF calves compared to BB calves can possibly be due to a larger contribution of pyloric ulcerations, which are suggested to be predominantly linked with infectious causes, as opposed to fundic ulcerations, which are predominantly linked with welfare factors (Bähler *et al.*, 2010, Pardon *et al.,* 2012). In the BB calves, 163 swabs were taken from abomasal ulcerations, while only 35 swabs were taken from healthy abomasa. This ratio was conversed in the HF calves. The influence of breed might therefore bias the interpretation of the culture. However, in contrast to ulcerations linked with welfare factors, there are no indications that the breed influences the pathogenesis of ulcerations linked to infectious causes. Since no *Helicobacter spp*. could be detected in the lesions and there was no difference between the presence of *C. perfringens* in abomasa from healthy calves as compared to ulcerations, the role of these bacteria in the pathogenesis of fundic ulceration is presumed to be limited, if existing.

Conclusions

In conclusion, the present study did not demonstrate any difference in the prevalence of *C. perfringens* in fundic ulcerations or normal abomasa. However, the significantly higher prevalence of fundic ulcerations in BB veal calves compared to HF veal calves implies that other factors than *C. perfringens* colonization need to explored.

CHAPTER 4

INTESTINAL CLOSTRIDIAL COUNTS HAVE NO DIAGNOSTIC VALUE IN THE DIAGNOSIS OF ENTEROTOXAEMIA IN VEAL CALVES

Even if the pathogenesis of enterotoxaemia stays unclear, its association with *C. perfringens* was already shown. In the field, clostridial quantification of intestinal content is the most commonly used diagnostic technique for enterotoxaemia. However, the technique could be impacted by post-mortem clostridial overgrowth. Moreover, the feed management can influence the basal clostridial counts, potentially biasing the value of clostridial counts as a diagnostic technique in veal calves fed very specific diets (large amounts of MR).

INTESTINAL CLOSTRIDIAL COUNTS HAVE NO DIAGNOSTIC VALUE IN THE DIAGNOSIS OF ENTEROTOXAEMIA IN VEAL CALVES

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ABSTRACT

Enterotoxaemia is an important cause of sudden death in veal calves. This study aimed to evaluate intestinal *C. perfringens* counts as a diagnostic tool for enterotoxaemia. Field necropsies were conducted on 48 sudden death cases in BB veal farms. In 31/48 suddenly deceased calves, the diagnosis of enterotoxaemia was made based on haemorrhagic lesions in the small intestines, while in seven of these cases, no clear-cut diagnosis could be made based on macroscopic appearance of the gut. In the 10 remaining calves, a definitive cause of death other than enterotoxaemia could be identified. Samples of the intestinal content were taken for quantification of *C. perfringens*. After matching cases and controls for diet, and the interval between death and sampling, no significant differences could be detected between the mean *C. perfringens* counts of the small intestines in enterotoxaemia cases and counts in the matching segments in the control group.

These results indicate that intestinal *C. perfringens* counts cannot be advised as a discriminative post-mortem diagnostic tool for enterotoxaemia in veal calves, not even when sampled within three hours after death.

INTRODUCTION

Enterotoxaemia, which is attributed to *C. perfringens*, is an important cause of sudden death in calves worldwide (Uzal *et al.,* 1994; Miyakawa and Uzal, 2005; Younan and Gluecks, 2007; Lebrun *et al.,* 2010). All breeds can be affected, but specifically in BB veal and suckler calves the incidence is high (Manteca *et al.,* 2002; Pardon *et al.,* 2012). A recent study showed that enterotoxaemia is still responsible for up to 20% of the total mortality in BB veal calves, occurring mostly towards the end of the production period, when the animals have a high economic value (Pardon *et al.,* 2012).

In practice, the diagnosis of enterotoxaemia is mainly based on the typical haemorrhagic enteritis at necropsy (Lebrun *et al.,* 2010; Muylaert *et al.,* 2010). However, despite that the macroscopic appearance of haemorrhagic enteritis in a case of sudden death is highly suggestive for enterotoxaemia, it is far from pathognomonic (Philippeau *et al.,* 2004; Lebrun *et al.,* 2010). At present the most frequently used laboratory test to diagnose *C. perfringens* enterotoxaemia in the field is quantification of *C. perfringens* colonies in the intestinal content at the lesion site. In suckler calves a cut-off value between 10^6 and 10^7 colony forming units (CFU) per ml of intestinal content has been recommended (Popoff, 1989; Manteca *et al.,* 2001; Phillipeau *et al.,* 2003).

However, since *C. perfringens* multiplies in high numbers in the intestines as part of the post mortem putrefaction process, the time-period between death and sampling is an important bias for quantitative bacterial analysis (Philippeau *et al.,* 2003, Lebrun *et al.,* 2010). Previous studies did not match enterotoxaemia-cases and control-cases based on this time-period, and for obvious practical reasons, sampling could not consistently be done shortly after death (Manteca *et al.,* 2001).

Furthermore, the type of diet influences *C. perfringens* growth. In humans and poultry a protein-rich diet can enhance intestinal *C. perfringens* growth and changes in the clinical manifestation of the infection (Wekell *et al.,* 1980, Goliamdekhordi *et al.,* 2006, Kalmendal *et al.,* 2011). BB veal calves are typically fed with a highly concentrated liquid diet based on milk protein. This specific diet might influence the intestinal *C. perfringens* numbers in comparison to suckling calves used in previous studies.

Therefore, the main objective of the present article was to evaluate intestinal *C. perfringens* counts as a diagnostic tool for enterotoxaemia in BB veal calves.

METHODS

STUDY DESIGN AND SAMPLING PROTOCOL

Veterinarians, active in the Flemish veal industry, were contacted to report cases of sudden death of BB veal calves. Calves were sampled on farm within 8 hours after death. A case anamnesis, including dietary details, was recorded. All calves were necropsied and macroscopic lesions were recorded. The abomasum, small intestine, caecum and colon were removed from the abdominal cavity. One loop of 5 cm from the caecum and the colon and 3 loops of 5 cm from the jejunum (proximal, mid and distal location), preferentially in affected areas, were selected. Loops were firmly ligated with Surgicryl PGA (SMI, St. Vith, Belgium). Abomasal content was collected in a fully filled airtight recipient. The ligated loops and the abomasal content were immediately cooled (4°C) and transported to the lab. For every selected segment the macroscopic aspect of mucosa and serosa was recorded and a sample was fixed in 4% phosphate buffered (wt/vol) formaldehyde. After fixation for 24 hours, the samples were embedded in paraffin wax. Five µm-thick transverse sections were cut and stained with HE for histology. Sections were examined by an experienced pathologist and evaluated for the presence of necrohaemorrhagic lesions on the villi tips, typical for enterotoxaemia in calves (Lebrun *et al.,* 2010). The test group included all calves with a clear-cut diagnosis of enterotoxaemia. The control group included all calves with a clear-cut diagnosis different from the gastro-intestinal tract. More details are provided in the 'data management section'. Additionally, to extend the control group, also BB veal calves suffering from chronic health problems were euthanized and subjected to an identical autopsy and sampling protocol.

POST-MORTEM EVOLUTION OF INTESTINAL *CLOSTRIDIUM PERFRINGENS* COUNTS

To gain insights in the normal post-mortem evolution of intestinal *C. perfringens* counts, three conventionally reared 4 month old HF calves without gastro-intestinal pathology were euthanized at the Faculty of Veterinary Medicine because of advanced arthritis. Immediately after euthanasia, the abdomen was opened on the right flank, and the proximal jejunum, the distal jejunum and the colon were exteriorized. A loop was firmly ligated with Surgicryl to keep the intestinal environment anaerobic and the intestinal content was collected. Thereafter the abdomen was closed and this procedure was repeated in a successive loop at 2, 4 and 6 hours after euthanasia, followed by bacteriological quantification of *C. perfringens* in intestinal content immediately after collection.

QUANTIFICATION OF *C. PERFRINGENS* IN INTESTINAL CONTENT

Of the intestinal content, 20 µl was suspended in 180 µl (dilution 10^{-1} or 1 in 10) of sterile phosphate buffered saline (PBS). Ten-fold dilutions were made in sterile PBS, each time bringing 20 µl of the 10^{-x} dilution in 180 µl of sterile PBS, resulting in the 10^{-x-1} dilution (x ranging between 1 and 6; i.e. dilutions down to 1 in 1 000 000). Subsequently, for each dilution 6 droplets of 20 µl were plated on Columbia agar (Oxoid, Basingstoke, UK) with 5% defibrinated sheep blood, containing 12 mg kanamycin sulphate and 30,000 U/l polymyxin B sulphate. Bacteria were allowed to grow overnight at 37°C in anaerobic conditions in HP11 jars (Oxoid). Identification of *C. perfringens* colonies was made on the basis of a dual haemolysis zone. Numbers of CFU *C. perfringens* per ml intestinal content were calculated based on the recovered colonies on the plates.

DATA MANAGEMENT AND STATISTICAL ANALYSIS

For comparison, calves were grouped based on the macroscopic necropsy findings. Cases of sudden death, which showed haemorrhagic small intestines, were diagnosed as enterotoxaemia. The other cases of sudden death were split into cases with a clear diagnosis different from enterotoxaemia and cases in which the diagnosis of enterotoxaemia could neither be confirmed nor excluded. In addition to these case

calves, also calves euthanized for chronic health problems were sampled. All intestinal *C. perfringens* counts are reported as the logarithm of the number of colony forming units per ml gut content (log CFU/ml). Differences in intestinal *C. perfringens* counts between the different intestinal segments or groups were determined by the Independent Sample Kruskal-Wallis test. Significance was set at $P<0.05$. All analyses were performed using SPSS Statistic 19 (IBM®). To determine the diagnostic value of intestinal counts for distinguishing enterotoxaemia cases from other causes of death, cases of enterotoxaemia were matched with control calves based on diet and time interval between death and sampling. Only those calves from which samples were taken and inoculated for quantification of *C. perfringens* within 3 hours after death and that received a diet based on MR were included in the comparative study on quantification of *C. perfringens* between calves with enterotoxaemia and calves that died or were euthanized with health problems other than enterotoxaemia.

RESULTS

CALF CHARACTERISTICS

A total of 68 calves, all BB or crossbreeds, were necropsied, of whom 48 sudden deaths and 20 calves euthanized for chronic health problems. The 31 sudden deaths that were diagnosed with enterotoxaemia had a mean age of 20.8 ± 6.7 (mean ± standard deviation) weeks and a mean death-sampling interval of 3.1 ± 2.0 hours. In 10 calves a definitive cause of death other than enterotoxaemia was identified (mean age 19.3 ± 7.4 weeks and mean death-sampling interval 3.2 ± 1.4 hours), and in 7 calves, no clear-cut diagnosis could be made (mean age 22.1 ± 6.3 weeks and mean death-sampling interval 2.9 ± 1.8 hours). All these calves received the standard diet based on MR. Of the 20 calves that were euthanized for chronic health problems, 12 calves received a diet based on MR and 8 mainly received a replacement diet based on concentrates and roughage. In this last group the mean age was 18.7 ± 6.8 weeks and the mean death-sampling interval was 1.2 ± 2.2 hours.

DISEASE PRESENTATION

In all 31 enterotoxaemia cases, the disease developed very fast, with a maximum of 5 hours between the first clinical symptoms and death. Only 12/31 animals (39%) were found dead without any previous symptoms noticed. In all other calves lateral recumbency and cold extremities were observed. Signs of colic were seen in 32.2% of the calves in this group, while forced breathing was noticed in 13% of the calves. Other observed symptoms were nervous symptoms (6.5%), distended abdomen (6.5%) and bloody diarrhea (3.2%). Of the suddenly deceased calves with a definite diagnosis other than enterotoxaemia 6/10 calves showed no symptoms, while 4 calves showed signs of shock and 3 calves showed forced breathing. In the group with unknown cause of death, calves showed either no symptoms (3/7 calves) or signs of shock (4 calves) and colic (1 calf). The 20 euthanized calves presented signs of chronic respiratory problems (35%), immobility (50%), disturbed equilibrium (10%) or melena and weakness (5%).

PATHOLOGICAL EXAMINATION

All 31 calves with enterotoxaemia had marked haemorrhagic intestines. In 9, 50 and 41% of the cases proximal, mid and distal jejunum was the worst affected segment, respectively. In the 10 calves where a definite cause of death other than enterotoxaemia was identified, severe pneumonia (5 calves), partial mesenterial torsion (2 calves), frothy ruminal bloat (2 calves) and gas gangrene (1 calf) were identified at necropsy. In the 20 euthanized calves lesions compatible with the underlying disease were found. None of these calves with a definite exclusion of enterotoxaemia had haemorrhagic intestines. For 7 calves no conclusive diagnosis could be made. Either there were no macroscopic lesions present (2 calves), or there was a slight hyperemia of the small intestines without clear haemorrhage (4 calves), or there were haemorrhagic intestines in combination with severe lethal lesions of pneumonia (1 calf). Twenty-one calves out of 31 with enterotoxaemia (68%) showed petechial haemorrhages on the heart, while this was not noticed in other calves. Furthermore the presence of abdominal fluid, often haemorrhagic or with fibrin clots, was striking in 13 (42%) of the calves with enterotoxaemia and in 3 calves without enterotoxaemia. On histology, the presence of necrohaemorrhagic lesions of the villi tips could not be evaluated with certainty due to advanced post-mortem decay of the superficial mucosa, even in samples fixed 30 minutes after death.

QUANTIFICATION OF *C. PERFRINGENS* IN INTESTINAL CONTENT

The *C. perfringens* counts of the intestinal content in the gut of euthanized HF animals increased with time post-mortem (figure 4.1).

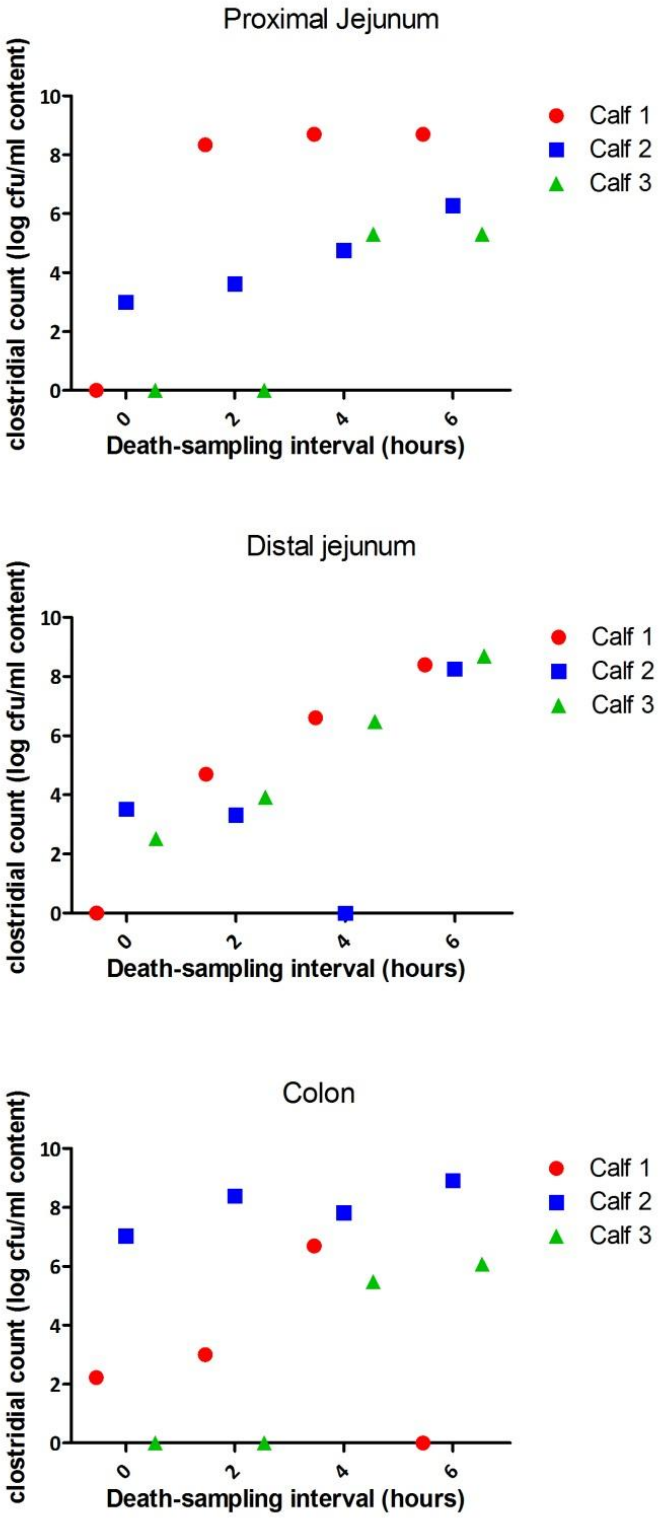

Figure 4.1: Evolution of post mortem *Clostridium perfringens* count in different gut segments of 3 conventional Holstein Friesian calves.

Nineteen calves diagnosed with enterotoxaemia fulfilled the criteria to be included in this comparative study (sampling within 3 hours after death and fed with MR). Fourteen calves diagnosed with a pathology other than enterotoxaemia also fulfilled the criteria, of which 5 sudden deaths with a known diagnosis other than enterotoxaemia and 9 calves euthanized for chronic health problems, and in the group with an unknown cause of death 4 calves met the criteria for death-sampling interval and diet.

In all groups (test-group, control group and the group with unknown cause of death) the bacterial counts in the small intestine were generally higher than in the abomasum or the large intestine ($P>0.05$) (figure 4.2). Figure 4.3 illustrates the range in the *C. perfringens* counts in the test and control group. The mean intestinal *C. perfringens* count of the enterotoxaemia group (n= 19; 3.83 ± 0.49 log cfu/ml) was slightly higher but not significantly different from the non-enterotoxaemia group (n= 14; 3.10 ± 2.46 log cfu/ml). The count in the group with unknown cause of death was 2.66 ± 0.68 log cfu/ml.

