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EMERGING THREAT OF NECROTIC ENTERITIS IN POULTRY AND ITS CONTROL WITHOUT USE OF ANTIBIOTICS: A REVIEW

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ABSTRACT

Necrotic enteritis (NE) is an emerging economically significant problem of poultry caused by a bacterium *Clostridium perfringens*. The rising incidence of Clostridium infections and development of NE in commercial chickens has been associated with the withdrawal of antibiotics. There is an urgent need to control NE after European ban on the use of antibiotics as growth promoters in animal feed. Prevention strategies include avoiding predisposing factors, such as coccidiosis, and in-feed supplementation with a variety of feed additives. Supplementation of poultry diet with pre and probiotics has proven to be efficient to increase broiler chickens performance (health, weight gain, feed conversion) and to prevent or reduce the incidence of diseases caused by pathogenic bacteria. However, vaccination with modified toxin or other secreted immunogenic proteins seems a logical preventive tool for protection against a toxin-producing bacterium. This review describes the recent developments in novel preventive treatments against *C. perfringens* induced NE and highlights the role of pre and probiotics, bacteriophages and vaccine for the better control of NE.

Keywords: Necrotic enteritis, *Clostridium perfringens*, Pathogenesis, Control measures.

INRODUCTION

Poultry has got prime importance as a source of animal protein for masses. Poultry production has undergone a substantial increase compared to the livestock industries since 1970. It contributes about 34 % in total share of meat produced in world (M'Sadeq *et al.* 2015). However, the industry worldwide is now facing challenges with the removal of in-feed antibiotics completely or gradually, as the once well-controlled poultry diseases have re-emerged to cause tremendous loss of production (Van Immerseel *et al.* 2016). Enteric diseases are an important concern to the poultry industry because of production losses, increased mortality, reduced welfare of birds, and increased risk of contamination of poultry products for human consumption (Hafez, 2011).

The intestinal ecosystem of poultry has been inevitably changed as a result of the ban of antimicrobial growth promoters (AGP). Necrotic enteritis (NE) is a major disease of poultry caused by infection with *C. perfringens* of which the typical hallmark is small intestinal necrosis (Moore, 2016; Umar *et al.* 2016). NE has increased in occurrence and severity over the years.The re-emergence of NE has been the most significant threat for the poultry industry, which, in clinical form, causes high mortality and in subclinical forms, affects growth and feed conversion. It is one of the most common and economically devastating bacterial diseases in modern broiler flocks in terms of performance, welfare and mortality. According to estimation, NE losses increased to approximately US$6 billion in 2015 as compared to US$2 billion in 2000 (Umar *et al.* 2016; Wade & Keyburn, 2015). NE in chickens was first reported in the 1950s and subsequently in periodic episodes over time, depending on the world region. NE is a multi-factorial disease process, in which a number of co-factors are usually required to precipitate an outbreak of the disease. Although, *C. perfringens* has been identified as the etiological agent of the disease, the predisposing factors that lead to over-proliferation of *C. perfringens* and the subsequent progression to disease are poorly understood. The onset of NE is associated with a shift in the microbiota present within the gastrointestinal tract (GIT) (Antonissen *et al.* 2016). Any factor that causes stress in broiler chicks could suppress the immune system and disturb the balance of the intestinal ecosystem, in such a way that the risk of a NE outbreak increases (Tsiouris, 2016). It has been clear that the use of antimicrobials and ionophorous coccidiostats have played a major role in keeping the disease under control for many years (Kaldhusdal *et al.* 2016). AGPs have been banned from animal feed in the European Union because of concern for the spread of antimicrobial resistance. It is thus not surprising that a search in the biomedical literature database of PubMed, using the terms “Chicken, *C. perfringens*”, shows an increasingly steep increase in the number of published papers starting from shortly after

the ban on AGPs in the European Union late in 2006 (Van Immerseel *et al.* 2016). One of the negative consequences associated with the prohibition of AGP in commercial poultry production is the increase in intestinal health problems, including NE, emerged after the AGP ban (Umar *et al.* 2016). The disease is regarded as a typical production disease related to predisposing factors associated with the way the birds are raised. Poultry management could significantly affect the pathogenesis of NE. In particular, feed restriction and coccidiosis vaccination can protect against NE, while extreme house temperature, feed mycotoxins and high stocking density predispose to NE (Antonissen *et al.* 2014; Moore, 2016; Tsiouris *et al.* 2015). Most of the experimental models use a high protein fishmeal supplemented diet and coccidiosis infection as predisposing factors (Wu *et al.* 2014). Timing of predisposing factors is important in NE models (Prescott *et al.* 2016; Van Waeyenberghe *et al.* 2016). Critical to the successful interpretation of all of the scientific studies is accuracy of diagnosis of the disease, so that researchers (1) are sure that the *C. perfringens* isolates they are working with are truly from NE lesions, or not, and (2) interpret correctly the disease outcome of either field- or laboratory- based bird studies designed to examine virulence factors or the effects of intervention strategies such as vaccines. Definitive diagnosis of the clostridial enteric diseases is fraught with difficulties, even for experienced veterinary pathologists, especially for (1) those not familiar with this disease and/or the other common enteric diseases of poultry such as coccidiosis, which can have a wide range of presentations (2) those without knowledge of the effects of and appearance of autolytic change of the intestine especially at the microscopic level, change which starts to develop within minutes of death and which is commonly mistaken for pathological change. Thus an effective multi-dimensional strategy is required to prevent the poultry from devastating NE.