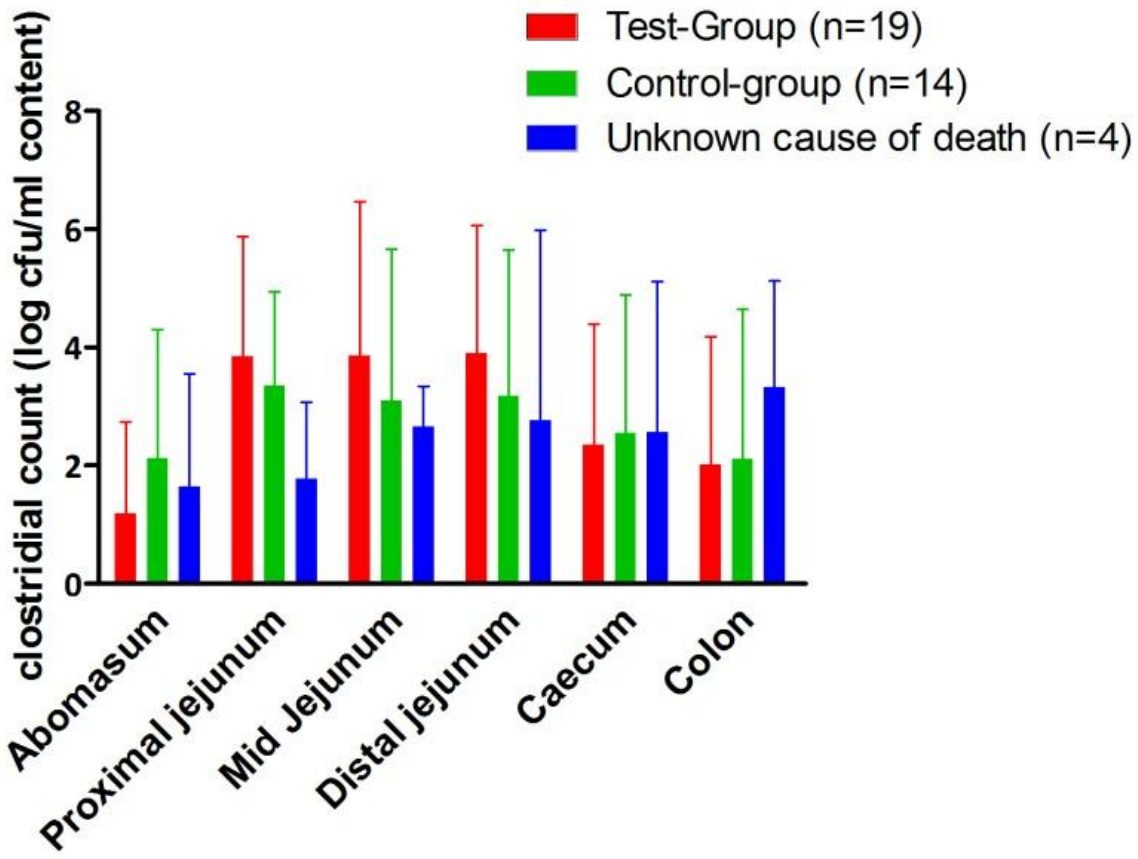

Figure 4.2: Mean logarithm of CFU *C. perfringens*/ml intestinal content in different intestinal segments in veal calves diagnosed with enterotoxaemia (test-group), with a definite exclusion of enterotoxaemia – either sudden death or euthanized due to chronic health problems – (control-group) and with an unknown cause of death, all sampled within 3 hours after death and fed with a diet based on milk replacer.

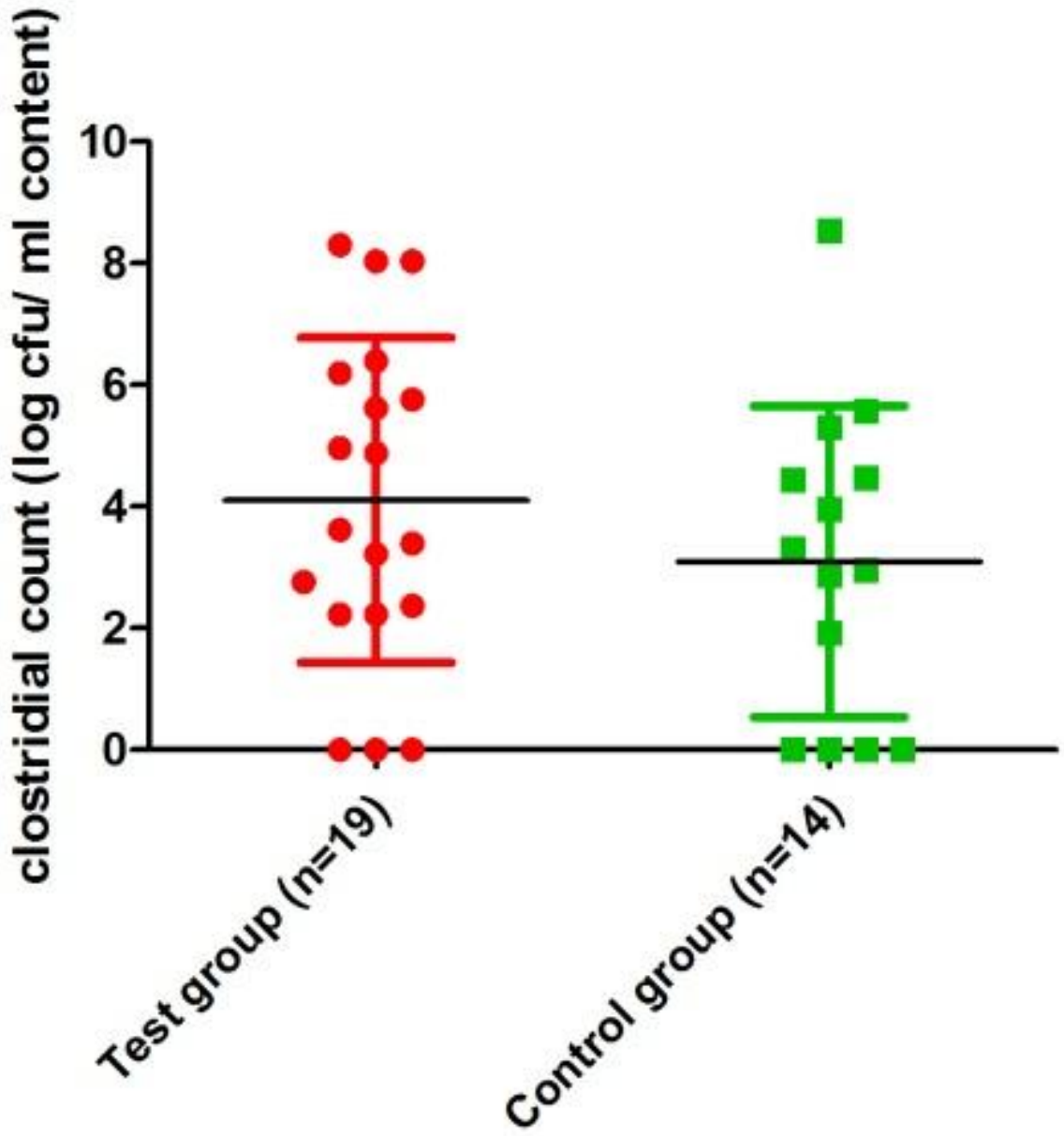

Figure 4.3: *Clostridium perfringens* counts of the contents of a haemorrhagic jejunal segment of calves diagnosed with enterotoxaemia (test group: mean death-sample interval 2.2 ± 1.2 hours; control group: mean death-sample inderval 1.8 ± 1.5 hours) compared to the matching segment of calves with a definite exclusion of enterotoxaemia – either sudden death or euthanized due to chronic health problems – (control group), sampled within 3 hours after death and fed with a diet based on milk replacer.

DISCUSSION

The present study shows that enterotoxaemia accounted for the largest proportion of sudden death cases in the small cohort of this study. It is remarkable that in 15% of the cases of sudden death, no clear-cut diagnosis could be made. A majority of these calves did present hyperemic intestines, yet without haemorrhagic lesions. It is not clear whether these calves died of enterotoxaemia before the onset of haemorrhagic enteritis, or whether they died due to another unidentified cause. Moreover, preceding symptoms (when present) were mostly unspecific, and there was an important overlap in symptoms between groups. Whereas the main symptom of enterotoxaemia is believed to be sudden death, close monitoring in the present study shows that most cases do display colic symptoms.

No statistical difference in intestinal *C. perfringens* counts could be detected between enterotoxaemia cases and veal calves dying from other causes, in contrast to what has been published previously in conventional calves (Manteca *et al.,* 2001; Philippeau *et al.,* 2004). Most likely the longer intervals between death and sampling (24h) and the lack of exact recording of the sampling interval in those previous studies have interfered with the results. In literature, a relevant maximum sampling interval of 3 hours is proposed to minimize the effect of post-mortem *C. perfringens* multiplication as part of the putrefaction process (Phillipeau *et al.,* 2004, Lebrun *et al.,* 2010). Also in the present study longer sampling intervals resulted in higher *C. perfringens* counts. In the first 2 hours after euthanasia the mean *C. perfringens* count in the jejunum already increased with 2.5 log CFU/ml intestinal content. Therefore, sampling shorter than 2 hours after death should be recommended. Unfortunately, the time of death is often unknown in field conditions and such fast sampling is not realistic in the field.

No significant differences between the bacterial counts of intestinal segments of enterotoxaemia cases (3.83 ± 0.49 log cfu/ml) and intestinal segments derived from animals that died from other causes (3.10 ± 2.46 log cfu/ml) could be detected. After matching the time interval post-mortem and potential diet differences, only a limited number of calves could be included in the analysis. However, the data clearly suggest that there is no difference between *C. perfringens* counts in the intestinal segments of the group of enterotoxemia cases and the control cases. All control cases were sick calves,

which might influence the clostridial counts, since several of these calves had been subjected to antibiotic treatment. Also, the intestinal motility is important in the clostridial overgrowth, therefore, the general illness itself might also influence the clostridial counts of the control cases, possibly biasing the statistical analysis. However, the variation in intestinal *C. perfringens* counts was very large within the groups and there was too much overlap between cases and controls, to support the value of quantifying *C. perfringens* in intestinal contents as a diagnostic test.

BB veal calves are fed with a specific protein and energy rich diet. Because in humans and in other animal species, the diet influences intestinal *C. perfringens* counts (Wekell *et al.,* 1980, Iwatsuku *et al.,* 2011, Kalmendal *et al.,* 2011), it might be possible that *C. perfringens* counts in the intestine of healthy veal calves are higher than in conventionally reared calves. This could explain the difference between the observations previously made in BB suckler calves compared to the veal calves in this study (Manteca *et al.,* 2001).

It is clear that other criteria are essential to accurately attribute sudden death to enterotoxaemia in veal calves. If the causal toxins of bovine enterotoxemia would be known, toxin detection in the intestinal content or bacterial culture followed by genotyping would be the most promising diagnostic technique (Uzal and Songer, 2008). This technique is commonly used with success in human medicine for *Clostridium difficile* infections (Planche *et al.,* 2008). Also, in poultry, the netB toxin has recently been identified as a critical virulence factor in the pathogenesis of necrotic enteritis, enabling a reliable diagnosis of the disease (Timbermont *et al.,* 2011). Identification of *C. perfringens* toxins that are causative for enterotoxemia are thus of utmost importance.

CONCLUSIONS

In conclusion, the present study demonstrates that quantification of *C. perfringens* in intestinal contents has no value in the diagnosis of enterotoxaemia in BB veal calves. More insights into the pathogenesis of enterotoxaemia in veal calves are necessary to obtain a reliable diagnostic method.

CHAPTER 5

LESION DEVELOPMENT IN A NEW INTESTINAL LOOP MODEL INDICATES THE INVOLVEMENT OF A SHARED *CLOSTRIDIUM PERFRINGENS* VIRULENCE FACTOR IN HAEMORRHAGIC ENTERITIS IN CALVES

In order to develop adequate diagnostic tests and better prevention tools for enterotoxaemia, it is essential to determine the causative virulence factors of *C. perfringens* in the pathogenesis of enterotoxaemia. In pursuance of identifying these causative toxins, a reproducible and practically achievable model is needed.

LESION DEVELOPMENT IN A NEW INTESTINAL LOOP MODEL INDICATES THE INVOLVEMENT OF A SHARED *CLOSTRIDIUM PERFRINGENS* VIRULENCE FACTOR IN HAEMORRHAGIC ENTERITIS IN CALVES

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ABSTRACT

C. perfringens associated enterotoxaemia is a highly fatal disease in fast growing suckler and veal calves. An intestinal loop model was developed to study the pathogenesis of the disease. Loops were injected with stationary and logarithmic *C. perfringens* cultures with, or without, a milk protein based commercial MR for calves. Following isolates were used: isolates from bovine enterotoxaemia cases and from calves without symptoms of enterotoxaemia; *netB* positive and negative isolates from poultry; a type C isolate from piglets and the human isolate JIR325. All tested isolates induced necrohaemorrhagic lesions in combination with MR, while all control loops (i.e. BHI medium plus MR) remained normal. In addition, time-course experiments were conducted using an isolate from an outbreak of bovine enterotoxaemia. Histological examination showed that lesion development was initiated by congestion of the capillaries, starting within 30 minutes after inoculation. Haemorrhages and mucosal necrosis started from the tips of the villi at 3 to 4 hours after bacterial inoculation. These lesions are similar to those observed in natural cases of bovine enterotoxaemia. It can thus be concluded that, in this model, necrohaemorrhagic lesions can be induced by *C. perfringens* isolates from diverse origins, suggesting that the lesions may be caused by the action of one or more virulence factors that are present in many *C. perfringens* isolates, emphasizing the importance of environmental factors in the development of the disease in the field.

INTRODUCTION

C. perfringens is associated with a variety of diseases including neonatal haemorrhagic enteritis, abomasitis and abomasal ulceration in calves (Katchuik, 1992; Songer and Miskimins, 2005; Marshall, 2009; VanImmerseel *et al.*, 2010). The most important *C. perfringens*-associated disease in calves is, however, enterotoxaemia (Songer, 1996; Manteca *et al.*, 2000). The latter causes sudden death, and at necropsy diffuse haemorrhagic small intestines, filled with bloody content are found (Mainil *et al.*, 2010). The most typical histological lesions are haemorrhages and necrosis in the intestinal mucosa, extending from the tips of the villi to the base of the crypts. Enterotoxaemia is predominantly seen in calves consuming cow's milk or MR based on milk protein, as opposed to SFs or MRs not containing milk protein (Mainil *et al.*, 2001; Lebrun *et al.*, 2010). Since the ban on antimicrobial growth promoters, enterotoxaemia has emerged as a major cause of mortality, especially in BB veal calves (Pardon, 2011). However, the pathogenesis of enterotoxaemia in calves is still not clear. The disease is associated with *C. perfringens* type A (Mainil *et al.*, 2010), and the β2-toxin it produces has been described as potentially implicated in the development of lesions (Mainil *et al.*, 2002; Schotte *et al.*, 2004; Lebrun *et al.*, 2007). A major problem in identifying disease-specific toxins is to determine whether isolates derived from enterotoxaemia cases and not from other sources, are capable of inducing lesions (Keyburn *et al.*, 2006; Timbermont *et al.*, 2009).

In order to study the pathogenesis of enterotoxaemia and identify pathogenic isolates and causative toxins, a reproducible *in vivo* model is needed. Mouse models have been used to study the role of toxins in clostridial diseases, but are not suitable to identify isolates or toxins which are pathogenic in cattle, due to possible species-specific activity of *C. perfringens* toxins (Uzal *et al.*, 2009). Intestinal loop models have been used in rabbits (Duncan *et al.*, 1968), lambs (Hauschil *et al.*, 1968), mice (Uzal *et al.*, 1999; Caserta *et al.*, 2011) and also calves (Mainil *et al.*, 2002; Morris *et al.*, 2011), in which the bacteria are inoculated into ligated small intestinal loops, ensuring close contact between the host mucosa and the bacteria. The advantage of intestinal loop models compared to oral challenge is that multiple factors can be tested in a single animal. However, for the study of bovine enterotoxaemia, intestinal loop models have only been used in limited preliminary studies and, to date, isolates originating only from diseased

animals have been inoculated (Mainil *et al.*, 2002; Morris *et al.*, 2011). The aim of the present study was to develop a reproducible intestinal loop model in calves to assess strain dependency and sequence of events in the development of the lesions.

METHODS

CLOSTRIDIUM PERFRINGENS ISOLATES AND CULTURE CONDITIONS

The isolates, used for inoculation of intestinal loops, are listed in table 5.1. Isolates from enterotoxaemic calves were cultured from jejunum samples within 6h after death. The selection criteria were sudden death, macroscopically visible haemorrhagic intestines and histological features typical for haemorrhagic enteritis cases (mucosal necrosis, haemorrhages). Bacteria were isolated on Columbia agar (Oxoid, Basingstoke, UK) supplemented with 5% defibrinated sheep blood, 12 mg kanamycin sulphate and 30,000 U/l polymyxin B sulphate. Plates were incubated for 24 h at 37°C in an anaerobic cabinet (Ruskinn Technology, Bridgend, UK) with 84% N2, 8% CO2 and 8% H2. *C. perfringens* colonies were identified by characteristic colony morphology and dual haemolysis. Typing was performed using multiplex and single PCR protocols for the detection of genes encoding alpha (cpa), beta (cpb), iota (ibp), epsilon (etx), beta2 (cpb2) and netB (netB) toxin (Gholamiandehkordi *et al.*, 2007; Keyburn *et al.*, 2008).