Antibiotics have been commonly used worldwide as growth promoters and for prophylactic treatment of *C.perfringens*-induced NE in poultry. However, with the European ban on antibiotics, alternatives to antibiotics became essential in order to prevent NE occurrence and the consequent economic losses for the poultry industry. However, identification of antibiotic-free, alternative disease control strategies has been hindered by the difficulty of experimentally reproducing NE by *C. perfringens* infection alone (Umar *et al.* 2016). Preventive treatment scan take the form of actions on predisposing factors, such as coccidiosis prevention, diet modifications, or improving overall cleanliness and hygiene. Alternatively they can directly target the causal agent of the disease by controlling the proliferation, colonization and persistence of virulent strains of *C. perfringens* or interfering with virulence and pathogenicity factors (Fig.1). *C. perfringens* infections can be reduced or abolished by using natural feed additives, such as probiotics (yeasts or bacteria), plants (Engberg *et al.* 2012), molecules of plant origin for example, essential oils (Liu *et al.* 2016; Timbermont *et al.* 2010) or Annatto extracts (Galindo-Cuspinera *et al.* 2003), organic acids (Geier *et al.* 2010; Timbermont *et al.* 2010), enzymes (Caly *et al.* 2015), lysozyme (Liu *et al.* 2010), or molecules of microbial origin, such as yeast extract and antimicrobial peptides (Fig.1). However, for a disease caused by a toxin-producing bacterium, it seems logical to explore whether vaccines can be developed, which may or may not be based on the causative toxins. Much work has been done in recent years in this area and proteins and toxins have been tested as vaccine candidates. In addition, the use of live vectors is under investigation and studies are being carried out on practical strategies for vaccination in the field. A major question is how birds can be protected by vaccination in the limited time span of 3 to 4 weeks before the lesions are most likely to develop. The disease thus develops at an age when maternal antibodies have declined. In addition, vaccination of young broilers is hampered by their immature immune systems and problems related to enhancing the immune system, because mass parenteral vaccination is possible at day 1 but, for practical reasons, not beyond this point

Here we give an overview of these preventive treatments, by focusing on micro-organisms and molecules or products of microbial origins that affects *C. perfringens* growth and pathogenicity.

This review encompasses the various aspects of molecular basis of pathogenesis and non-conventional control of NE.

Molecular basis of necrotic enteritis pathogenesis: At molecular level, pathogenesis of NE is governed by various exotoxins produced by *C.perfringens*. *C. perfringens* isolates are toxinotyped by the presence of four major toxins (α, β, ε and ι toxins) various strains can also produce other so-called minor toxins such as CPE, β2 toxin, perfringolysin O (θ-toxin) and collagenase (κ-toxin); the term minor toxin does not refer to the importance or level of production of these toxins, but rather to the fact that they are not part of the toxinotyping scheme (Rood *et al.* 2016; Uzal *et al.* 2014). Following part of review is a glimpse of structure and functional activities of α and β toxins.

Alpha toxin: For many years, the chromosome-encoded alpha toxin, a membrane active phospholipase, was considered to be the major toxin associated with NE (Ellemor *et al.*, 1999) before the discovery of NetB toxins (β-pore-forming toxins) which are now considered the principal virulence factor essential for pathogenesis

(Keyburn *et al*. 2008). Almost all *C. perfringens* strains produce α-toxin, a major extracellular toxin that has been shown to be essential for clostridial myonecrosis (Awad *et al*. 1995). Earlier work indicated that secreted products from *C. perfringens* were able to cause lesions typical of NE in chickens and since α-toxin was known to be a major secreted component, it was inferred that it was responsible. The limitation of this interpretation is that it does not take into account other secreted toxins that the bacteria may have produced. Alpha toxin is composed of two domains which are associated with phospholipase C activity (N-domain, 1–250 residues) and membrane recognition (C-domain, 251–370 residues), respectively. The C-terminal domain contributes to maintaining the active form of the toxin and mediates interactions with membrane phospholipids in a calcium-dependent manner. Individually, these domains are nontoxic but immunogenic in mice, resulting in the generation of antibody that reacts with the holotoxin. However, only immune responses against the C-domain provided protection against a subsequent challenge, possibly due to the blocking effects on the initial membrane-binding event. Therefore, the C-terminal domain of the alpha toxin has been studied extensively as a vaccine against *C. perfringens* infection, delivered as a purified protein or by live attenuated Bacteria (Jiang *et al.* 2015). Role of Alpha toxin in pathogenesis of NE is shown in Fig.2.

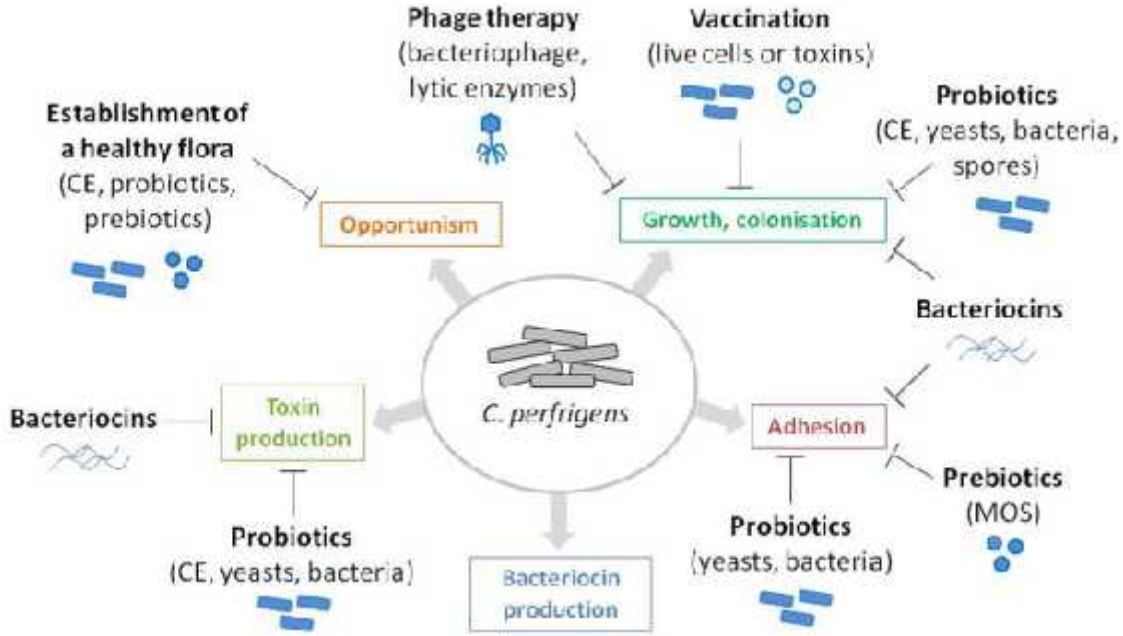

Fig.1. Identification of *C. perfringens* virulence and pathogenicity factors as potential targets for NE prevention. *C. perfringens* virulence and pathogenicity factors are represented as colored boxes. Antagonistic action of the microbes and microbe-derived products discussed in this review are represented by flat-end arrows (Caly *et al.* 2015).

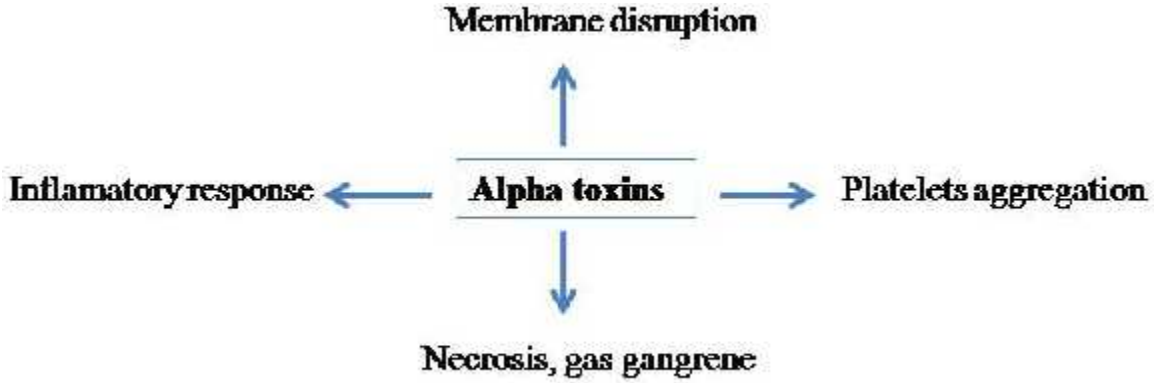

Fig 2. Role of Alpha toxin in pathogenesis of NE (Cooper & Songer, 2009).

Net B toxin: Recent studies have identified a novel toxin linked to NE, designated NetB toxin. It was first identified in an Australian *C.perfringens* type A strain (Keyburn *et al.* 2008) and it has been detected in the vast majority of NE-associated strains throughout the world (Jiang *et al.* 2015; Rood *et al.* 2016). Thus, it is now considered to be the most critical virulence factor for the development of NE in poultry. NetB is a pore-forming toxin encoded on a large conjugative plasmid (approximately 85 kilobase [kb]) within a 42-kb pathogenicity locus (NELoc-1) (39), showing similarity to *C. perfringens* b-toxin (38% identity) (Keyburn *et al.* 2010; Keyburn *et al.* 2008). Moreover, NetB toxin shows 30% identity with alpha toxin of *Staphylococcus aureus* and 29% identity with *Bacillus cereus* haemolysin II toxin. Presence of the netB gene is highly correlated with NE strain. Carriage of the netB gene distinguishes virulent strains of *C.perfringens* that are capable of inducing NE in poultry from strains that do not cause this syndrome. Surveys of *C. perfringens* isolates from chickens have generally found a higher prevalence of netB carriage in strains from diseased birds compared to strains from healthy birds (Rood *et al.* 2016), although a Danish survey found a higher netB incidence in isolates from healthy birds (Abildgaard *et al.* 2010). The isolation of netB positive strains from healthy birds indicates that simple infection is not sufficient to induce disease. The need for predisposing factors to be present in order for disease to develop is well recognized (Moore, 2016; Shojadoost *et al.* 2012). NetB induced morphological changes in chicken leghorn male hepatoma (LMH) cells, resulting in significant rounding and cell lysis. These morphological changes and subsequent cellular lactate dehydrogenase release were able to be blocked by polyethylene glycol (PEG) 1000 and PEG 1500, indicating that these changes were caused by the toxin-dependent formation of pores in the plasma membrane. Osmotic stabilizers such as PEG can inhibit the lysis of target cells if they are unable to pass through the pore generated by a pore-forming toxin. Based on the estimated Stokes radii of various PEG molecules, the results suggested that NetB formed a hydrophilic pore with a functional diameter of 1.6-1.8 nm in the cell membrane. Subsequent determination of the crystal structure of the NetB pore complex revealed an internal pore diameter of ca. 26 Å (Savva *et al.* 2013), which was consistent with this initial finding. The structure of the NetB monomer has been determined to a resolution of 1.8 Å (Yan *et al.* 2013), which revealed that it had the β-sandwich, latch, rim and prestem domains that are typical of proteins that belong to the α-haemolysin family of β-pore-forming toxins (Fig.3a). It has an almost identical structure to δ-toxin from *C. perfringens* despite the fact that these toxins have very different amino acid sequences. NetB has a four amino acid deletion in the rim domain, in a region that in other β-pore-forming toxins is responsible for lipid binding, suggesting that NetB has a different cell surface target. Finally, analysis of the ability of NetB to form pores in planar phospholipid bilayers revealed that the NetB pore channels have a preference for cations over anions (Yan *et al.* 2013). The structure of the NetB pore, without the first 20 N-terminal amino acids, has been solved to a resolution of 3.9 Å after solubilization and purification of the complex from lipid vesicles (Savva *et al.* 2013). Like the monomer, the pore structure shows similarity to staphylococcal α-haemolysin (Figure 3b). It comprises seven NetB monomers in a ring structure, with the predicted hydrophobic transmembrane domain spanning residues I121 to V146 (numbering from the N-terminal amino acid of the secreted toxin). Liposome studies showed that oligomerization of NetB suggested that NetB formed a hydrophilic pore with a functional diameter of 1.6-1.8 nm in the cell membrane. Subsequent determination of the crystal structure of the NetB pore complex revealed an internal pore diameter of ca. 26 Å (Savva *et al.* 2013), which was consistent with this initial finding. 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An Increase Collagenolytic activity of matrix

metalloproteinase (MMP-2) in intestinal tissues of the birds, affected with *C. perfringens* is thought to be associated with morphological changes at the level of basal and lateral domains of enterocytes (Olkowski *et al.* 2008). Favorable environment for the growth of *C. perfringens* is produced by mucosal damage inducing factors such as parasitism (coccidiosis) high fiber diets, poor hygienic and housing conditions in addition to toxins. Moreover, excessive use of AGP enhance the capability of *C. perfringens* to induce disease.