INOCULA

The bacteria were incubated overnight in 25 ml brain heart infusion broth (BHI, Oxoid, UK) at 37°C in an anaerobic cabinet, as described above. When logarithmic phase cultures were used as the inoculum, 1/100 dilutions of the overnight cultures were made by adding 250 µl culture to 25 ml of BHI broth in 50 ml falcon tubes (BD, Franklin Lakes, USA). The dilutions were made in the anaerobic cabinet 2 hours before inoculation into the intestinal loop, to allow further growth for 2 hours. When using stationary phase isolates, the overnight cultures were used for inoculation without further dilution. The falcon tubes were closed in the anaerobic cabinet before transportation. In some trials the cultures were administered together with other compounds. When using MR as co-inoculum, this was prepared by dissolving 5 grams of commercial MR (Vitaspray, Vitamex®, Drongen, Belgium) in 20 ml of sterile 0.9% NaCl

solution. In the first two experiments, soluble starch (Sigma-Alldrich®, Bornem, Belgium), trypsin (Sigma-Alldrich®, Bornem, Belgium) and trypsin-inhibitor (Sigma-Aldrich®, Bornem, Belgium) were added to some inocula. The starch solution was prepared and injected using the same method and in the same concentration as described for MR. When trypsin or trypsin-inhibitor were used, 500 µg were added per ml of culture, followed by an additional anaerobic incubation of 30 minutes at 37°C before inoculation.

ANIMALS

Thirteen HF calves were used, varying in age between 3 and 8 months. For the development of an intestinal loop model for haemorrhagic enteritis in veal calves, conventional ruminating calves of 4 to 8 months of age were kept in group housing on straw, and fed with hay and concentrates. For the remaining experiments 3 to 5 months old veal calves were used, originating from commercial veal herds. These calves received a commercially available MR based on vegetable protein, and only a minimum of corn-silage as SF. The day before the experiment they were moved to the animal experimentation unit, individually housed on straw and fed MR up to 12 hours before the start of the experiment, using the same MR in the same concentration and amount as in the herd of origin.

ANAESTHESIA AND PREPARATION OF LOOPS

The experiments were carried out with approval (EC2011_024) of the Ethical committee of the Faculties of Veterinary Medicine and Bioengineering (Ghent University), in conformity with the Belgian Royal Decree of April 6, 2010. Calves were premedicated with fentanyl (2 µg kg^{-1} IV) and midazolam (0.1 mg kg^{-1} IV) and anaesthesia was induced with propofol (2-4 mg kg^{-1} IV). After intubation, anaesthesia was maintained by inhalation of isoflurane in an oxygen-air mixture (inspiratory O2 fraction 55%) and a fentanyl infusion (0.1 µg kg^{-1} min^{-1}). Body temperature, oxygen saturation in arterial blood, arterial and venous blood pressure, electrocardiogram, airway gases and airway pressure were monitored and recorded every 15 minutes. After opening of the abdomen, the small intestines were exteriorized. Intestinal loops of approximately 10 cm in length were firmly ligated with Surgicryl PGA (SMI, St. Vith, Belgium), and a 5 cm

interspace was left between the loops. The first loops were ligated 50 cm aboral from the ileocecal junction, and construction of loops was continued in the direction of the abomasum. In calves 1 and 2 all loops were injected. In calves 3 to 13 only half of the loops were injected, thus each time leaving one bufferloop to avoid leakage between sampled loops. Bufferloops were not sampled. The inocula differed between experiments and were injected into the antimesenteric edge using a 20 ml syringe and a 22 gauge needle. When both bacterial culture (or sterile medium in the control loops) and extra components (mostly MR) were injected in the lumen of the loops, first the culture (or sterile medium) was injected, followed by a second injection with solution containing MR, into the antimesenteric edge, using a new 20 ml syringe and a 22 Gauge needle. After injection of the loops the abdomen was closed, and the calves were maintained under deep general anesthesia until euthanasia.

INTESTINAL LOOP ASSAYS

EXPERIMENT 1: DEVELOPMENT OF AN INTESTINAL LOOP MODEL FOR THE IN VIVO REPRODUCTION OF HAEMORRHAGIC ENTERITIS IN CALVES

In three calves (calves 1, 2 and 3), 9 different isolates, with or without trypsin or trypsin inhibitor (as possible toxin activators), and starch or MR (as possible growth stimulants) were inoculated into individual loops. In total, respectively 24, 76 and 30 loops were injected per calf. The tested isolates, growth phase and added components are shown in table 5.1.

Table 5.1. Description of the isolates used in experiment 1.

Isolate	Source	Reference	Present genes
BCP20	HF calf, abomasal ulcer	Van Immerseel *et al.*, 2010	*cpa*
BCP62	BB calf, haemorrhagic enteritis	this study	*cpa*
BCP281	HF calf, rectal swab	this study	*cpa*
JIR325	human	Lyristis *et al.*, 1994	*cpa*
JIR4107	human	Lyristis *et al.,* 1994	*cpa*
CP56	chicken	Gholamiandehkordi *et al.*, 2006	*cpa, netB*
CP24	chicken	Gholamiandehkordi *et al.*, 2006	*cpa*
NE18	chicken	Keyburn *et al.,* 2006	*cpa, netB*
JF3721	pig	Gurtner *et al.*, 2010	*cpa, cpb*

Abbreviations: HF= Holstein Friesian; BB= Belgian Blue; *cpa*= gene for alpha toxin; *netB*= gene for netB toxin; *cpb*= gene for beta toxin

In the first calf the abdomen was reopened every hour after the inoculation to macroscopically judge the condition of the serosal side of the intestines until euthanasia at 12 hours post-inoculation. Calves 2 and 3 were euthanized and sampled after 6 hours. In the three calves control loops were included, i.e. non-inoculated loops injected with sterile BHI-medium for each condition (trypsin, trypsin inhibitor, starch, MR and their respective combinations) and noninoculated loops injected with BHI-medium only.

EXPERIMENT 2: VALIDATION OF THE INTESTINAL LOOP MODEL USING AN ISOLATE OBTAINED FROM A CASE OF ENTEROTOXAEMIA

To determine that the developed protocol was reproducible, and to analyze the efficiency (number of loops with lesions per number of inoculated loops). the haemorrhagic enteritis isolate BCP62 was used to inoculate intestinal loops. Two calves (calves 4 and 5) were used in this experiment. In each calf, a total of 60 loops was prepared which included 30 non-inoculated buffer loops, alternated with 30 injected loops. The latter included 10 loops inoculated with a culture of BCP62 in logarithmic phase, 10 loops with a culture of BCP62 in logarithmic phase supplemented with MR, 5

loops with BHI-medium and 5 loops with BHI-medium supplemented with a MR. The sequence of the loops in the intestine was randomized using Microsoft Office Excel 2007 (Microsoft®). At 6 hours post-inoculation the animals were killed humanely and samples collected.

EXPERIMENT 3: LESION DEVELOPMENT AFTER INOCULATION OF ISOLATES FROM DIFFERENT SOURCES

In calves 6 and 7 bovine haemorrhagic enteritis case isolates (BCP62, BCP134, BCP510, BCP544), an isolate derived from an abomasal ulcer (BCP20) and isolates from healthy calves (BCP506, BCP281, BCP334 and BCP447) were used. In calf 8, isolates from non-bovine origin, CP24, NE18, CP56 (three poultry isolates), JIR325 (human isolate) and JF3721 (porcine isolate) were used. In each calf, 3 intestinal loops per isolate were injected with a logarithmic phase culture in combination with MR. In every calf, three loops were injected with BHI-medium supplemented with a MR as control loops. In calves 6 and 7, each time 30 loops were injected, and in calf 8, 36 loops were injected. The sequence of injection was randomized using Microsoft Office excel 2007 (Microsoft®). At 6 hours post-inoculation the animals were euthanized and sampled (see further).

EXPERIMENT 4: LESION DEVELOPMENT OVER TIME

In calves 9 and 10 the protocol was similar to the protocols described above, with the exception that all loops were ligated at the start, but the injection was done at time intervals of 30 minutes, so that euthanasia at 5 hours would yield intestinal loops that had been in contact with the inocula for different time periods, ranging from 30 to 300 minutes (5 hours). A bovine enterotoxaemia isolate, BCP62, was used. In calf 9, at 30 minute intervals up to 5 hours BCP62 was inoculated in three loops, together with MR. In addition, three control loops were inoculated at the start of the experiment with BHI medium supplemented with MR, making a total of 33 loops. The protocol used in calf 10 was similar, but at every 30 minute injection interval only 2 loops (instead of 3) were injected with a logarithmic culture of BCP62 with MR and 1 intestinal loop was injected with BHI-medium to which MR was added (30 injected loops in total).

EXPERIMENT 5: INOCULATION OF INTESTINAL LOOPS WITH BACTERIA-FREE SUPERNATANT

A total of eight loops, divided over three calves (calf 11, 12 and 13; respectively 2, 3 and 3 loops per calf) were injected with bacteria-free supernatant from isolate BCP62. Supernatant was made by centrifuging an overnight culture of BCP62 (5000 x g, 15', 37°C) and filtering the resulting supernatant with a 0.2µm filter (Merck Millipore, Billerica, Massachusetts) to make it sterile. In every loop, 20 ml of the supernatant was injected in combination with 10 ml MR. At 6 hours post-inoculation the animals were euthanized and sampled (see further).

SAMPLING, PROCESSING OF SAMPLES AND SCORING LESIONS

The intestines were exteriorized, and exposed to allow rapid sampling. The calves were euthanized under continued generalized anesthesia using pentobarbital before sampling. Immediately after fall in arterial and venous blood pressure to 0 mmHg a team of experienced veterinary surgeons excised, opened and inspected the injected intestinal loops, followed by swiftly submerging the whole intestinal loops in 4% phosphate buffered (wt/vol) formaldehyde. In experiment 1, development of the model, samples were removed within 30 minutes after death. For the remaining experiments, samples were removed within 10 minutes of death, as to avoid postmortal decay. After fixation in formaldehyde for 24 hours, for the samples of the first 8 calves the mucosa was subjected to visual inspection. The macroscopic appearance of the mucosa was scored on a scale of 1 to 3, with 1 representing the normal non-pathological appearance, score 2 moderate lesions, and score 3 representing severe mucosal damage (figure 5.1).

Figure 5.1 Macroscopical lesion score of the intestinal mucosa: A) score 1, intact mucosa; B) score 2, integrity of mucosa partially affected; C) score 3, diffuse extensive mucosal damage

After fixation for 24 hours, the samples were processed routinely to paraffin wax, sections cut at 5µm and stained with haematoxylin and eosin. Sections were evaluated for presence of necrosis, presence of erythrocytes outside the vascular system (haemorrhages) and the presence of rod-shaped *C. perfringens*-like bacteria.

RESULTS

STRAIN CHARACTERISTICS

A description of the characteristics of the inocula in experiment 1 is presented in table 5.2.

Table 5.2. Inocula used for experiment 1 (the development of an intestinal loop model for haemorrhagic enteritis in veal calves). Per calf, the table provides information about the used strains, growth phase and the added components.

Strain	Phase	0	T	TI	MR	MR + T	MR + TI	S	S + T	S + TI
Calf 1										
BHI (neg control)	/	x	x	x						
BCP20	stat	x	x	x						
BCP62	stat	x	x	x						
BCP156	stat	x	x	x						
BCP226	stat	x	x	x						
BCP273	stat	x	x	x						
BCP274	stat	x	x	x						
Calf 2										
BHI (neg control)	/	x	x	x	x			x		
BCP20	stat	x	x	x	x					
BCP62	stat	x	x	x	x					
BCP156	stat	x	x	x	x					
BCP256	stat	x	x	x	x					
CP56	stat	x	x	x	x					
JIR325	stat	x	x	x	x					
BCP20	log	x	x	x						
BCP62	log	x	x	x	x	x	x	x	x	x
BCP156	log	x	x	x						
BCP256	log	x	x	x	x	x	x	x	x	x
CP56	log	x	x	x						
JIR325	log	x	x	x						
Calf 3										
BHI (neg control)	/	x	x	x	x	x	x			
BCP62	log	x	x	x	x	x	x			
BCP256	log	x	x	x	x	x	x			
CP56	log	x	x	x	x	x	x			
CP325	log	x	x	x	x	x	x			

Abbreviations: BHI= Brain Heart Infusion Broth (negative control); stat= stationary phase; log= logarithmic phase; 0= culture alone; T= culture plus trypsine; TI= culture plus trypsininhibitor; M= culture plus milk replacer; M+T= culture plus milk replacer and trypsin; M+TI= culture plus milk replacer and trypsininhibitor; S= culture plus starch; S+T= culture plus starch and trypsin; S+TI= culture plus starch and trypsininhibitor

EXPERIMENT 1: DEVELOPMENT OF AN INTESTINAL LOOP MODEL FOR THE IN VIVO REPRODUCTION OF HAEMORRHAGIC ENTERITIS IN CALVES

From the results from the first calf it could be concluded that severe dilatation of some loops was observed from 6 hours post-incubation onwards, and subsequently calves were killed at 6 hours. When using stationary cultures to inoculate the small intestinal loops, lesions were not observed. Also, in the absence of MR in the inoculum, lesions were not observed. However, using logarithmic cultures in combination with MR, necrohaemorrhagic lesions could be induced with isolates CP56, BCP62 and BCP256. Using BCP20, BCP156, BCP274 and JIR325, congestion of the capillaries could be induced, but without haemorrhages or necrosis. No lesions were induced in this experiment using BCP226 and BCP273. Trypsin and trypsin inhibitor had no effect on the induction of lesions.

EXPERIMENT 2: VALIDATION OF THE INTESTINAL LOOP MODEL USING AN ISOLATE OBTAINED FROM A CASE OF ENTEROTOXAEMIA

Similar results were observed in both calves. 18 loops (90%) injected with a logarithmic culture of BCP62 together with MR, produced macroscopic lesions. Macroscopically, congestion of the mucosa and the presence of necrotic debris in the lumen was observed. Only 10% of the loops injected with a culture of BCP62 without MR or BHI medium without BCP62 or MR showed macroscopic mucosal lesions. None of the loops injected with a combination of BHI medium and MR showed macroscopic lesions. The observed macroscopic lesions were congestion of the mucosa and the presence of necrotic debris in the lumen. Histologic lesions are presented in table 5.3. Necrosis of the tips of the villi only occurred when injecting the combination of a culture of BCP62 and MR (90%). Mucosal haemorrhages were always present in loops injected with BCP62 culture and MR (100%), as opposed to 70% of loops injected with BCP62 culture without MR, and 20 to 30% of control loops (BHI either or not supplemented with MR). Numerous rod-shaped bacteria were attached to cellular debris and to the mucosa in the majority of intestinal loops inoculated with BCP62 culture and MR, while this was only visualized in a minority of control loops and loops injected with BCP62 culture without MR.

EXPERIMENT 3: LESION DEVELOPMENT AFTER INOCULATION OF ISOLATES FROM DIFFERENT SOURCES

In the experiments comparing isolates of different origin, all tested *C. perfringens* isolates, either from bovine or non-bovine origin, were able to induce necrohaemorrhagic lesions in one or more injected intestinal loops as described above (table 5.3). In the control loops (BHI + MR) none of the above mentioned necrohaemorrhagic lesions were detected.

EXPERIMENT 4: LESION DEVELOPMENT OVER TIME

In both time courses a similar sequence of lesion development was observed. Congestion of the capillaries at the tips of the villi was present in some loops that were incubated for 30 minutes, and in all loops that were incubated for more than one hour. Subsequently haemorrhages were observed from 3 to 4 hours onwards. More intensive haemorrhages were found after 4 to 5 hours post-inoculation. Demarcating necrosis started at 3.5 to 4 hours at villus tips, extending to involve complete villi later on. For all time periods, large rodshaped bacteria were observed binding to cell debris and plasma proteins in the lumen and attached to the mucosa in several loops. Necrosis or haemorrhages were not seen in control loops (figure 5.2).