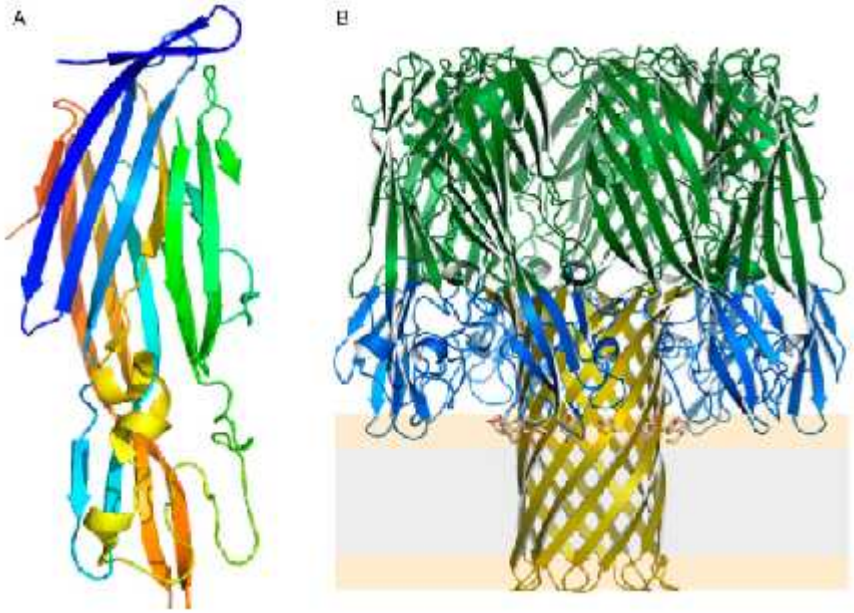

Fig. 3. The crystal structure of NetB. (a) Structure of the NetB monomer. (b) Structure of the NetB pore. The β-sandwich domain is shown in green, the rim domain in blue and the stem domain in yellow (Rood *et al.* 2016; Savva *et al.* 2013; Uzal *et al.* 2014).

Intestinal pathology and diagnosis: The gross lesions of NE due to *C. perfringens* vary in both extent and severity and may be focal, multifocal to coalescing, or in severe cases may be diffuse, affecting the entire small intestine. While lesions are most commonly found in the proximal part of the jejunum, any part of the small intestine as well as the caecum may be affected. Necrotizing and fibrinonecrotizing lesions can be recognized as tan discolouration of the mucosa (Shojadoost *et al.* 2012). In some cases, the mucosa becomes thickened due to build-up of adherent fibrin and necrotic debris, and has a marked coarsely granular texture and may be moderately firm and adherent or soft and moist. Ulcers may be recognized as depressed foci with a roughened red exposed surface and there may be some limited haemorrhages at the margins or in the lumen (Smyth, 2016). In birds with NE, all crater-like lesions are commonly classified as ulcers, when in fact many of these represent areas of mucosal thinning due to sloughing of necrotic mucosa. Such areas are characterized by having a smooth, often glistening, surface which is depressed compared to the surrounding mucosa. Often the rim of mucosa surrounding these re-epithelialized craters and ulcers is necrotic, again evidenced by tan discolouration. Ulcers and re-epithelialized craters are often visible from the serosal surface. Sloughing of large areas of mucosa together with loss of smooth muscle tone result in a thin flaccid intestinal wall. Foul-smelling gas and sloughed necrotic material may accumulate in the intestinal lumen (Smyth, 2016). Frequently, abnormally thick dark-green coloured bile discolours the mucosal surface or luminal content in the duodenum and proximal jejunum. Histologically, lesions of NE have a very characteristic appearance, but this appearance is not pathognomonic for *C. perfringens*; other clostridia, for example, *Clostridium colinum* (Swayne *et al.* 2013) and *Clostridium sordellii* (Rimoldi *et al.* 2015) can produce very similar pathology. Therefore, identification of the characteristic intestinal lesions together with cultural or immunohistochemical confirmation of the presence of *C. perfringens* is essential for the diagnosis of NE. Lesions can affect scattered single villi, or one or more clusters of villi, or may affect all the villi in a section. There is usually a variably thick superficial zone of intensely eosinophilic necrotic material which is coated by a thick mat of clostridia and which is sharply demarcated from healthy tissue by an intense zone of predominantly heterophilic infiltrate. Sometimes necrotic lamina propria and bacteria are found underlying relatively normal epithelium. Large clumps of bacteria may also be found within the necrotic coagulum. The re-epithelialized craters previously described at the

gross level, are evident as areas with markedly reduced villus height and increased crypt depth. Changes such as separation of epithelium from the lamina propria, denuded villi, strips of detached epithelial cells and dissociated cells must be interpreted with caution from histologic features of autolysis which can be erroneously interpreted as pathology and/or evidence of NE (Smyth, 2016). To reach an accurate diagnosis of NE or not-NE, diagnosticians and investigators must be familiar with the appearance of the normal intestine in both freshly dead poultry and poultry that have been dead for a period of hours or days. Moreover, thorough inspection of the intestine for evidence of the characteristic lesions and demonstration of *C. perfringens* in the lesions is essential for accurate diagnosis.

Control of necrotic enteritis: Three general strategies have been proposed to control NE in poultry: (1) reduction of environmental exposure (biosecurity measures), (2) an increase in poultry's host resistance to reduce pathogenic *Clostridium* species carriage in the gut (e.g., competitive exclusion, vaccination, and host genetics selection), and (3) the use of antimicrobial alternatives to reduce and even eliminate *Clostridium* species from colonised chickens (e.g., bacteriophage therapy and bacteriocin treatment). The genetics of birds appears to have some influence on susceptibility to NE as different lines of birds have different degrees of susceptibility (Jang *et al.* 2013) to NE and this may result from subtle difference in immune responses to C. perfringens (Hong *et al.* 2014; Kim *et al.* 2014). Due to legistlative constraints on the use of antibiotics in feed, control of C. perfringens through natural approaches of intervention has become urgent for chicken production (Liu *et al.* 2016). Thus keeping in view the scenario of post AGP era, now it's the need of time to look forward for the control of N.E using all possible means, other than antibiotic compounds.