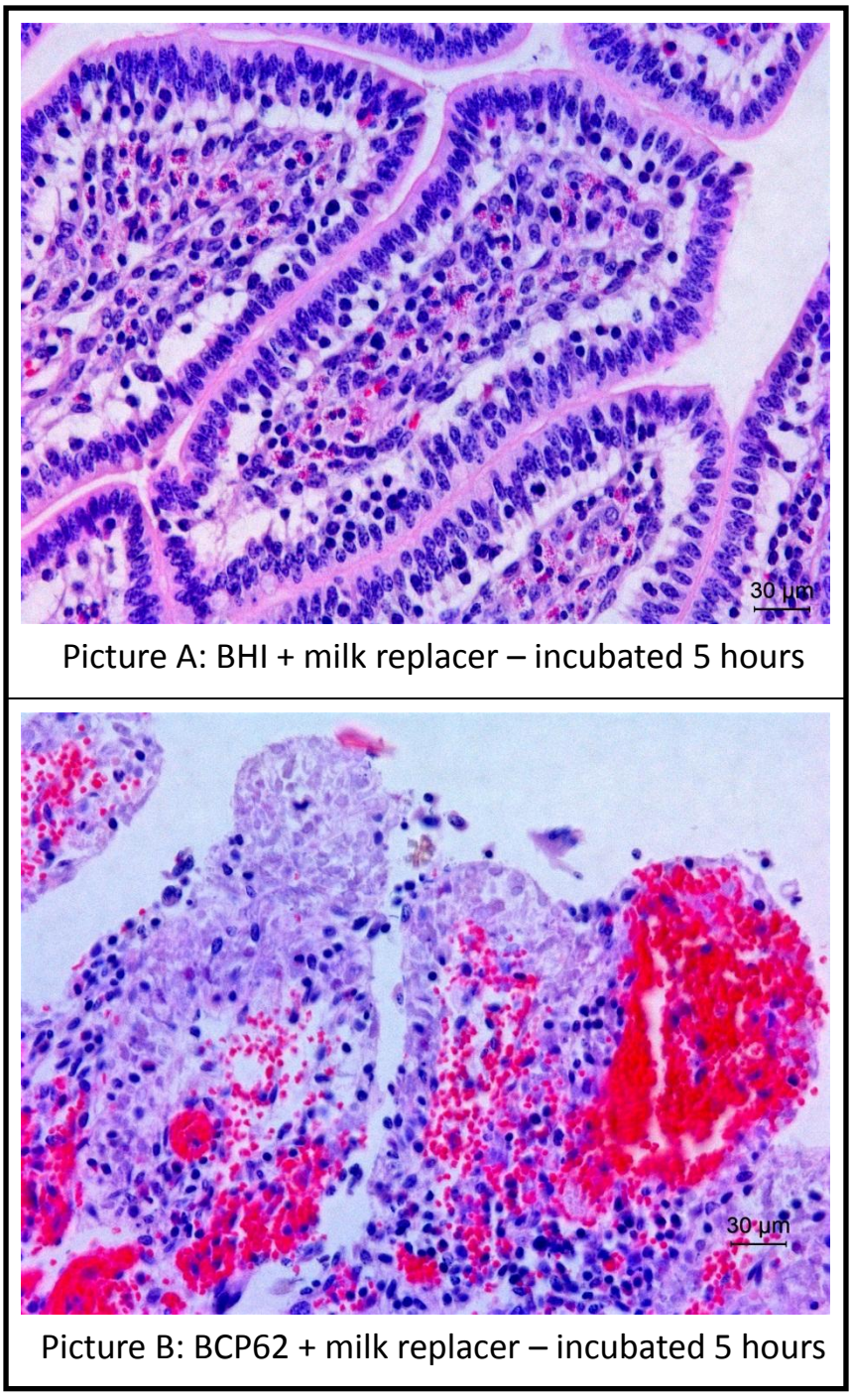

Figure 5.2 Histological sections of intestinal villi of a loop in calf 10, injected with isolate BCP62 and milk replacer (picture B) or injected with sterile medium and milk replacer (picture A) and incubated for 5 hours. Picture A shows the negative control without specific lesions. Picture B shows necrosis of the intestinal villi, capillary congestion and haemorrhage (HE-staining; bar = 30 µm).

EXPERIMENT 5: INOCULATION OF INTESTINAL LOOPS WITH BACTERIA-FREE SUPERNATANT

No necrohaemorrhagic lesions were detected in the loops injected with sterile supernatant (table 5.3).

Table 5.3. Number of loops showing specific lesions in the validation of the model, lesion development after inoculation of isolates from different sources and inoculation of intestinal loop with bacteria-free supernatant

Inoculans	Origin of isolate	Extra component	Grossly visible mucosal damage[1]	Haemorrhage[2]	Necrosis of villus tips[2]	Presence of large rods[2]
Experiment 2: Validation of the intestinal loop model using an isolate obtained from a case of enterotoxaemia (calves 4 and 5)						
BHI	/	/	1/10	4/10	0/10	0/10
BHI	/	MR	0/10	3/10	0/10	1/10
BCP62	BB calf, haemorrhagic gut	/	2/20	15/20	0/20	2/20
BCP62	BB calf, haemorrhagic gut	MR	18/20	20/20	18/20	13/20
Experiment 3: Lesion development after inoculation of isolates from different sources (calves 6, 7 and 8)						
BHI	/	MR	0/9	0/9	0/9	0/9
BCP62	BB calf, haemorrhagic gut	MR	3/6	2/6	2/6	2/6
BCP134	HF calf, haemorrhagic gut	MR	3/6	4/6	3/6	1/6
BCP506	BB calf, haemorrhagic gut	MR	3/6	2/6	4/6	1/6
BCP510	BB calf, haemorrhagic gut	MR	4/6	2/6	3/6	2/6
BCP544	BB calf, haemorrhagic gut	MR	2/6	3/6	3/6	2/6
BCP20	HF calf, abomasal ulcer	MR	6/9	7/9	7/9	5/9
BCP281	HF calf, rectal swab	MR	6/9	5/9	5/9	5/9
BCP334	BB calf, rectal swab	MR	4/6	3/6	3/6	3/6
BCP447	BB calf, healthy gut	MR	3/6	4/6	4/6	3/6
CP24	chicken	MR	3/3	2/3	3/3	3/3
CP56	chicken	MR	3/3	0/3	3/3	3/3
NE18	chicken	MR	2/3	2/3	2/3	2/3
JIR325	human	MR	3/3	2/3	3/3	2/3
JF3721	pig	MR	3/3	2/3	3/3	3/3
Experiment 5: Inoculation of intestinal loops with bacteria-free supernatant (calves 11, 12 and 13)						
BHI	/	MR	0/9	0/9	0/9	1/9
BCP62	BB calf, haemorrhagic gut	MR	4/8	5/8	4/8	3/8
Sterile supernatant of BCP62	/	MR	0/8	0/8	0/8	0/8

Abbreviations: BB= Belgian Blue, HF= Holstein Friesian, MR= milk replacer, BHI= Brain Heart Infusion broth (negative control)
References: 1= macroscopy, 2= histology

DISCUSSION

The model developed in this study was successful in inducing necrohaemorrhagic lesions in the small intestine, comparable to the lesions seen in field cases of enterotoxaemia. The use of intestinal loop models to study the pathogenesis of infections and inflammatory processes is described in several animal species, including young calves (Haralambiev *et al.*, 1979; Yamagishi *et al.*, 1987; Mainil *et al.*, 2002; Stevens *et al.*, 2002; Timbermont *et al.*, 2009; Caserta *et al.*, 2011; Morris *et al.*, 2011). In the present model, the entire procedure was performed under general anesthesia, ensuring minimal stress and suffering for the animals. It provides an *in vivo* intestinal environment with intact neural and vascular systems (Griebel *et al.*, 2001). Large numbers of variables can be studied in one animal, thus reducing the number of experimental animals and providing excellent reference material for comparative studies using different pathogenic isolates or conditions in 1 animal (Griebel *et al.*, 2001).

MR appeared to be an important predisposing component as an additive to the *C. perfringens* inoculum to induce typical necrohaemorrhagic lesions in the intestinal loops. In previous loop models developed for different clostridial diseases in different animal species, the addition of a nutrient-rich supplement was needed to induce lesions (Niilo, 1986; Titball *et al.*, 2009). The necessity of the addition of a MR fits in with information from the veal industry, where more enterotoxaemia cases occur after intake of large quantities of MR without a proper adaptation period (Mainil *et al.*, 2010). However, in a natural situation the MR is first predigested by pepsin and other proteases in the abomasum, while in the loop experiment the MR is injected undigested directly in the small intestine. Therefore, the protein is still intact and unavailable for the host, consequently increasing its availability for the clostridia. The MR used in this experiment contained a high amount of animal protein. This MR is typically used in the BB veal industry, which has a high prevalence of enterotoxaemia (9.75%) (Pardon *et al.*, 2011). In the dairy veal industry, where a MR based on mainly vegetable protein is used, enterotoxaemia has a low prevalence (0.89%) (Pardon *et al.*, 2011). Abrupt changes in feed composition are known to trigger enterotoxaemia in conventional beef calves (Lebrun *et al.*, 2010; Lewis, 2011). The nature of the components of the MR triggering the development of lesions is unknown. MR contains large amounts of high quality

protein which may stimulate the growth of *C. perfringens*, a bacterium that is auxotrophic for multiple amino acids (Titball *et al.*, 2009). Trypsin and trypsin inhibitor did not contribute to the development of lesions in the intestinal loops, indicating that the causative toxins are not susceptible to trypsin-mediated cleavage that would result in either inactivation or activation of the toxins. Starch fermentation also did not contribute to the development of lesions.

The sequence of microscopical events during lesion development in the loop model provides new insights into the pathogenesis of calf enterotoxaemia. In an early stage congestion of the capillaries at the tips of the intestinal villi was noted. Certain toxins produced by *C. perfringens* such as beta toxin, are known to disrupt the endothelium by immediate cytopathic effects (Gurtner *et al.*, 2010). This could influence the blood supply and contribute to the typical haemorrhages and to microvascular thrombosis leading to reduced tissue perfusion, hypoxia, and subsequent necrosis, as seen in the villi tips in a later stage (Bryant, 2003; Gurtner *et al.*, 2010). Strains that did not contain the beta toxin gene induced haemorrhages and necrosis in this study, but other toxins can potentially have similar effects.

In most clostridial diseases the presence of the causative toxin determines the potential of an isolate to cause lesions (Niilo, 1988; Lewis, 1998; Moore *et al.*, 2008; Timbermont *et al.*, 2009). The causative trigger for enterotoxaemia in calves remains to be identified. This model offered the opportunity to screen isolates of different origin. However, in contrast to other *C. perfringens* associated diseases in other host species (Timbermont *et al.*, 2009; Gurtner *et al.*, 2010), all injected isolates were capable of inducing necrohaemorrhagic lesions when injected in combination with MR, independent of their origin. A possible explanation for this phenomenon is that the causative toxin for enterotoxaemia in calves is present in many *C. perfringens* isolates, and that the development of bovine enterotoxaemia is determined by environmental triggers, such as nutrition. Eligible toxins could be, among others, alpha toxin and perfringolysin, as these toxins are present in most, if not all, isolates of *C. perfringens* (Rood *et al.*, 1991). In order to identify causative toxins, non-toxinogenic mutants should be included to assess their pathogenicity.

CONCLUSIONS

To conclude, in this study a reproducible intestinal loop model for calf enterotoxaemia was developed. Early pathogenesis is typified by congestion of the capillaries, resulting in haemorrhages and finally necrosis of the mucosa within 3 to 4 hours. Additionally, this study indicates that, when injected in combination with MR most *C. perfringens* isolates, independent of their origin, are capable of eliciting lesions in this model, resembling those seen in bovine enterotoxaemia, suggesting that the virulence factors of *C. perfringens* responsible for enterotoxaemia are present in most, if not all, *C. perfringens* isolates.

CHAPTER 6

LACK OF PRODUCTION OF ANTIBODIES AGAINST *CLOSTRIDIUM PERFRINGENS* ALPHA TOXIN AS A POTENTIAL EXPLANATION FOR THE SUSCEPTIBILITY TO ENTEROTOXEMIA IN CALVES

The intestinal loop model described in chapter 5 was used by Verherstraeten *et al.* (2014) to identify alpha toxin and perfringolysin as the most important *C. perfringens* virulence factors for enterotoxaemia. With this knowledge, the road is open for the development of better vaccines against enterotoxaemia. In order to develop good vaccination schemes, it is important to chart the natural occurrence of antibodies against the toxins, and the duration of maternal immunity. Since the feed management can influence the contact of the calves with *C. perfringens* and its toxins, the natural occurence of antibodies might differ between different production types and breeds. This information might be useful in the development of various preventive measures against enterotoxaemia.

LACK OF PRODUCTION OF ANTIBODIES AGAINST *CLOSTRIDIUM PERFRINGENS* ALPHA TOXIN AS A POTENTIAL EXPLANATION FOR THE SUSCEPTIBILITY TO ENTEROTOXEMIA IN CALVES

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ABSTRACT

Enterotoxaemia is a disease with a high associated mortality rate, affecting beef and veal calves worldwide, caused by *C. perfringens* alpha toxin and perfringolysin. The present paper describes the results of two studies. A longitudinal study was conducted to determine the evolution of antibodies of calves between 2 and 26 weeks of age against alpha toxin and perfringolysin in 528 calves on beef (n= 4) and veal (n= 15) farms. The second study aimed to determine the effect of SF intake in veal calves on the production of antibodies between 2 and 26 weeks against alpha toxin and perfringolysin. The control group only received MR, whereas in the test group SF was provided next to the MR. Maternal antibodies for alpha toxin were present in 45% of the veal calves and 66% of the beef calves. In beef calves, a fluent transition from maternal to active immunity was observed for alpha toxin, whereas almost no veal calves developed active immunity. Perfringolysin antibodies significantly declined both in veal and beef calves between the age of 2 and 8 weeks. In the second study all calves were seropositive for alpha toxin throughout the experiment and SF intake did not alter the dynamics of alpha and perfringolysin antibodies. In conclusion, the present study shows that veal calves on a traditional MR diet have significantly lower alpha toxin antibodies compared to beef calves in the risk period for enterotoxaemia, whereas no differences were noticed for perfringolysin antibodies.

INTRODUCTION

Enterotoxaemia is a fatal disease of young cattle, particularly in intensive production systems, and may be characterized by sudden death and necro-haemorrhagic enteritis (Valgaeren *et al.*, 2013). The pathogenesis of enterotoxaemia in calves has long been unclear. Recently, *C. perfringens* alpha toxin and perfringolysin have been identified as the key virulence factors involved in the development of bovine necro-haemorrhagic enteritis (Verherstraeten *et al.*, 2013).

BB calves are predisposed to enterotoxaemia. In BB veal calves up to 20% of the total mortality, especially in the last weeks before slaughter, can be attributed to enterotoxaemia, whereas in HF or crossbred veal calves the incidence is significantly lower (Manteca *et al.*, 2001; Pardon *et al.*, 2012). High mortality rates due to enterotoxaemia have also been reported in suckler calves, whereas enterotoxaemia is less frequent in beef production systems with immediate separation from the dam (Griner and Bracken, 1953; Niilo *et al.*, 1974; Manteca and Daube, 1994). In addition to a possible genetic predisposition, the differences in diet between veal and beef calves might play a role (Pardon *et al.*, 2014). Whereas veal calves are mainly raised on MR and receive only limited amounts of SF, beef calves are fed a limited amount of MR, are weaned at an early age, and are thereafter predominantly fed with SFs (Schofield, 1955; Griesemer, 1962). Similar to other animal species, the protein- and energy-rich diet of veal calves has been implicated in predisposition to enterotoxaemia, but there is no scientific evidence for this statement (Uzal and Kelly, 1996; Berghaus *et al.*, 2005; Timbermont *et al.*, 2011).

In other clostridial diseases, maternal immunity against exotoxines is generally protective (Veschi *et al.*, 2008). In calves, experimental studies have shown that maternal antibodies against *C. perfringens'* epsilon and alpha toxin are detectable up to 200 days after birth (Troxel *et al.*, 1997; Heier *et al.*, 2001). In small ruminants, vaccination against the most important virulence factors (beta toxin and epsilon toxin), yields good results in field situations. However, there is a significant interference with antibody production when neonatal lambs from vaccinated ewes are vaccinated (Reynolds and Griffin, 1990). Currently, new vaccines are being developed using recombinant *C. perfringens* toxoids, aiming to induce protective immunity against

enterotoxaemia in cattle (Jiang *et al.*, 2014). To date, no information on maternal antibody decline and acquisition of active immunity against alpha toxin and perfringolysin in calves is available. Such information may help to elucidate the epidemiology of bovine enterotoxaemia and is of crucial importance for the development of schemes for alpha toxin and perfringolysin based vaccines in the field, in order to avoid interference with maternal immunity. We hypothesized that these antibody dynamics differ between breeds and production systems, thereby partially explaining differences in susceptibility for enterotoxaemia.

Therefore the primary objective of the first study was to determine antibody dynamics in beef calves and veal calves, and the secondary aim was to determine antibody dynamics in different breeds in veal calves. The objective of the second study was to determine the effect of SF intake on the production of alpha toxin and perfringolysin antibodies in veal calves.

MATERIALS AND METHODS

All experiments were conducted with permission of the local ethical committee (EC2012/118 and EC2014/016).

STUDY 1: DYNAMICS OF ANTIBODIES AGAINST ALPHA TOXIN AND PERFRINGOLYSIN IN VEAL AND BEEF CALVES

A prospective longitudinal cohort study was conducted to determine the effect of production system (veal or beef) on the alpha toxin and perfringolysin antibody dynamics. Secundary, within the veal calves, the effect of breed on the alpha toxin antibody dynamics in veal calves was tested.

ANIMALS

The study was conducted in 528 animals, housed in 19 conveniently selected farms (15 veal farms [5 BB, 5 HF, 5 crossbred (HFxBB)] and 4 beef farms (BB) (table 6.1). Male veal calves were sampled between October 2007 and October 2009, and beef calves were sampled between November 2013 and August 2014. Veal calves received a commercial all liquid diet, with minimal amounts of SF (<200 g/day). On the conventional BB farms calves were weaned (deprived of MR) between 14 and 26 weeks of age, whereas veal calves received MR throughout the study. Details on the rations and housing conditions in veal and beef farms are provided in table 6.1.

None of the farms had a history of vaccination against enterotoxaemia. On the beef farms, calves received fresh colostrum from their own unvaccinated mother. For the veal farms, vaccination history of the dams was unknown.

Table 6.1: Housing conditions and feeding regime of the herds included in the first study on antibody dynamics of *C. perfringens* alpha toxin and perfringolysin

Herd	Number of calves in study	Production system	Breed	Floor	Feed			
					Milk replacer		Weaning	Solid feed
					% NP	% SMP		
1	25	Veal	HF	Slatted floor	95	5	No	< 200g/d
2	25	Veal	HF	Slatted floor	95	5	No	< 200g/d
3	25	Veal	HFXBB	Slatted floor	95	5	No	< 200g/d
4	25	Veal	BB	Slatted floor	30	70	No	< 200g/d
5	25	Veal	HFXBB	Slatted floor	95	5	No	< 200g/d
6	25	Veal	BB	Slatted floor	30	70	No	< 200g/d
7	25	Veal	HF	Slatted floor	95	5	No	< 200g/d
8	26	Veal	HF	Slatted floor	95	5	No	< 200g/d
9	25	Veal	HF	Slatted floor	95	5	No	< 200g/d
10	24	Veal	BB	Slatted floor	30	70	No	< 200g/d
11	30	Veal	BB	Slatted floor	30	70	No	< 200g/d
12	39	Veal	HFXBB	Slatted floor	95	5	No	< 200g/d
13	38	Veal	BB	Slatted floor	30	70	No	< 200g/d
14	41	Veal	HFXBB	Slatted floor	95	5	No	< 200g/d
15	39	Veal	HFXBB	Slatted floor	95	5	No	< 200g/d
16	21	Beef	BB	Straw bedded	0	100	Yes	Ad lib
17	20	Beef	BB	Straw bedded	0	100	Yes	Ad lib
18	27	Beef	BB	Straw bedded	0	100	Yes	Ad lib
19	23	Beef	BB	Straw bedded	0	100	Yes	Ad lib

Abbreviations: BB= Belgian Blue, HF= Holstein Friesian, HFXBB= Crossbred calves, SMP= Milk replacer containing whey powder and skimmed milk powder, NP= Nill product (Milk replacer based on whey powder, without skimmed milk powder, casein fraction replaced by vegetable protein), Ad lib= unrestrained provision.

SAMPLING

Serum samples were taken from the jugular vein from all calves at the age of 2, 8, 14 and 26 weeks. All serum samples were kept frozen (-18°C) until analysis.

ANTIBODY DETERMINATION

ALPHA TOXIN ANTIBODY ELISA

A commercial serum blocking ELISA kit for *C. perfringens* alpha toxin (BIO K 291, Bio-X, Jemelle, Belgium) was used according to the manufacturer's guidelines. The percentage inhibition for a sample was calculated by the following formula based on the optical density (OD):

% inhibition sample = [(OD neg – OD sample)/OD neg]*100

A calf was considered seropositive if the inhibition percentage was higher than 40%. A calf was considered to have seroconverted if the inhibition percentage increased with 40% or more in consecutive sampling points.

EXPRESSION AND PURIFICATION OF PERFRINGOLYSIN

The pTrcHisA plasmids encoding native perfringolysin (Department of Microbiology and Immunology, College of Medicine, University of Oklahoma, USA) were transformed in chemically competent *E. coli* Tuner™(DE3)pLysS cells (Novagen, Darmstadt, Germany). The proteins were expressed in Terrific Broth (24% Tryptone (Oxoid, Hampshire, UK), 42% Yeast extract (BD), 4% glycerol, 0.72M Na_2HPO_4, 0.16M NaH_2PO_4) and the protein expression was induced with 0.5mM isopropyl β-D-thiogalactopyranoside (IPTG, Promega, Madison, WI) [22, 28, 29]. The *E. coli* cells were lysed by sonication in a lysis buffer (pH 7.4) containing 20 mM $NaPO_4$, 0.5 M NaCl, 20mM Imidazole, 1 mg/ml lysozyme (Sigma-Aldrich) supplemented with protease-inhibitor cocktail (Sigma-Aldrich, St Lois, MO). The supernatant was then loaded on a Cobalt-affinity column (His GraviTrap; GE Healthcare, Brussels, Belgium). The column was washed with buffer (20 mM $NaPO_4$, 0.5 M NaCl) containing 40mM Imidazole and the histidine-tagged proteins were eluted from the column with buffer containing 0.3M imidazole. The eluted proteins were dialyzed against phosphate-buffered saline (PBS - 137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 1.8mM KH_2PO_4; pH 7.4) overnight at 4°C. Protein concentrations were determined with the Pierce BCA protein Assay (Thermo Scientific) using bovine serum albumin (Sigma-Aldrich, St Lois, MO) as a standard. Protein purity was analyzed

by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with Coomassie Brilliant Blue Staining (Sigma-Aldrich, St Lois, MO). PageRuler Unstained Protein Ladder (Thermo Scientific, Waltham, MA) was used as a protein standard.

PERFRINGOLYSIN ANTIBODY ELISA

A direct ELISA was developed to detect antibodies against perfringolysin in serum samples. Immunoplates (Nunc-Immunoplate Polysorp) were coated with 20 μg/ml of purified perfringolysin diluted in carbonate buffer (pH 9.5) and incubated overnight at 4°C. After washing the plates with phosphate-buffered saline (PBS) containing 0.1% Tween 20 (Washing Buffer, WB), test and control sera diluted in PBS containing 0.05% Tween 20 (Dilution Buffer, DB) were added to the plates and incubated for 1 h at 37 °C. The plates were washed between steps with WB, pH 7.4. A hyperimmune serum from vaccinated calves diluted in 1:100,000 DB was used as positive control. Testsera and negative control serum from a neonatal calf deprived of colostrum were diluted 1:50. All serum dilutions were performed in duplicate. The plates were incubated for 2 hours at 37°C with the sera and subsequently incubated for 1.5h at 37°C conjugated with α Bovine horseradish peroxidase (HRP), diluted 1:30,000, and again incubated for 1.5h at 37°C. The reaction was developed with 3,3′ ,5,5′ -Tetramethylbenzidine (TMB; Sigma–Aldrich, St Louis, MO) as chromogen substrate and the intensity of staining was read at 450 nm after 30 min of incubation. All reagents were purchased from Sigma–Aldrich, St Louis, MO. The perfringolysin antibody ELISA was only done on samples from BB calves.

STATISTICS

First, values were checked for normal distribution. To determine the effect of breed and production system (beef vs. veal) on alpha toxin ELISA inhibition percentage and perfringolysin ELISA optical density, a linear mixed model with repeated measurements was used (PROC MIXED). Sampling point was added as the repeated effect and herd as a random effect to account for clustering of calves within a herd. Test variables were breed (BB, HF or crossbred) and production system (beef vs. veal). Pairwise comparisons of significant main effects were made using bonferroni corrections. Significance was set at $P<0.05$. All analyses were done in SAS 9.4 (SAS Institute, Cary, NC).

STUDY 2: EFFECT OF SOLID FEED INTAKE ON ALPHA TOXIN AND PERFRINGOLYSIN ANTIBODY DYNAMICS

STUDY DESIGN

A longitudinal experimental study to determine the effect of SF intake on perfringolysin and alpha toxin antibody dynamics in veal calves was conducted between May 2014 and November 2014. To detect a difference of 25% in calves that are seropositive against alpha toxin with 95% confidence and 80% power, a sample size of 12 calves per group was necessary. A total of 15 calves per group were sampled to account for possible mortality. Serum samples were taken at the age of 2, 8, 14 and 26 weeks. Calves were weighed at the age of 2 and 26 weeks.

ANIMALS, HOUSING AND FEEDING

The trial was performed at a commercial veal fattening unit. Thirty male HF calves were randomly assigned to one of two treatment groups at the day of arrival at two weeks of age. All calves were kept on slatted floor without bedding. Both groups were fed the same amount of MR (21.2% crude protein, 17.7% crude fat at dry matter base; Nill product, Vilatca nv, Geel, Belgium) in the same concentration, twice a day. At the age of 2 weeks the calves were fed 220g MR/feeding, increasing to 1260g/feeding at the age of 26 weeks. Group 2 also received increasing amounts (32 to 710 g/feeding) of commercial veal SF (a mixture of barley, corn, hulled wheat and 10% straw; ash 2.4%, crude protein 9.6%, crude fat 2.9%, crude fiber 8%) twice daily. The calves were not vaccinated. The vaccination status of the dams was unknown.

LABORATORY ANALYSIS

Alpha toxin and perfringolysin antibody ELISAs were performed on all samples as described above.

STATISTICS

First, values were checked for normal distribution. The effect of the treatment group (SF or not) on alpha toxin ELISA % inhibition and perfringolysin antibody ELISA OD was

determined by a repeated measurement linear mixed model (PROC MIXED) as described above, but without a random effect.

RESULTS

STUDY 1: ANTIBODY DYNAMICS AGAINST ALPHA TOXIN AND PERFRINGOLYSIN IN VEAL AND BEEF CALVES

ALPHA TOXIN ANTIBODY DYNAMICS

Figure 6.1 and table 6.2 give an overview of the mean inhibition of the optical density (OD) and table 6.2 of the prevalence of *C. perfringens* alpha toxin antibodies in the different groups at the different ages. At the age of two weeks, the seroprevalence of alpha toxin was 45% and 66% in veal and conventional BB respectively. This difference was not significant. In beef calves, there were no significant differences between the different ages. In contrast, veal calves demonstrated a significant decline in alpha toxin antibodies between the ages of two and eight weeks as well as between the ages of 8 and 14 weeks. Only 5% ± 4% of the veal calves seroconverted for alpha toxin over the course of this study. The difference between subjects (production system beef vs. veal) was significant at every time point ($P<0.001$). At the age of 26 weeks, the percentage of animals with alpha toxin antibodies was significantly higher in beef (85%) than in veal calves (16%).

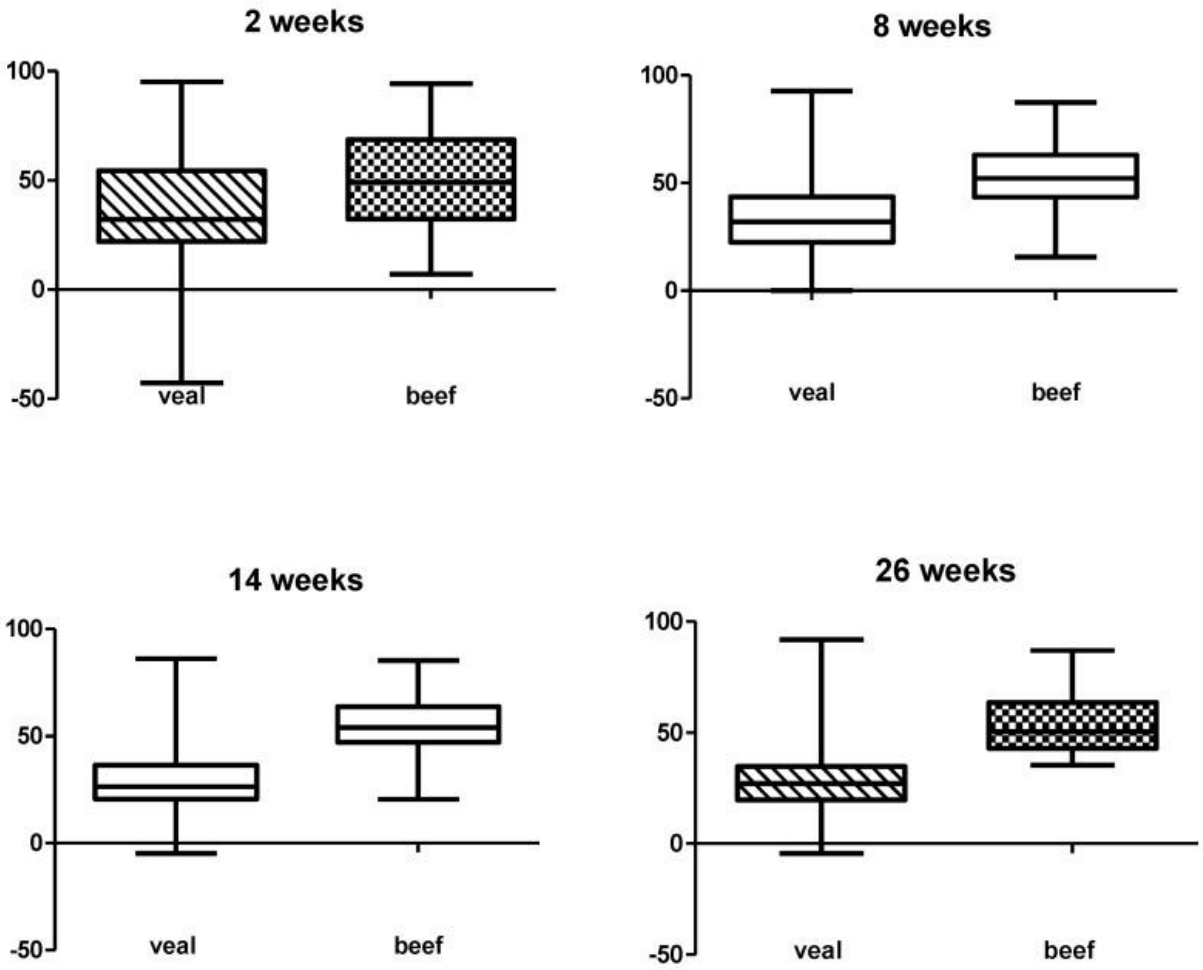

Figure 6.1 Results of the *Clostridium perfringens* alpha toxin antibody blocking ELISA in study 1, presented as the mean percentage inhibition of the OD for veal and beef calves at every sampled age.

Within the group of veal calves, there was no overall significant effect of breed on % inhibition of OD. At the age of 8 and 14 weeks crossbred calves had significantly higher % inhibition of OD (more antibodies) than HF calves ($P<0.01$).

Table 6.2. Results of the *C. perfringens* alpha toxin antibody blocking ELISA

		Mean % inhibition of OD ± standard deviation				Seroprevalence ± standard deviation			
		Age (in weeks)				Age (in weeks)			
	n	2	8	14	26	2	8	14	26
Veal total	443	[a]43±21	[b]35±17	[c]30±15	[c]30±15	46±13	29±14	17±13	16±9
Veal cross	167	[d]45±22	[e]40±18	[f]35±15	[f]33±15	49±18	45±11	28±12	20±12
Veal HF	121	[g]40±21	[h]29±12	[h]23±10	[h]29±17	43±10	17±4	6±10	17±8
Veal BB	155	[i]42*±20	[j]34*±17	[k]29*±24	[k]26*±13	45±13	26±10	16±4	9±6
Beef BB	85	[l]51*±23	[l]52*±16	[l]55*±13	[l]53*±12	66±40	76±28	91±8	85±14
All calves	528	[m]44±21	[n]37±18	[n]32±16	[n]33±17	44±22	37±18	32±16	33±17

Abbreviations: BB= Belgian Blue, HF= Holstein Friesian, cross= crossbred, OD= optical density

Remarks: Statistic analyses were performed on the Mean % inhibition of OD. Values indicated with * are significantly different ($P<0.05$) between veal and beef Belgian Blue calves at a given time point (columns). Within subjects effect was significant in the veal groups. Values with a different letter are significantly different over time within the same subject (rows) ($P<0.05$). A calf was considered positive when the % inhibition of the OD was higher than 40, as was suggested by the test producer.

PERFRINGOLYSIN ANTIBODY DYNAMICS

Results are summarized in figure 6.2. Perfringolysin antibodies decreased significantly between the age of two and eight weeks (P<0.01) in beef calves and between the age of two and fourteen weeks in veal calves. Veal calves had significantly higher perfringolysin antibody titers than beef calves at the age of 8 weeks and at the age of 26 weeks (P<0.01).

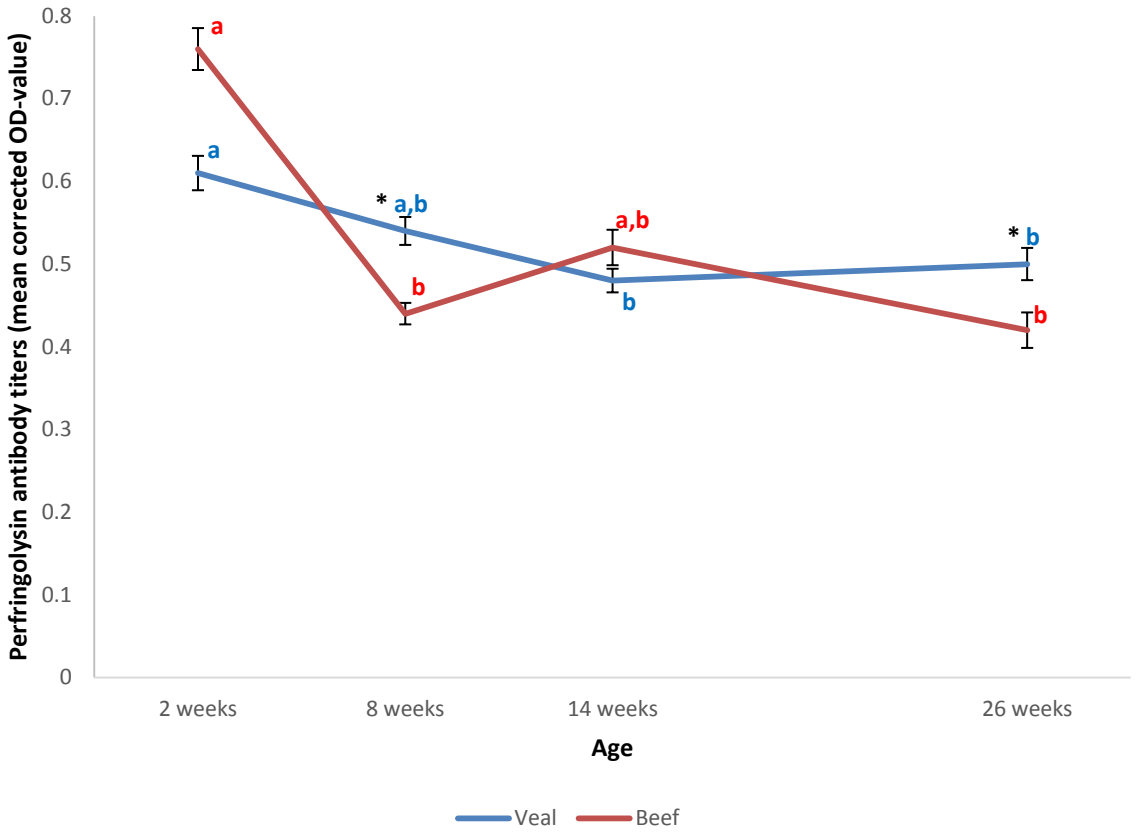

Figure 6.2: C*lostridium perfringens*' perfringolysin antibody dynamics in 153 Belgian Blue veal and 84 beef calves. Values indicated with * are significantly different (*P*<0.05) between veal and beef at a given time point. Within subjects effect was significant in both groups. Values with a different letter are significantly different within subject over time (*P*<0.05). Error bars represent standard error of the mean.