Dietary control of Necrotic enteritis: Dietary control of NE provides excellent opportunity to cope up with the losses associated with devastating disease in a manner compatible with the demands of the post AGP era. Dietary ingredients play important role in eruption of NE. Tsiouris and coworkers showed that feed restriction of broiler chicks limited the severity of NE lesion and reduced the C. perfringens population in the caecum in an NE experimental model (Tsiouris *et al.* 2014). The protective effect of the feed restriction against NE was attributed to the neuroendocrine and immune system influence, as well as to the absence of nutrients in the intestinal tract. Furthermore, feed restriction improves blood circulation to the intestinal mucosa and may protect it from becoming necrotic (Tsiouris *et al.* 2014). Feeding the birds with diets based on wheat, rye oats and barley increases the incidence of NE in the birds than maize based diets (Annett *et al.* 2002). Maize is considered an excellent ingredient in broiler diet due to its high energy value however it also has privilege over wheat and barley as high inclusion of maize in diet reduces the incidence of NE in birds (Moore, 2016). Cereal grains have high level of indigestible non-starch polysaccharides (NSP). Large amount of NSPs in cereals increases viscosity of digesta and gut passage time (Moore, 2016) which in turn leads to the bacterial colonization in the intestine. The ultimate results of increase viscosity and intestinal stasis predisposes the birds to NE. Thus reducing the amount of these cereal grains would be helpful in controlling the NE. High levels of animal protein in the diet, particularly fishmeal, have been used as a predisposing factor in experimental disease models (Cooper & Songer, 2010; Keyburn *et al.* 2006; Wu *et al.* 2014). The high protein levels in the GIT can induce C. perfringens growth and change microbiota composition; effects modulated by the supply of nutrients and possibly by the increase in the pH throughout the GIT.

Supplementation of the broilers' diet with one or several beneficial bacteria has proven to be efficient to prevent the overgrowth of pathogens and the subsequent diseases. Several bacterial strains have been shown to increase broiler chickens performance (health, weight gain, feed conversion) and to prevent or reduce the incidence of diseases caused by pathogenic bacteria (Caly *et al.* 2015). Probiotics, or direct-fed microbials, and competitive exclusion (CE) cultures are thus commonly used in broiler farms. There are several commercially available products that have been shown to be efficient against *C. perfringens* and NE in poultry (Table1). A probiotic is defined as "a live microbial food supplement that beneficially affects the host by improving the intestinal microbial balance" (Caly *et al.* 2015). Indeed, probiotics can interact with the host to improve immunity and intestinal morphology or stimulate the metabolism, thus reducing the risk of infection by opportunistic pathogens. Probiotic bacteria have also been shown to produce molecules with antimicrobial activities, such as bacteriocins, that target specific pathogens, or even inhibit the adhesion of pathogens or the production of pathogenic toxins (Schoster *et al.* 2013). Moreover, beneficial bacteria can act as competition against pathogenic strains within the host. The exact mechanisms of action of CE remain unclear. However, it is now well known that implanting a "healthy" flora in the early days of life accelerates the establishment of the intestinal flora and creates a competition for nutrients within the intestine, thus preventing colonization by pathogens. Yeasts are also known to have antimicrobial properties, which were recently reviewed (Hatoum *et al.* 2012). In addition the cell wall is, for many types of yeast, rich in beta- glucans, which have immunomodulatory properties (Alizadeh *et al.* 2016). On top of the beneficial effects

they have on the host, yeasts can constitute a protection against pathogens by (i) producing mycocins, (ii) secreting enzymes that degrade bacterial toxins, (iii) preventing adhesion to epithelial cells, or (iv) by acting as a competitive exclusion agent. Prebiotics are additives that will stimulate the commensal flora and enhance the beneficial effects of probiotics within the host and are mostly indigestible oligosaccharides (Patel & Goyal, 2012). Numerous molecules have been described, with mannan- oligosaccharides (MOS) being the main prebiotic of microbial origin. MOS are components within the yeast cell wall and constitute the main active ingredient of yeast extract (YE) for disease control. They are often used as feed additives in broiler diets (Table1) where they have been shown to improve intestinal health and immune response, and also inhibit pathogen colonization by reducing adhesion (Kim *et al.* 2011). The addition of MOS to broiler feed was shown to improve overall performance as measured by productivity and weight gain (Fowler *et al.* 2015).

Bacteriophages are highly species-specific viruses that infect and kill bacteria. Upon replication within the bacterial cell, phages produce endolysins, which target peptidoglycans and lyse the bacterial cell wall, freeing the phages and allowing them to spread to other cells (Nakonieczna *et al.* 2015). Many bacteriophages of *C. perfringens* have been described and sequenced (Caly *et al.* 2015), including several that were isolated from strains of poultry origin and that had specific anti- *Clostridium* activity (Volozhantsev *et al.* 2011). The use of bacteriophages to limit *C. perfringens* infection has proven efficient in field trials. The use of bacteriophages to control NE is a promising alternative; however, the application can be problematic. Indeed, it is hard to predict the behavior of the molecule *in vivo* and many factors can interfere. Recently, encapsulated carvacrol (essential oil) has been proved effective in controlling NE (Liu *et al.* 2016). Moreover, organic acid supplementation in feed can also inhibit growth of harmful bacteria and maintain intestinal heath by modification of host-pathogen interactions (Timbermont *et al.* 2010).

Table 1. Examples of commercially available microbial feed additives for NE prevention in poultry (Caly *et al.* 2015).

Product	Company	Composition	Origin	Activity	Reference
Aviguard®	MSD Animal Health	Over 200 bacterial species	Healthy, adult chickens	Competitive exclusion	Hofacre *et al.*, 1998
BROILACT®	Nimrod Veterinary products	Complex mixture of bacteria	Intestine of a normal adult fowl	Competitive exclusion	Kaldhusdal *et al.*, 2001
PoultryStar®	Biomin	6 bacterial species and 1 prebiotic (FOS)	Unknown	Competitive exclusion	McReynolds *et al.*, 2009
MSC™	Continental Grain Co.	Bacteria	Caeca and caecal sections	Competitive exclusion	Craven *et al.*, 1999
Finelact™	QTI Animal Health	*L.reuteri*	Live, healthy chicken	Probiotic	Tested in a field trial (manufacturer's product data)
FloraMax® B-11	Pacific Vet Group, USA	11 lactic acid bacteria and inactivated *Saccharomyces cerevisiae*	Poultry intestine	Probiotic	Layton *et al.*, 2013
NuPro®	Alltech Inc.	Yeast extract	Yeast	Immunostimulation, antimicrobialactivity	Thanissery *et al.*, 2010
SafMannan®	Phileo Lesaffre Animal Care	Yeast Extract	*S.cerevisiae*	Immunostimulation, antimicrobialactivity	Abudabos and Yehia, 2013

Control of coccidial infections: Coccidial infection has multiple effects on the GIT that predispose birds to NE. Physical damage to the GIT epithelium compromises gut integrity which (i) opens direct access to the intestinal basal layer, which may be an important site in the early stages of disease; (ii) may expose extracellular matrix molecules, such as collagen, that have a role in *C. perfringens* adherence (Wade & Keyburn, 2015); (iii) causes serum leakage into the gut which acts as a rich nutrient source for *C.perfringens* growth; and (iv) induces

mucus production, providing another protein-rich nutrient source to support *C.perfringens* proliferation (Moore, 2016). Therefore, control of coccidiosis will ultimately help to reduce NE in poultry.

Immune interventions: Current situation augments the use of immunization as an alternate to the antibiotics for the control of NE. The immune response to *C. perfringens* infection, including immune recognition of the pathogen and its secreted proteins and toxins, is still poorly understood. In several vaccination studies, a mucosal IgA response against alpha toxin, NetB and other immunogenic proteins was reported in chickens partially protected against NE (Caly *et al.* 2015; Jang *et al.* 2012; Kulkarni *et al.* 2007). In intestinal washings from experimentally infected birds, however, only weak reactivity of mucosal IgA against proteins of *C. perfringens* was found. This might indicate that a serum IgY response plays a more important role in immunity to NE than does mucosal IgA. After systemic immunization with recombinant immunogenic proteins, serum IgY still reaches the mucosal surface under inflammatory conditions caused by *C. perfringens* (Kulkarni *et al.* 2007; Mot *et al.* 2014). Unfortunately there is no effective vaccine to be used in broilers yet. Different attempts made to develop effective vaccine against NE resulted into variable level of protection. This section encompasses different vaccine types that are attempted to be used against NE. Possible preventive measures to control NE has been shown in Fig.4.

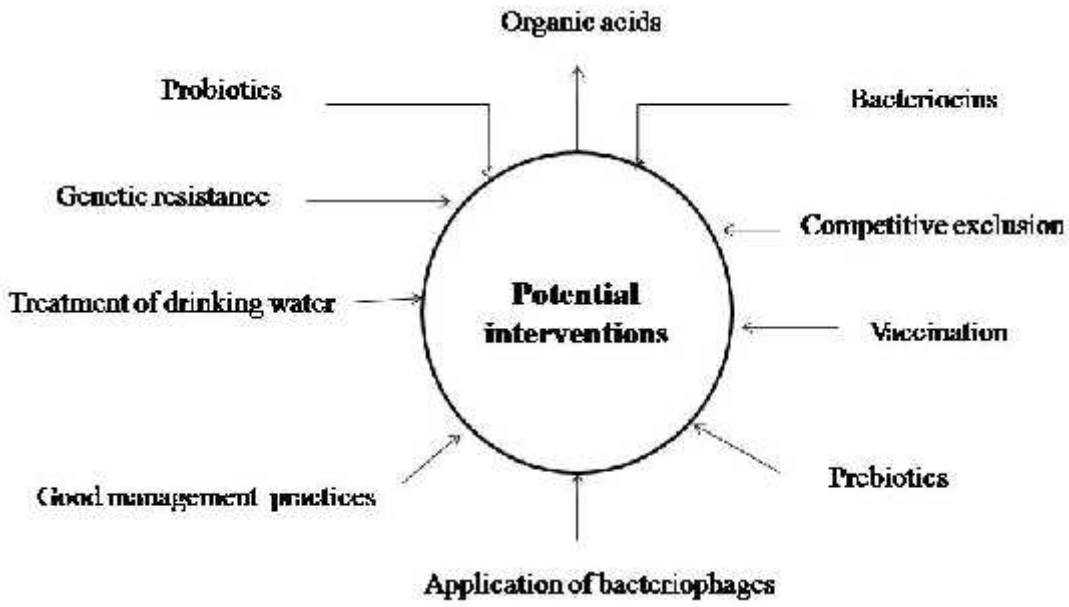

Fig. 4. Possible interventions of NE in Poultry

The reader is also directed to a recent review by the Van Immerseel lab (Mot *et al.* 2014). Several strategies have been used to vaccinate broilers against *C.perfringens* to include use of live bacteria or inactivated toxins. Vaccines can be delivered by spraying chicks upon hatching, by addition to the feed or the drinking water, or even injected *in ovo* (Mot *et al.* 2014). Vaccination using non- virulent *C.perfringens* strains have proven to be inefficient, and it has been shown that strains used in vaccines need to remain mildly virulent. Thompson *et al*. (2006) showed that strains with a mutation in the gene coding for the alpha toxin that were still virulent (but less than the wild-type) were able to protect chickens against NE, where as an avirulent strain of *C. perfringens* did not have any immunizing effects (Thompson *et al.* 2006). Several trials have shown that chickens could be protected against *C. perfringens*-induced NE by injection with inactive and active toxins (Jang *et al.* 2012; Kulkarni *et al.* 2007) and antigenic proteins (Jiang *et al.* 2015). Protein-based vaccines are used because they are safer and better characterized when compared with live vaccines, while still providing protection (Unnikrishnan *et al.* 2012). They include toxoids (inactivated bacterial toxins) and subunit vaccines, often based on virulence factors or secreted toxins. DNA vaccines that express Clostridium toxins, but not *C. perfringens* toxins, have also been tested as vaccine candidates (Jin *et al.* 2013; Li *et al.* 2011). Attenuated or avirulent bacteria can be used as vehicles for the effective delivery of vaccine candidates (Rappuoli *et al.* 2011). Attenuated Salmonella strains are often used in poultry for the control of salmonellosis and they can serve as safe and effective oral carrier vaccines to prevent NE by expressing heterologous antigens (Jiang *et al.*