Remark: The OD-value was corrected by substracting the OD-value of the negative control from the OD-value of the test sample.

STUDY 2: EFFECT OF SOLID FEED PROVISION ON ALPHA TOXIN AND PERFRINGOLYSIN ANTIBODY DYNAMICS

MORTALITY, INCIDENCE OF ENTEROTOXAEMIA AND AVERAGE DAILY GAIN

There was no morbidity during the trial, so no calves had to be excluded from the experiments; nor were there any fatalities or enterotoxaemia cases.

Average body weight at two weeks of age was 53 ± 6 kg in group 1 and 52 ± 3 kg in group 2. Average daily gain was significantly higher in the calves that received SF (836 ± 118 g vs. 1002 ± 120 g; $P<0.05$, two way ANOVA).

ALPHA TOXIN AND PERFRINGOLYSIN ANTIBODY DYNAMICS

There were no significant differences in alpha toxin and perfringolysin antibody levels between both groups (table 6.3 and figure 6.3). For alpha toxin, antibodies were high in both groups during the whole trial, and the overall seroprevalence (% of calves with a % of inhibition of OD>40) was 100%. For perfringolysin, there was a significant decrease in antibody titers between the age of 2 weeks and the age of 8 weeks in both groups ($P<0.05$).

Table 6.3. Results of study 2: effect of solid feed provision

Alpha toxin serology (% inhibition of OD ± standard deviation)

	Age in weeks			
	2	**8**	**14**	**26**
Group 1 (MR)	72±14	67±11	67±8	70±5
Group 2 (MR+SF)	64±13	66±8	68±6	67±5
Total	68±14	66±10	68±7	69±5

Group 1 was fed with only milk replacer, while group 2 received milk replacer and solid feed. There were no significant differences between groups or within a group between time points.

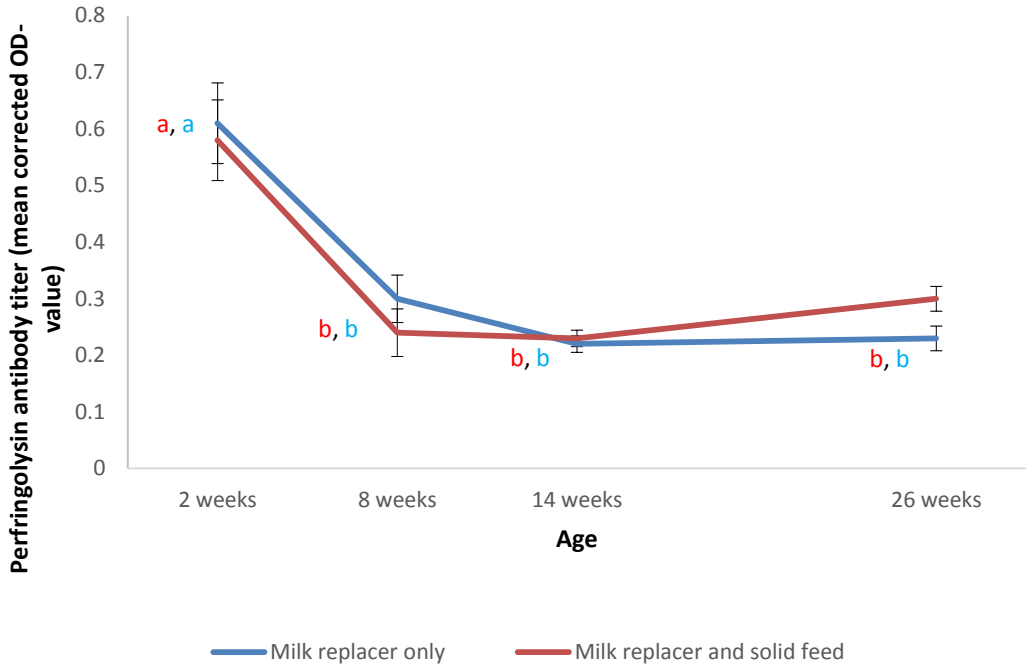

Figure 6.3. Dynamics of *Clostridium perfringens*' perfringolysin antibody titers for Holstein Friesian veal calves fed only with milk replacer or with milk replacer and solid feed. Within subjects effect was significant in both groups. Values with a different letter are significantly different within subject over time ($P<0.05$). Error bars represent standard error of the mean.

DISCUSSION

To be able to protect calves from enterotoxaemia by vaccination, it is crucial to obtain insights in the presence and decline of maternal antibodies against the toxins included in the vaccine (Veschi *et al.*, 2008). In the present study, maternal antibodies against alpha toxin were common in calves destined for beef and veal production, as has also been described for epsilon toxin in goats (Veschi *et al*., 2008). In veal calves, there was a significant decrease in antibodies against alpha toxin until the age of 14 weeks and the antibody levels remained low until the end of the study when the calves were 26 weeks of age. At the age of 14 weeks, only 17% of the veal calves were seropositive for alpha toxin, possibly indicating that, from that moment on, the majority of these animals could have been vaccinated with alpha toxin-based vaccines without interference with maternally derived antibodies. Since in veal calves, enterotoxaemia mainly occurs from the age of 20 weeks onward, it might be possible to immunize them prior to the period of greatest risk, using such vaccines. Further studies, including field trials and protection studies in experimentally infected calves are, however, necessary to try and develop efficient vaccination schedules.

We hypothesized that differences exist between veal and beef calves in dynamics of alpha toxin and perfringolysin antibodies, which might help to explain the higher incidence of enterotoxaemia in veal calves (Pardon *et al.*, 2012). This hypothesis was confirmed for alpha toxin. Indeed, only 5% of the veal calves, raised with the traditional all-liquid diet, seroconverted for alpha toxin and at the age of 26 weeks, only 16% of the veal calves were seropositive for alpha toxin, as opposed to 85% of the beef calves at the same age. Based on the absence of a breed effect within the same nutritional management (= veal), it was hypothesized that the predisposition of veal calves might be associated with the diet rather than the breed. One possible explanation might be the effect of the diet on the production of alpha toxin by *C. perfringens* in the gastro-intestinal tract. Prolonged production of low amounts of toxin in the gastro-intestinal might be influenced by dietary factors and may lead to seroconversion and possibly protection against enterotoxaemia. This has also been suggested for acquisition of antibodies against tetanus toxin and *C. perfringens* epsilon toxin (Dastur *et al.*, 1981; Ehrengut *et al.*, 1983; Veronesi *et al.*, 1983; Matzkin and Regev, 1984; Haghighi *et al.*, 2006; Veschi *et al.*, 2008).

Our hypothesis was not confirmed for perfringolysin antibodies, since at the ages of 8 and 26 weeks, veal calves had significantly higher antibody titers against this toxin than beef calves. It has been shown that alpha toxin and perfringolysin act synergistically in the development of necrohemorrhagic enteritis in a calf intestinal loop model, possibly by targeting the endothelial cells (Verherstraeten *et al*., 2014). The relative importance of antibodies against both toxins for protection requires further study.

Since the most striking difference between the diet in veal and beef calves is the availability of SF, we studied the influence of SF provision. Unfortunately, we encountered several unexpected observations which made the interpretation of this trial more difficult. First, using the same ELISA, all of the studied calves had high levels of alpha toxin antibodies at the age of two weeks, in contrast to the field observations made between 2009 and 2011. A straightforward explanation was not found. It is possible that differences in colostrum provision or in the presence of antibodies against alpha toxin of the dams may have played a role. Secondly, in contrast to the first study, the veal calves on the all-liquid diet produced alpha toxin antibodies. Differences in MR composition may help to explain this observation. At the time of the first study (2009–2011), veal calves were predominantly fed with high amounts of highly concentrated MR. The second study was conducted five years later, and alternative feeding regimes claiming improved animal welfare had then become common practice in the local veal industry. The current MRs, including the product used in our second study, contain lower concentrations of whey proteins than MRs generally used at the time of our first field study in veal calves. Moreover, the current feeding regimes also provide lower amounts of MR compared to the previous regimes. Contact with milk protein has been observed to decrease alpha toxin production by *C. perfringens*, which may influence antibody production against this toxin (Adams *et al.*, 1947, confirmed by unpublished data). Since the provision of SF did not influence the antibody dynamics of *C. perfringens* alpha toxin and perfringolysin, differences in quantity and quality of MR used in veal calves compared to beef calves might lead to different exposure to alpha toxin, possibly explaining the difference in antibody production observed in both studies. Further research is required to unravel the dietary risk factors for enterotoxaemia (quantity, concentration and composition of both MR and SF), in order to be able to prevent the disease through nutritional management.

CONCLUSION

This study explored antibody dynamics for alpha toxin and perfringolysin in beef and veal calves. A clear difference in the antibody production for alpha toxin, but not for perfringolysin, was observed between veal and beef calves. In beef calves a fluent transition from maternal to active immunity for alpha toxin was observed, whereas in veal calves on a traditional all-liquid diet a significant decline of alpha antibodies was noted. Breed did not influence the antibody dynamics. These observations indicate that dynamics of alpha toxin antibodies may influence the occurrence of enterotoxaemia in calves.

CHAPTER 7

GENERAL DISCUSSION

This thesis provided new insights in the involvement of CPA in both abomasal ulcerations as in enterotoxaemia, and in the influence of diet in the pathogenesis of enterotoxaemia. The most important conclusions and their implications are listed below.

NEW INSIGHTS IN THE INVOLVEMENT OF CLOSTRIDIUM PERFRINGENS TYPE A IN ABOMASAL ULCERATIONS IN VEAL CALVES

C. perfringens was not associated with abomasal ulcerations in veal calves in this study. Nevertheless, Van Immerseel *et al.* (2010) isolated a clonal population CPA from a beef calf with a diffuse ulcerative abomasitis and haemorrhagic enteritis. The diet has an important influence on the pathogenesis of abomasal ulcerations, and conclusions made for the veal population might not be valid for beef calves. It can also not be excluded that, when abomasal lesions are present, caused by dietary, environmental or infectious factors different from *C. perfringens*, an environment is created which favours this bacterium. Bleeding ulcerations lead to the availability of serum proteins, predisposing for clostridial overgrowth (Annett *et al.*, 2002), explaining the presence of high clostridial counts near ulcerative or haemorrhagic lesions. The study described in chapter 3 did not check for clonality. However, even the presence of clonality does not prove causality. Some *C. perfringens* strains can produce growth-inhibiting proteins, leading to intra-species growth-inhibition (Timbermont *et al.,* 2011), presumably predisposing for clonal overgrowth of the growth-inhibiting protein producing strains in certain cases.

DIFFICULTIES IN STUDYING ENTEROTOXAEMIA

It is important to understand the limitations and difficulties of studying a rare event disease caused by an ubiquitous pathogen, as is the case for enterotoxaemia. This heading provides information necessary to understand the selected methods.

ENTEROTOXAEMIA IS A RARE EVENT DISEASE CAUSED BY AN UBIQUITOUS PATHOGEN

C. perfringens is ubiquitous in the environment and part of the intestinal microbiota of most animals. However, the associated diseases are relatively rare, and often require complex interactions between different predisposing environmental factors, strain dependent virulence factors and the immunity of the animals. This thesis focused predominantly on enterotoxaemia in calves, where the enterotoxaemia incidence varies strongly between the different populations. In Belgian white veal calves the mortality risk due to enterotoxaemia within a production round (on average 200 days) was estimated at 0.5% from the calves at risk, with large differences between HF (0.2%) and BB veal calves (1.3%) (Pardon *et al.*, 2012). There are no scientific data available about the incidence of the disease in the other production systems, but empirically, enterotoxaemia is thought to be extremely rare in weaned calves and dairy calves. In BB suckler calves, the disease is occasionally seen, but not nearly as often as in BB veal calves. Also, in other comparable populations, such as the north-american feedlots, the incidence of enterotoxaemia is generally a lot lower than in veal calves (Glock and DeGroot, 1998). Allthough morbidity is low (about 1,3% in BB veal calves), mortality rate in diseased animals is close to a 100% and enterotoxaemia stays the second most important cause of mortality in BB veal calves. Therefore, the economic losses are very large, especially because the disease affects the most valuable, heaviest, fastest growing animals. Despite the obvious financial losses, compared to other diseases, enteroxaemia has to be regarded as a rare event pathology, which has important consequences for the method by which this disease can be studied. As an example for a rare event pathology, there has been elaborative research and documentation on how to deal with epidemiosurveillance of brucellosis (Saegerman, 2005). Similar to enterotoxaemia, caused by *C. perfringens*, brucellosis is caused by a bacterium, namely *Brucella abortus*. However, the two diseases demand a completely different approach. For brucellosis, both the disease and the causative agent are extremely rare, while for enterotoxaemia

only the disease is rare, and the causative agent (*C. perfringens*) is a commensal in the intestinal microbiota. This means that for brucellosis, surveillance is possible by detecting contact with *Brucella abortus* by, for example, serological testing. In contrast, it is not possible to use the same techniques for studying the epidemiology of enterotoxaemia. Detection of *C. perfringens* via culture or PCR does not give information on the incidence of enterotoxaemia. Commercial ELISA kits are available for the detection of antibodies against *C. perfringens* alpha toxin (Bio-X, Jemelle, Belgium). Although the presence of antibodies against alpha toxin does prove contact with *C. perfringens*, it can also not be used as a measure of the incidence of enterotoxaemia. To be able to gather correct data about the incidence of a disease, a reliable and easy diagnostic tool is needed.

PROBLEMS WITH DIAGNOSIS

Although the symptoms and lesions are rather typical (sudden death with haemorrhagic intestines), the disease is most likely overdiagnosed when a structured necropsy is not performed (Vanneste *et al.,* 2013). Differential diagnosis of sudden death in veal calves includes acute or chronic pneumonia, cardiac problems, perforated abomasal ulcerations and mineral disturbances (hypomagnesemia, iron intoxication) (Glock and DeGroot, 1998; Pardon *et al.,* 2012). As described in the first chapter, at present there are no reliable diagnostic techniques available, complicating surveillance studies on this disease.

In practice, confirmation of a clinical suspicion of enterotoxaemia was predominantly done by clostridial counts of the content of affected intestinal segments. When conducting studies on commercial farms, it is difficult to obtain good negative control groups for comparative studies with enterotoxaemia-cases. It is not ethical nor economically feasible to euthanize and sample healthy animals in the same environment, on the same feed and of the same age as the affected animals. Alternatively, slaughterhouse samples can be used. However, these animals are older, as they have reached the end of the production cycle, and fasted in the hours before slaughter. Another possibility is the use of deceased or euthanized animals without gastro-intestinal problems (for example calves with pneumonia). However, these animals are prone to intestinal stasis and changes in feed intake caused by general

illness, or might be subject to antimicrobial therapy. This hampers the interpretation of comparative studies between enterotoxaemia-cases and control calves.

PROBLEMS WITH PATHOGENESIS STUDIES

At the start of this PhD study, there were no models available to induce enterotoxaemia in calves. The disease is rare and only occurs in combination with very specific predisposing conditions, which are only known to a limited extent. Therefore, developing a model is challenging. It is important to gain further insight in the predisposing factors in order to facilitate this process. Similarly, the assessment of different preventive measures is impeded for this reason. In diseases where the incidence is high, field studies are often conducted, in which the incidence of the treated group is compared, depending on the study design, to itself before and after treatment, or to a comparable control group (Phillipe *et al.*, 2014; Person *et al.*, 2015). However, for rare event diseases, in order to get statistically significant differences between the incidence of the two populations, a very high number of calves would be needed.

In the field, multiple pre- and probiotic supplements are marketed by different players. Required sample sizes to evidence or contradict the effects of these products on mortality due to enterotoxaemia are very large. Also for preventive measurements such as vaccination or feed adaptations this issue exists. Alternatively, *in vitro*-tests or *in vivo* models can be used, such as the intestinal loop model described in chapter 6. The used methods in this thesis and their limitations have led to the following new insights in the pathogenesis of enterotoxaemia.

NEW INSIGHTS IN THE PATHOGENESIS OF CPA IN ENTEROTOXAEMIA IN CALVES

ASSOCIATION OF CLOSTRIDIAL COUNTS AND ENTEROTOXAEMIA

Intestinal clostridial counts were not suitable as a diagnostic technique in enterotoxaemia, most importantly due to the rapid post-mortem multiplication of *C. perfringens*. Also, the variability of intestinal clostridial counts was very high. Both in enterotoxaemia cases and in control cases, intestinal counts ranged from negative to more than 10^8 cfu/g intestinal content. There are numerous possible reasons for negative results in the enterotoxaemia-cases. In contrast to the study conducted by Manteca *et al.*, 2007, the study described in chapter 4.1 only included calves in which the time of death was certain, therefore, a lot of the included calves were found premortem with symptoms of colic and shock, indicating enterotoxaemia. Some of these calves might have been treated with antibiotics in an ultimate attempt to treat the disease, leading to negative *in vitro* cultures. Also, unsuitable preservation or cultivation might have led to negative results. However, despite the overlap between groups, *C. perfringens* counts of the contents of a haemorrhagic jejunal segment of calves diagnosed with enterotoxaemia (test group) were only slightly higher compared to the matching segment of calves in the control group. The difference was not significant, and therefore, an association between intestinal clostridial counts and enterotoxaemia could not be proven. However, only a limited number of calves could be included, due to reasons of matching time of death and diet, and moreover, the control group consisted of sick calves. These calves might also have been treated with antibiotics, had decreased feed intakes, or developed intestinal ileus caused by their general illness. All these factors can have an influence on the clostridial counts, potentially biasing the detection of a possible association between clostridial counts and enterotoxaemia in this study. In conclusion, under practical circumstances clostridial counts are of no use due to the large variation and rapid post-mortem overgrowth. Therefore, a reliable model was needed in order to study the pathogenesis of enterotoxaemia, and the association with CPA.

INVOLVEMENT OF CPA IN ENTEROTOXAEMIA

In order to further study the pathogenesis of enterotoxaemia, an intestinal loop model was developed as described in chapter 5. CPA induced necro-haemorrhagic lesions, similar to the lesions observed in natural enterotoxaemia cases. Also, identical strains could be isolated from the lesions, fulfilling the third and fourth of Koch's postulates. Therefore, using this intestinal loop model, we could conclude that CPA was associated with enterotoxaemia.

Moreover, this model made it possible to screen the role of different environmental conditions and bacterial strains for their lesion-inducing potency. Incubation of numerous strains from different origin and toxinotypes induced similar necro-haemorrhagic lesions. This indicates a shared virulence factor, and therefore points to chromosome linked toxins, as alpha toxin and perfringolysin (Hatheway, 1986). No differences were observed between the necrosis-inducing potency of both β2-positive and negative strains. Therefore, in contradiction with earlier literature, β2-toxin seems to be not or of secondary importance in the pathogenesis of the necro-haemorrhagic lesions in the intestinal loop model (Lebrun *et al.,* 2007).

Additionally, the time course experiments indicated that the primary lesions in the pathogenesis of necrohaemorrhagic enteritis were loss of epithelium and congestion of the capillaries, both present within 30 minutes after incubation with a strain isolated from an enterotoxaemia case. This is followed shortly by necrosis of the intestinal villi and haemorrhages. These observations suggest that both intestinal epithelium and endothelium are primary targets in the initial stage of enterotoxaemia, either as a target for a bacterial toxin or for another virulence factor, or target for other predisposing factors (as is for example the case for epithelial damage by coccidiosis in broiler chickens). In contrast to other *C. perfringens* associated diseases in other host species (Timbermont *et al.*, 2009; Gurtner *et al.*, 2010), all isolates were capable of inducing necrohaemorrhagic lesions when injected in combination with MR, independent of their origin. A possible explanation for this phenomenon is that the causative toxin for enterotoxaemia in calves is present in many, if not all, *C. perfringens* isolates, and that the development of bovine enterotoxaemia is determined by environmental triggers, such as nutrition.

NEW INSIGHTS IN THE ROLE OF FEED MANAGEMENT IN THE PATHOGENESIS OF ENTEROTOXAEMIA IN VEAL CALVES

The impact of feed is an essential point in the unravelment of the pathogenesis of enterotoxaemia. Previous studies have suggested that protein- and energy-rich diets predispose for this disease (Lebrun *et al.,* 2010). In cattle, changes in diet or pasture are often noted 24 to 36 hours prior to death by enterotoxaemia (Manteca *et al.,* 1999). Despite the fact that a predisposing role of feed on enterotoxaemia is generally accepted, there are no studies focussing on the exact role of feed in the pathogenesis of this disease, and the interaction with *C. perfringens* in the intestinal micro-environment. The studies described in this thesis did not examine a direct effect of nutrition on the pathogenesis of enterotoxaemia. Therefore, the information on the impact of feed on the pathogenesis of enterotoxaemia derived from this thesis is limited to the predisposing effect of MR in the intestinal loop model (chapter 5) and the observation that within the same breed (BB), veal calves produce less antibodies against *C. perfringens* alpha toxin than beef calves. This effect was not due to a difference in the provision of SF (chapter 6). In succession to this information, the effect of SF provision on the faecal excretion of *C. perfringens* was studied. Supplying SF decreases the faecal clostridial excretion in veal calves (unpublished data). The mechanism behind this effect is not completely understood. Veal calves fed exclusively MR only develop a rudimental rumen (Suarez *et al.*, 2007). In veal calves offered roughage, even a limited amount of 300g per feeding induces relevant ruminal development and an active ruminal function (Webb *et al.*, 2013). This might lead to an intestinal environment less favorable for Clostridia, possibly by favoring bacteria with a direct *C. perfringens* growth-inhibiting effect. Although the association between FCCs and enterotoxaemia risk remains to be determined in calves, the provision of SF to veal calves might be a useful preventive tool against enterotoxaemia. This opens perspectives for further dietary management of the disease. It remains to be determined whether the composition of the SF can be optimized in order to reduce FCC's even further. The impact of feed on clostridial disease is best studied in necrotic enteritis in broiler chickens, a disease with important analogies with enterotoxaemia in calves. The following paragraphs describe the effect of the different feed components on *C. perfringens* and the current knowledge on expected influences on the pathogenesis of enterotoxaemia.

ROLE OF DIFFERENT NUTRIENTS

ROLE OF PROTEIN

C. perfringens is lacking many genes necessary for amino acid biosynthesis. Therefore, this bacterium can not grow in an environment where amino acid supply is limited (Shimizu *et al.*, 2002). Feeding a protein-rich diet induced an increased ileal and caecal concentration of *C. perfringens* in broiler chickens (Drew *et al.*, 2004). The protein source seems to influence the effect of a high dietary protein level. Also, in chickens, feeding a fishmeal-based diet stimulates the intestinal *C. perfringens* overgrowth, and subsequently predisposes to necrotic enteritis (Drew *et al.,* 2004; Kaldusdhal *et al.,* 1996; Shojadoost *et al.,* 2012). Fishmeal is characterized by a higher level of the amino acids glycine and methionine compared to soy protein concentrate, positively correlated with the *C. perfringens* population in the intestine (Drew *et al.,* 2004). It is suggested that not only the level of individual amino acids but also the balance of amino acids is important for maximum growth of *C. perfringens* (Fuchs and Bonde, 1957). In veal diets, similar to fish meal in broiler diets, the whey present in MR contains high quality, readily available amino-acids, potentially predisposing for clostridial overgrowth.

The predisposing effect might also be associated with an effect on the bacterial toxin production. Contact of *C. perfringens* with certain components might increase or decrease the production of specific toxins. For example, the presence of casein in culture media decreases alpha toxin activity in the supernatant (Adams, 1947). Therefore, one might hypothesise that the presence of skimmed milk powder in the MR containing casein could possibly lead to a decreased expression of alpha toxin in the intestinal lumen. If this is true, calves fed skimmed milk powder might have less contact with alpha toxin, leading to the absence of active immunity, and thus potentially leaving the calves unprotected against enterotoxaemia. However, in chapter 6, there was no difference in antibodies against alpha toxin between veal calves from different breeds, while the BB veal calves were fed predominantly MR containing skimmed milk powder, and the HF veal calves were fed predominantly with nill product, without skimmed milk powder or casein. Nevertheless, a similar effect on toxin production might exist for whey proteins, present in both skimmed milk powder and nill product. This should be further investigated and could explain the lack of difference in antibody dynamics of *C. perfringens* toxins between BB and HF veal calves, fed diets with and without casein.

Indeed, when *C. perfringens* is cultured in the presence of MR, this does not alter the bacterial growth curves, and yet both protein expression, and alpha toxin activity of the supernatant are significantly lower compared to a negative control cultured without MR (Goossens 2012, unpublished data).

In conclusion, the role of (milk) protein in the pathogenesis of enterotoxaemia seems controversial. On one hand milk protein might predispose for enterotoxaemia by facilitating clostridial overgrowth, leading to toxin production and necro-haemorrhagic enteritis. On the other hand, milk protein might predispose for enterotoxaemia by limiting basal contact with toxins, leading to the absence of antibodies, and leaving the calves unprotected.

ROLE OF DIGESTIBLE CARBOHYDRATES

High dietary levels of digestible carbohydrates that exceed the digestion and absorption capacity of the intestinal mucosa can be utilized by *C. perfringens* to proliferate (Allaart *et al.,* 2013), because the bacterium can produce a variety of carbohydrate degrading enzymes (Shimizu *et al.,* 2002). An increased growth of *C. perfringens* was observed *in vitro* by adding digestible carbohydrates to the growth medium, including lactose (Fuchs and Bonde, 1957; Labbe and Duncan, 1975). Since MR contains high amounts of easily digestible lactose, a predisposing effect on clostridial overgrowth is to be expected.

ROLE OF NON-STARCH POLYSACCHARIDES

In cattle, high fiber diets are often believed to protect from gastro-intestinal disease, and in practice the provision of hulled wheat, containing up to 10% fiber, is believed to protect against enterotoxaemia in calves. Indeed, in contrast to digestible carbohydrates, the addition of indigestible carbohydrates such as raffinose or cellobiose did not increase the *C. perfringens* growth rate (Allaart *et al.*, 2013; Fuchs and Bonde, 1957; Labbe and Duncan, 1975; Sakurai and Duncan, 1979). However, in broiler chickens, diets with high levels of indigestible, water-soluble non-starch polysaccharides (NSP) increase the viscosity of the digesta and are a risk factor for necrotic enteritis. The increased intestinal viscosity can prolong the intestinal transit time associated with a

greater intestinal clostridial count (Annett *et al.*, 2002). Besides, NSP also interact with glycoproteins on the epithelial surface to increase mucin production, and thus indirectly stimulate *C. perfringens* proliferation (Sjojadoost *et al.*, 2012; Kleessen *et al.,* 2003). This is in contrast to the observation that SF provision to veal calves lowers the faecal clostridial excretion (unpublished data). However, based on the results presented in chapter 6, it can be concluded that an increase in SF provision does not influence the humoral immunity against clostridial toxins. The provision of (large amounts of) MR (without fibres) seems to be a more important risk factor than the absence of SF (containing high fibre). This is suggested by the results of chapter 5, in which MR strongly predisposes for necrotic lesions in an intestinal loop model, and of chapter 6, in which the development of antibodies against alpha toxin or perfringolysin was not influenced by the provision of forage rich SFs.

ROLE OF OTHER NUTRITIONAL FACTORS

Other nutritional factors can also influence the intestinal proliferation of *C. perfringens* and predispose for enterotoxaemia. For example, the type of fat source is likely to indirectly influence the intestinal microbiota. It was demonstrated that dietary fat of animal origin increases intestinal *C. perfringens* count compared to vegetable oil in broiler chickens (Knarreborg *et al.,* 2002). There are no studies determining the effect of fat on enterotoxaemia in calves.

Trypsin inhibition is also a well-established predisposing factor to necrotic enteritis, since trypsin induces cleavage of *C. perfringens* toxins in the small intestine (Allaart *et al.*, 2013; Sato *et al.,* 1978). The ability of trypsin to degrade alpha toxin is for example reduced by a high dietary level of zinc and the trypsin inhibitor activity of potato protein concentrate, and of the trypsin inhibitor activity in soy beans (Sato *et al.,* 1987, Baba *et al.*, 1992), two likely components of commercial MRs.

In addition, empirically more enterotoxaemia is observed when calves have been deprived of water for some time, and then drink large amounts of water in a short period of time. A potential explanation for this observation is the dilatation of the abomasum, and dilution of the available proteases. When a calf drinks milk or MR short after the ingestion of large amounts of water, this might hypothetically lead to

inadequate predigestion of proteins, potentially leading to free proteins in the lumen of the small intestine available for clostridial overgrowth.

In conclusion, the role of feed in the pathogenesis of enterotoxaemia can not be attributed to one specific factor, but to a very complex interaction of influences from a broad variety of feed components.

ROLE OF INTESTINAL HOMEOSTASIS

Enterotoxaemia is more frequently observed in veal calves and suckler calves, and also neonatal calves are prone to neonatal haemorrhagic enteritis and abomasitis (Griner and Bracken, 1953; Niilo *et al.*, 1974; Lebrun *et al.*, 2010; Pardon *et al.*, 2012; Garcia *et al.*, 2013). A common dietary factor in these populations is the high proportion of cow's milk or MR in the diet, supporting the hypothesis that this is an important risk factor for enterotoxaemia. On one hand, the hypothesis that contact with MR decreases toxin expression seems paradoxal with the observation that high MR diets predispose for enterotoxaemia. On the other hand, veal calves are often fed very large amounts of MR, up to more than 10L per feeding (Pardon *et al.,* 2010). This will lead to volume overload of the abomasum, which can only contain up to 7 liters of content in a 12 week old calf (Schmidt and Zsédely, 2011). Consequently, there might be an overflow of the abomasum, predominantly towards the rumen, predisposing for ruminal putrefaction and tympany, but more importantly, there might also be a limited overflow of unpredigested protein toward the intestinal lumen. An excess of free protein in the intestinal lumen can predispose for clostridial overgrowth.

However, it should be taken into account that when MR is ingested, some predigestion of the protein occurs in the abomasum under influence of pepsin and other gastric proteases, and no intact proteins enter the small intestine under natural circumstances. The concentration of casein in the MR and the pH of the MR both determine the ability to form a curd in the abomasum under the influence of rennin. When a casein curd is formed, this curd temporarily blocks the casein from pepsin digestion, leaving more pepsin for the digestion of whey protein. Therefore, MR which allows curd formation is more easily digested (Leary and Sheib, 1917). Nill products do not contain any skimmed milk powder, and therefore no casein, and do not allow curd formation. This might lead

to an enhanced probability of leaking unpredigested protein into the small intestine. However, enterotoxaemia is seen more in BB veal calves, where MR are used which do contain casein, and less in HF veal calves, which are fed exclusively with nill product. Therefore, the effect of curd formation and predigestion is likely subordinate to other predisposing effects.

In suckler calves, farmers report a peak in enterotoxaemia when dams are moved from the stable to the pasture (Manteca *et al.,* 1999; Lebrun *et al.*, 2010). A possible explanation for this observation is the change in composition of the cow's milk in response to differences in cow feeding between the indoor and outdoor period (Lerch *et al.,* 2012). Also the relocation itself can provoke stress in both cow and calf, leading to irregular feeding and potentially intestinal ileus. A similar situation is routinely created in veal calves by feeding large amounts of MR, only twice daily. In the period between feedings, the gastro-intestinal tract is relatively empty and deprived of nutrients. Immediately after feeding, due to reasons mentioned above, there is an excess of nutrient in the intestinal lumen. This diurnal variation might create an unstable intestinal microbiota, which could potentially predispose for enterotoxaemia. The implementation of more frequent, smaller feedings, may help to reduce this risk. In this perspective, the use of automated calf feeding stations could be an adequate preventive measure for enterotoxaemia. However, these automated stations are associated with an increased risk of repiratory disease and a reduced growth rate (Wolfger *et al.*, 2015). Moreover, the system is difficult to disinfect, and the biofilm present in used teats might increase the oral ingestion of clostridial spores, potentially even predisposing for enterotoxaemia.

Gastro-intestinal stasis, as can be induced by a broad variety of nutritional and infectious gastro-intestinal disorders, decreases the flushing effect of bacteria and nutrients, and can contribute to bacterial (clostridial) overgrowth (Wernery *et al.,* 1991; Manteca *et al.,* 2004). Stasis is simulated in the intestinal loop model described in chapter 5. In these experiments necro-haemorrhagic lesions could only be induced by injecting intact bacteria. The injection of high doses of supernatant of *C. perfringens* cultures was not capable of inducing lesion. Also, clusters of large rods, presumably *Clostridia*, could be found associated with the necrotic lesions, suggesting a role of the vegetative bacteria next to the produced exotoxins. The association of the bacteria with

the affected mucosae might be an explanation for the segmental aspect of the lesions, often observed in enterotoxaemia. Remarkably, *C. perfringens* counts are higher at the location of the lesions than more proximal or more distal in the intestine (Manteca *et al.,* 2001). Clostridial overgrowth is influenced by many factors, such as competition and bacteriocin production from other intestinal bacteria, nutrient availability, or even the presence of antibodies (Niilo, 1986; Wernery *et al.,* 1991; Geeraerts *et al.,* 2015). A favourable micro-environment is needed for growth and toxin production. The creation of such micro-environment is subject to many external factors, including among others diet, use of certain feed additives, use of antibiotics, presence of enteritis, or stasis. Once a favourable micro-environment is created, this can lead to local necrohaemorrhagic intestinal lesions. This process can become more generalized in reaction to the haemorrhages leading to massive availability of amino acids, predisposing for clostridial overgrowth (Niilo, 1986). The segmental aspect of the disease and concomitant differences in clostridial counts contribute to the difficulties in diagnosis based on quantification of *C. perfringens* in intestinal content, as described earlier in this discussion.

CONCLUSIONS

In conclusion, the primary new insight into the pathogenesis of abomasal infection in veal calves was the observation that *C. perfringens* does not have a primary role in the development of fundic abomasal ulcerations in veal calves.

Apart from the role of *C. perfringens* in fundic abomasal ulcerations, the relation between *C. perfringens* and enterotoxaemia was studied. This thesis revealed that intestinal clostridial counts, which are currently used as a diagnostic tool for enterotoxaemia, are not suitable due to the large variation. In order to develop reliable diagnostic tools, knowledge of the causative toxins is essential. Therefore, an intestinal loop model was developed, which allowed the *in vitro* study of enterotoxaemia. The intestinal loop experiments revealed an important predisposing role for MR in the development of enterotoxaemia-like lesions after inoculation of *C. perfringens*. Moreover, the intestinal loop experiments demonstrated that the pathogenicity of a strain was independent of the origin of the strain, suggesting a universal virulence factor. Since it is not the virulence of the strain that determines occurrence of the disease, this thesis looked into other factors potentially determining the enterotoxaemia risk, such as the immunity of the host. This thesis concludes that veal calves have significantly lower alpha toxin antibody levels compared to beef calves in the risk period for enterotoxaemia, potentially explaining the difference in enterotoxaemia susceptibility. This difference could not be attributed to a difference in SF intake between veal and beef calves.