2015). The belief that alpha-toxin was important in disease misdirected vaccine efforts for many years but the recent advances made in our fundamental understanding of the basis of pathogenesis is now enabling the development of effective vaccines (Moore, 2015). Since the discovery of its role in NE, the NetB toxin has been intensively studied with regards to vaccination, with some promising results (da Costa *et al.* 2013; Keyburn *et al.* 2013a; Keyburn *et al.* 2013b). Several groups have shown that vaccination with NetB induces an immune response that delivers a degree of protection from the development of NE. Protection has been shown both in directly vaccinated birds (da Costa *et al.* 2013; Jang *et al.* 2012; Jiang *et al.* 2015; Keyburn *et al.* 2013b) and in chicks derived from vaccinated hens (Keyburn *et al.* 2013a). It is difficult to compare these studies as they each used different vaccination schedules, different challenge models and different scoring procedures, but in general it appears that protection levels of 70–80% can be achieved using recombinant NetB, native NetB, or toxoids and bacterin vaccines containing NetB at levels sufficient to induce strong immune responses. The challenge for NE vaccine development is to provide an efficacious vaccine that is safe, affordable and is compatible with the general management practices applied to broiler flocks. It appears that NetB will be an important antigen to include in an effective commercially viable vaccine.

Conclusion: NE is a continuously increasing problem after ban over use of AGPs. A better understanding of host-pathogen interactions and the identification of the nature of host immune responses that are critical for protection against NE will contribute to the development of logical intervention strategies against NE. Dietary control of the disease and protective immunization are too pronged strategies that may be exploited to tackle the issue in post AGPs regimen. Optimization of poultry farm management practices including husbandry, strict biosecurity and poultry house sanitation protocols, diet-related strategies and nutriceutical alternatives (probiotics, prebiotics, herbs, organic acids and enzymes) have become more relevant. With the recent development of a reliable NE experimental model system, future studies focused on the immunobiology of host–pathogen interactions will contribute to the development of novel control strategies against this disease, including second-generation recombinant vaccines, new delivery vectors, and novel adjuvants. The development of drug-free biocontrol approaches to reduce the burden of bacterial pathogens on food animal production systems will become important for NE control and may include innate immune molecules with antimicrobial function, such as antimicrobial peptides, defensins, bacteriophages, bacteriophage lysins, hyperimmune therapeutic antibodies, pre- and probiotics, bioactive phytochemicals, and other anti-virulence biotherapeutic alternatives. It is likely that NetB will be an important antigen to include in an effective, commercially viable, NE vaccine.

REFERENCES

Abildgaard, L., T. E. Sondergaard, R. M. Engberg, A. Schramm, and O. Højberg. (2010). In vitro production of necrotic enteritis toxin B, NetB, by netB-positive and netB-negative Clostridium perfringens originating from healthy and diseased broiler chickens. Vet. Microbiol. 144(1): 231-235.

Alizadeh, M., J. Rodriguez-Lecompte, A. Rogiewicz, R. Patterson, and B. Slominski. (2016). Effect of yeast-derived products and distillers dried grains with solubles (DDGS) on growth performance, gut morphology, and gene expression of pattern recognition receptors and cytokines in broiler chickens. Poult. Sci: pev362.

Annett, C., J. Viste, M. Chirino-Trejo, H. Classen, D. Middleton, and E. Simko. (2002). Necrotic enteritis: effect of barley, wheat and corn diets on proliferation of Clostridium perfringens type A. Avian. Pathol. 31(6): 598-601.

Antonissen, G., V. Eeckhaut, K. Van Driessche, L. Onrust, F. Haesebrouck, R. Ducatelle, R. J. Moore, and F. Van Immerseel. (2016). Microbial shifts associated with necrotic enteritis. Avian. Pathol. 45(3): 308-312.

Antonissen, G., F. Van Immerseel, F. Pasmans, R. Ducatelle, F. Haesebrouck, L. Timbermont, M. Verlinden, G. P. J. Janssens, V. Eeckhaut, and M. Eeckhout. (2014). The mycotoxin deoxynivalenol predisposes for the development of Clostridium perfringens-induced necrotic enteritis in broiler chickens. PLoS One. 9(9): e108775.

Awad, M. M., A. E. Bryant, D. L. Stevens, and J. I. Rood. (1995). Virulence studies on chromosomal α-toxin and Θ-toxin mutants constructed by allelic exchange provide genetic evidence for the essential role of α-toxin in Clostridium perfringens-mediated gas gangrene. Mol. Microbiol. 15(2): 191-202.

Caly, D. L., R. D'Inca, E. Auclair, and D. Drider. (2015). Alternatives to Antibiotics to Prevent Necrotic Enteritis in Broiler Chickens: A Microbiologist's Perspective. Front. Microbiol. 6: 1336. doi: 10.3389/fmicb.2015.01336

Cooper, K. K., and J. G. Songer. (2009). Necrotic enteritis in chickens: a paradigm of enteric

infection by Clostridium perfringens type A. Anaerobe. 15(1): 55-60.

Cooper, K. K., and J. G. Songer. (2010). Virulence of Clostridium perfringens in an experimental model of poultry necrotic enteritis. Vet. Microbiol. 142(3): 323-328.

da Costa, S. P. F., D. Mot, M. Bokori-Brown, C. G. Savva, A. K. Basak, F. Van Immerseel, and R. W. Titball. (2013). Protection against avian necrotic enteritis after immunisation with NetB genetic or formaldehyde toxoids. Vaccine. 31(37): 4003-4008.

Engberg, R. M., K. Grevsen, E. Ivarsen, X. Fretté, L. P. Christensen, O. Højberg, B. B. Jensen, and N. Canibe. (2012). The effect of Artemisia annua on broiler performance, on intestinal microbiota and on the course of a Clostridium perfringens infection applying a necrotic enteritis disease model. Avian. Pathol. 41(4): 369-376.

Fowler, J., R. Kakani, A. Haq, J. Byrd, and C. Bailey. (2015). Growth promoting effects of prebiotic yeast cell wall products in starter broilers under an immune stress and Clostridium perfringens challenge. J. Appl. Poult. Res. 24(1): 66-72.

Galindo-Cuspinera, V., D. C. Westhoff, and S. A. Rankin. (2003). Antimicrobial properties of commercial annatto extracts against selected pathogenic, lactic acid, and spoilage microorganisms. J. Food. Prot. 66(6): 1074-1078.