FUTURE PROSPECTS

This thesis provides the basics of preventive strategies against enterotoxaemia, enabling both better vaccine development and nutritional management.

The availability of an achievable *in vitro* model for enterotoxaemia opens up the road for pathogenesis studies, in order to identify causative toxins. Knowledge on the causative toxins would be an important step towards the development of more efficient vaccines and reliable diagnostic techniques (i.e. toxin detection tests).

Besides the elucidation of the bacterial virulence factors, more attention should be given towards feed regimes that are protective against enterotoxaemia. More insights are needed in the exact role of the different feed components on both the development of immunity as on the establishment of microbial homeostasis. However, this thesis suggests that other factors than SF provision are important in the pathogenesis of enterotoxaemia in veal calves, and that MR predisposes for enterotoxaemia. Therefore, future research focus should be on the role of the composition, concentration and administration of milk powder on both host and *C. perfringens* growth and toxin production.

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SUMMARY

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Enterotoxaemia is one of the most frustrating diseases known to cattle farmers and their veterinarians. The disease typically strikes the most vivid, fast growing and therefore most valuable calves. The rapid disease progress and inevitable death leave both farmer and veterinarian helpless. Although the disease is described worldwide and in all breeds, predominantly beef type animals, such as Belgian Blues (BB), are affected. In **chapter 1.1** the structure and management of the Belgian veal industry is explained, with the focus on feed management.

Chapter 1.2 gives an overview of the diseases currently associated with *C. perfringens* in cattle. The main CPA associated diseases in veal calves are enterotoxaemia and abomasal ulcerations. However, the role of CPA in the development of these diseases remains to be determined. Despite a lot of empiric experience with the nutritional management of enterotoxaemia outbreaks in cattle, no evidence on what dietary factors elicit the disease is available.

Therefore, the overall objective of the present doctoral thesis was to gain new insights in the role of *C. perfringens* in haemorrhagic enteritis (enterotoxaemia) and abomasal ulceration in veal calves and in the pathogenesis of enterotoxaemia (**chapter 2**).

The study described in **Chapter 3** determines the prevalence of fundic ulcerations in common veal production systems and the association with *Clostridia*. The prevalence of fundic ulcerations was estimated at 11% in dairy veal. BB veal calves tended towards a higher prevalence of fundic ulcerations compared to dairy veal, most likely due to dietary differences (higher animal protein/vegetable protein ratio in MR for BB calves), although a breed effect (e.g. susceptibility to stress) cannot be excluded. Since no *Helicobacter spp*. could be detected in the lesions and there was no difference between the presence of *C. perfringens* in abomasa from healthy calves as compared to ulcerations, the role of these bacteria in the pathogenesis of fundic ulceration is presumed to be limited, if existing.

The aim of **Chapter 4** was to elucidate the association between intestinal *C. perfringens* counts and enterotoxaemia. Field necropsies were conducted on sudden death cases in BB veal farms. In 65% of suddenly deceased calves, the diagnosis of enterotoxaemia was

made based on haemorrhagic lesions in the small intestines, while in 15% of these cases, no clear-cut diagnosis could be made based on macroscopic appearance of the intestine. In the remaining calves, a definitive cause of death other than enterotoxaemia could be identified. Samples of the intestinal content were taken for quantification of *C. perfringens*. After matching cases and controls for diet, and the interval between death and sampling, no significant differences could be detected between the mean *C. perfringens* counts of the small intestines in enterotoxaemia cases and counts in the matching segments in the control group. These results indicate that intestinal *C. perfringens* counts cannot be advised as a discriminative post-mortem diagnostic tool for enterotoxaemia in veal calves, not even when sampled within three hours after death.

Chapter 5 describes the development of an intestinal loop model to study the pathogenesis of the disease. Loops were injected with *C. perfringens* cultures from different origin with, or without, a commercial MR. All tested isolates induced necrohaemorrhagic lesions in combination with MR, whereas all controls remained normal. In addition, time-course experiments were conducted using an isolate from an outbreak of bovine enterotoxaemia. Histological examination showed that lesion development was initiated by congestion of the capillaries, starting within 30 minutes after inoculation. Haemorrhages and mucosal necrosis started from the tips of the villi at 3 to 4 hours after bacterial inoculation. These lesions are similar to those observed in natural cases of bovine enterotoxaemia. It can be concluded that, in this model, necrohaemorrhagic lesions can be induced by *C. perfringens* isolates from diverse origins, suggesting that the lesions may be caused by the action of one or more virulence factors that are present in many *C. perfringens* isolates.

The intestinal loop model described in the previous paragraph contributed to the identification of *C. perfringens* alpha toxin and perfringolysin as primary toxins in the pathogenesis of enterotoxaemia. Insight in the prevalence and transition of maternal to acquired immunity for these toxins is essential for further clarification of the pathogenesis and for development of effective vaccination shaemes. In **chapter 6** a longitudinal cohort study to determine the dynamics of naturally acquired antibodies against alpha toxin and perfringolysin is presented. The effect of breed and production system (veal vs. beef) on the transition from maternal to acquired immunity

was investigated. In a second study, the potential effect of SF intake on the development of acquired antibodies was determined. Maternal antibodies for alpha toxin were present in 52% of the calves. In beef calves a fluent transition from maternal to active immunity for alpha toxin was observed, whereas in veal calves a significant decline of alpha toxin antibodies was noted. Perfringolysin antibodies significantly declined for both veal and beef calves. There was no meaningful breed effect on the antibody dynamics in veal calves. SF intake did not alter alpha toxin and perfringolysin dynamics in veal calves. In conclusion, the present study showed that veal calves have significantly lower alpha toxin antibody levels compared to beef calves in the risk period for enterotoxaemia. This difference could not be attributed to a difference in SF intake between veal and beef calves.

Chapter 7 summarizes the most important new insights into the pathogenesis of enterotoxaemia derived from this PhD-study, discusses them in the light of current scientific knowledge, and formulates future research prospects and practical implementation in preventive strategies. This thesis provides the basis for future preventive strategies against enterotoxaemia, consisting of vaccine development and nutritional management.

The availability of an achievable *in vitro* model for enterotoxaemia opens up the road for pathogenesis studies, in order to identify causative toxins. This would be an important step towards the development of more efficient vaccines and reliable diagnostic techniques (i.e. toxin detection tests). Besides this, more attention should be given to feed regimes protective for enterotoxaemia. More insights are needed in the exact role of the different feed components on both the development of immunity as on the establishment of microbial homeostasis. This thesis suggests that other factors than SF provision are important in the pathogenesis of enterotoxaemia in veal calves, and that MR predisposes for enterotoxaemia. Therefore, future research focus should be on the effect of the composition and concentration of milk powder on both host and pathogen.

SAMENVATTING

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Enterotoxemie is één van de meest frustrerende ziekten voor rundsveehouders en hun dierenartsen. De ziekte slaat vooral toe bij de levendigste, snelst groeiende en daarom ook meest waardevolle kalveren. De snelle ziekte-evolutie en onvermijdelijke sterfte leiden tot machteloosheid bij zowel veehouder als dierenarts. Hoewel de ziekte wereldwijd bij alle rassen voorkomt, treft ze voornamelijk vleesrunderen zoals het Belgisch Wit-Blauw rund (BWB). In **hoofdstuk 1.1** wordt de structuur en de bedrijfsvoering van de Belgische witvleeskalverindustrie besproken.

Hoofdstuk 1.2 geeft een overzicht van de ziektes bij runderen die met *C. perfringens* geassocieerd worden. Bij witvleeskalveren zijn voornamelijk lebmaagulcerations en enterotoxemie belangrijk. Het verband tussen *C. perfringens* en deze gastro-intestinale ziektes is echter nog niet bevestigd en er zijn geen causale toxines bekend. Op basis van empirische ervaringen wordt een te intensieve voeding bij BWB witvleeskalveren beschouwd als een belangrijke risicofactor voor enterotoxemie, maar desondanks is er geen duidelijke indicatie welke voedingsfactoren hiervoor verantwoordelijk zijn.

Daarom was de voornaamste doelstelling van deze doctoraatsthesis om nieuwe inzichten te krijgen in de rol van *C. perfringens* bij haemorragische enteritis (enterotoxemie) en lebmaagulcerations bij witvleeskalveren en in de pathogenese van enterotoxemie (**hoofdstuk 2**).

De studie die in **hoofdstuk 3** wordt beschreven bepaalde de prevalentie van fundusulcerations in de meest conventionele witvleeskalf-productiesystemen en de associatie van deze ulcerations met *Clostridia*. Het voorkomen van fundusulcerations werd bij melktype kalveren op 11% geschat. Bij BWB witvleeskalveren was er, in vergelijking met melktype kalveren, een trend naar een hogere prevalentie van de aanwezigheid van fundusulcerations. Dit kan verklaard worden door de belangrijke verschillen in de voeding (een hogere verhouding van dierlijk proteïne ten opzichte van plantaardige proteïne in de melkvervangers voor BWB kalveren). Desondanks kan ook het belang van rasgebonden kenmerken (b.v. gevoeligheid voor stress) niet uitgesloten worden. Er konden geen *Helicobacter spp.* gedetecteerd worden in de letsels en er was geen verschil in de aanwezigheid van *Clostridia* in de lebmagen van gezonde kalveren in

vergelijking met de aanwezigheid in lebmaagulcerations. De rol van deze bacterie in de pathogenese van fundusulcerations lijkt dus beperkt.

Het doel van **hoofdstuk 4** was om het verband tussen de telling van het aantal *Clostridia* in darminhoud en enterotoxemie te onderzoeken. Daartoe werden lijkschouwingen uitgevoerd binnen de 3 uur na sterfte bij acuut gestorven witvleeskalveren op praktijkbedrijven. In 65% van de plots gestorven kalveren werd de diagnose van enterotoxemie gesteld, gebaseerd op de aanwezigheid van een haemorragische enteritis, terwijl in 15% van de gevallen geen duidelijke diagnose kon worden gesteld gebaseerd op het macroscopische uitzicht van de darmen. In de overige kalveren werd een andere definitieve doodsoorzaak dan enterotoxemie geïdentificeerd. Het aantal *Clostridia* werd gekwantificeerd in de darminhoud genomen in verschillende segmenten van het gastro-intestinaal stelsel. Ondanks matching op vlak van voeding en tijd tussen sterfte en staalname, waren er geen significante verschillen tussen de telling van *Clostridia* in de darminhoud van enterotoxaemie-gevallen en controle-kalveren. Deze resultaten tonen aan dat kwantificatie van *Clostridia* in darminhoud niet kan worden aangeraden als een diagnostische test voor enterotoxemie bij witvleeskalveren, zelfs niet wanneer het staal binnen de drie uur na het intreden van de dood genomen wordt.

Hoofdstuk 5 beschrijft de ontwikkeling van een darmlusmodel om de studie van de pathogenese van enterotoxemie mogelijk te maken. Darmlussen werden geïnjecteerd met *C. perfringens* culturen van verschillende herkomst, met en zonder commerciële melkvervanger. Alle geteste isolaten veroorzaakten necrohaemorrhagische letsels in combinatie met melkvervanger, terwijl alle controlelussen (d.w.z. BHI medium plus melkvervanger) normaal bleven. Daarenboven werden tijdsreeks experimenten uitgevoerd met een isolaat van een enterotoxemiegeval bij een kalf. Histologisch onderzoek toonde aan dat de letselontwikkeling begon met stuwing van de capillairen binnen de 30 minuten na de inoculatie. Na 3-4 uur incubatie ontstonden bloedingen en necrose van de mucosa ter hoogte van de toppen van de villi. Deze letsels zijn gelijkaardig aan de letsels die in natuurlijke gevallen van enterotoxemie bij runderen gezien worden. Daarom kan er besloten worden dat in dit model necrohaemorrhagische letsels kunnen worden veroorzaakt door *C. perfringens* isolaten van verschillende herkomst. Dit wijst op de aanwezigheid van een of meerdere virulentiefactoren in de

pathogenese van de letsels, aanwezig bij alle *C. perfringens* isolaten en benadrukt het belang van predisponerende omgevingsfactoren in de ontwikkeling van enterotoxemie.

Het darmlusmodel dat beschreven werd in de vorige paragraaf droeg bij tot de identificatie van het *C. perfringens* alfa toxine en perfringolysine als voornaamste toxines in de pathogenese van enterotoxemie. Inzicht in het voorkomen van maternale antistoffen en de overgang van maternale naar verworven immuniteit voor deze toxines is essentieel voor verdere uitdieping van de pathogenese en de ontwikkeling van efficiënte vaccins. **Hoofdstuk 6** beschrijft een longitudinale cohort studie om de dynamiek van natuurlijk verworven antistoffen tegen alfa toxine en perfingolysine te bepalen, alsook het effect van ras en productiesysteem (conventioneel vleesvee of witvleeskalf). In een tweede experiment werd het potentiële effect van verstrekking van kalverbrok op de ontwikkeling van verworven immuniteit bepaald. Maternale antistoffen voor alfa toxine waren aanwezig in 52% van de kalveren. Bij conventioneel opgekweekte kalveren werd een vlotte overgang van maternale naar actieve immuniteit voor alfa toxine waargenomen, terwijl in witvleeskalveren een belangrijke daling van alfa toxine antistoffen werd vastgesteld. Perfringolysine antistoffen daalden bij zowel conventionele als bij witvleeskalveren. Binnen de witvleeskalveren waren er geen significante verschillen tussen de verschillende rassen. De opname van kalverbrok had geen invloed op de dynamiek van antistoffen tegen beide toxines. Dit onderzoek toont aan dat witvleeskalveren in de risicoperiode voor enterotoxemie een aanzienlijk lagere hoeveelheid antistoffen tegen alfatoxine bezitten dan conventioneel opgekweekte kalveren. Dit verschil kon niet toegeschreven worden aan een verschil in opname van vast voer.

Hoofdstuk 7 vat de belangrijkste inzichten samen uit dit doctoraatsonderzoek, bespreekt ze in het licht van de huidige wetenschappelijke kennis en formuleert toekomstige onderzoeksvooruitzichten en praktische implementaties in preventieprotocols. Deze thesis vormt de basis voor preventieve strategieën tegen enterotoxemie, zowel op vlak van vaccin-ontwikkeling als op vlak van een geschikter voedermanagement. De beschikbaarheid van een efficiënt *in vitro* model voor enterotoxemie opent de weg voor onderzoek naar de verdere pathogenese om de oorzakelijke toxines te identificeren. Dit zou een belangrijke stap kunnen zijn in de richting van efficiëntere vaccins en betrouwbare diagnostische technieken (bijvoorbeeld

toxine-detectie). Daarnaast zou er meer aandacht moeten geschonken worden aan voedingsregimes die beschermen tegen enterotoxemie. Er is meer inzicht nodig in de exacte rol die de verschillende voedingscomponenten spelen in zowel de ontwikkeling van immuniteit als in het bewerkstelligen van een stabiele microbiota. Deze thesis suggereert dat ook andere factoren dan de verschaffing van vaste voeders belangrijk zijn in de pathogenese van enterotoxemie bij witvleeskalveren, en dat de aanwezigheid van melkvervanger in het darmlumen predisponeert voor enterotoxemie. Daarom zou de focus voor toekomstig onderzoek naar enterotoxaemie moeten liggen op het effect van zowel de samenstelling en de concentratie van de gebruikte melkvervangers op gastheerfactoren (zoals immuniteitsontwikkeling en darmschade) als op de groei en toxine-productie van *C. perfringens*.

CURRICULUM VITAE

Bonnie Valgaeren werd geboren op 15 december 1986 te Herentals. Na het beëindigen van het secundair onderwijs aan het St-Lambertusinstituut in Westerlo begon zij in 2004 met de studie diergeneeskunde aan de Universiteit Gent. In 2010 behaalde ze het diploma van dierenarts (optie herkauwers) met grote onderscheiding. Haar afstudeerwerk (Bioveiligheid in een paardenkliniek) werd bekroond met de prijs voor de beste masterproef m.b.t. onderzoeksonderwerpen buiten optie onderzoek. Naast de reguliere opleiding in de Diergeneeskunde volgde zij een AILO (Academische Initiële Leraren Opleiding) in de Diergeneeskunde, waarvan zij eveneens in 2010 afstudeerde.

Onmiddellijk na het afstuderen trad zij in dienst van de vakgroep Interne Geneeskunde en Klinische Biologie van de Grote Huisdieren (UGent) als doctoraatstudent op een IWT landbouwproject (Pathogenese en bestrijding van enterotoxaemie bij kalveren), onder begeleiding van prof. Dr. Deprez, prof. Dr. Van Immerseel en Dr. Pardon. In 2013 tenslotte trad ze in dienst als voltijds assistent aan diezelfde vakgroep. In deze functie was ze betrokken bij het werk in de kliniek en de opleiding van de studenten uit de masterjaren. Ze nam eveneens deel aan de nacht- en weekenddiensten van de vakgroep. In 2015 vervolledigde ze het trainingsprogramma van de Doctoral School of Life Sciences and Medicine van de Universiteit Gent.

Bonnie Valgaeren is auteur en coauteur van meerdere wetenschappelijke publicaties in nationale en internationale wetenschappelijke tijdschriften, gaf presentaties op verschillende internationale congressen en trad op als reviewer voor verschillende veterinaire tijdschriften.

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