Geier, M., L. Mikkelsen, V. Torok, G. Allison, C. Olnood, M. Boulianne, R. Hughes, and M. Choct. (2010). Comparison of alternatives to in-feed antimicrobials for the prevention of clinical necrotic enteritis. J. Appl. Microbiol. 109(4): 1329-1338.

Hafez, H. M. (2011). Enteric diseases of poultry with special attention to Clostridium perfringens. Pakistan Vet. J.

Hatoum, R., S. Labrie, and I. Fliss. (2012). Antimicrobial and probiotic properties of yeasts: from fundamental to novel applications. Front. Microbiol. 3.

Hong, Y. H., H. Dinh, H. S. Lillehoj, K.-D. Song, and J.-D. Oh. (2014). Differential regulation of microRNA transcriptome in chicken lines resistant and susceptible to necrotic enteritis disease. Poult. Sci. 93(6): 1383-1395.

Jang, S. I., H. S. Lillehoj, S.-H. Lee, K. W. Lee, E. P. Lillehoj, Y. H. Hong, D.-J. An, W. Jeong, J.-E. Chun, and F. Bertrand. (2012). Vaccination with Clostridium perfringens recombinant proteins in combination with Montanide™ ISA 71 VG adjuvant increases protection against experimental necrotic enteritis in commercial broiler chickens. Vaccine. 30(36): 5401-5406.

Jang, S. I., H. S. Lillehoj, S.-H. Lee, K. W. Lee, E. P. Lillehoj, Y. H. Hong, D.-J. An, H.-Y. Jeoung, and J.-E. Chun. (2013). Relative disease susceptibility and clostridial toxin antibody responses in three commercial broiler lines coinfected with Clostridium perfringens and Eimeria maxima using an experimental model of necrotic enteritis. Avian. Dis. 57(3): 684-687.

Jiang, Y., H. Mo, C. Willingham, S. Wang, J.-y. Park, W. Kong, K. L. Roland, and R. Curtiss III. (2015). Protection against necrotic enteritis in broiler chickens by regulated delayed lysis Salmonella vaccines. Avian. Dis. 59(4): 475-485.

Jin, K., S. Wang, C. Zhang, Y. Xiao, S. Lu, and Z. Huang. (2013). Protective antibody responses against Clostridium difficile elicited by a DNA vaccine expressing the enzymatic domain of toxin B. Hum. Vaccin. Immunother. 9(1): 63-73.

Kaldhusdal, M., S. L. Benestad, and A. Løvland. (2016). Epidemiologic aspects of necrotic enteritis in broiler chickens–disease occurrence and production performance. Avian. Pathol. 45(3): 271-274.

Keyburn, A. L., R. W. Portela, M. E. Ford, T. L. Bannam, X. X. Yan, J. I. Rood, and R. J. Moore. (2013). Maternal immunization with vaccines containing recombinant NetB toxin partially protects progeny chickens from necrotic enteritis. Vet. Res. 44(1): 1.

Keyburn, A. L., R. W. Portela, K. Sproat, M. E. Ford, T. L. Bannam, X. Yan, J. I. Rood, and R. J. Moore. (2013). Vaccination with recombinant NetB toxin partially protects broiler chickens from necrotic enteritis. Vet. Res. 44(1): 1.

Keyburn, A. L., S. A. Sheedy, M. E. Ford, M. M. Williamson, M. M. Awad, J. I. Rood, and R. J. Moore. (2006). Alpha-toxin of Clostridium perfringens is not an essential virulence factor in necrotic enteritis in chickens. Infect. Immun. 74(11): 6496-6500.

Keyburn, A. L., X.-X. Yan, T. L. Bannam, F. Van Immerseel, J. I. Rood, and R. J. Moore. (2010). Association between avian necrotic enteritis and Clostridium perfringens strains expressing NetB toxin. Vet. Res. 41(2): 1-8.

Keyburn, L., J. Boyce, and P. Vaz. (2008). Bannam. TL, Ford, ME, Parker, D., Di Rubbo, A.,. Rood, JI, Moore, RJ: 1-11.

Kim, D. K., H. S. Lillehoj, S. I. Jang, S. H. Lee, Y. H. Hong, and H. H. Cheng. (2014). Transcriptional profiles of host-pathogen responses to necrotic enteritis and differential regulation of immune genes in two inbreed chicken lines showing

disparate disease susceptibility. PLoS One. 9(12): e114960.

Kim, G., Y. Seo, C. Kim, and I. Paik. (2011). Effect of dietary prebiotic supplementation on the performance, intestinal microflora, and immune response of broilers. Poult. Sci. 90(1): 75-82.

Kulkarni, R., V. Parreira, S. Sharif, and J. Prescott. (2007). Immunization of broiler chickens against Clostridium perfringens-induced necrotic enteritis. Clin. Vaccine Immunol. 14(9): 1070-1077.

Li, N., Y.-Z. Yu, W.-Y. Yu, and Z.-W. Sun. (2011). Enhancement of the immunogenicity of DNA replicon vaccine of Clostridium botulinum neurotoxin serotype A by GM-CSF gene adjuvant. Immunopharmacol. Immunotoxicol. 33(1): 211-219.

Liu, D., Y. Guo, Z. Wang, and J. Yuan. (2010). Exogenous lysozyme influences Clostridium perfringens colonization and intestinal barrier function in broiler chickens. Avian. Pathol. 39(1): 17-24.

Liu, X., M. S. Diarra, Y. Zhang, Q. Wang, H. Yu, S.-P. Nie, M.-Y. Xie, and J. Gong. (2016). Effect of encapsulated carvacrol on the incidence of necrotic enteritis in broiler chickens. Avian. Pathol. 45(3): 357-364.

M'Sadeq, S. A., S. Wu, R. A. Swick, and M. Choct. (2015). Towards the control of necrotic enteritis in broiler chickens with in-feed antibiotics phasing-out worldwide. Anim. Nutr. 1(1): 1-11.

Moore, R. J. (2015). Necrotic enteritis in chickens: an important disease caused by Clostridium perfringens. Microbiol. Australia. 36(3): 118-119.

Moore, R. J. (2016). Necrotic enteritis predisposing factors in broiler chickens. Avian. Pathol. 45(3): 275-281. doi: 10.1080/03079457.2016.1150587

Mot, D., L. Timbermont, F. Haesebrouck, R. Ducatelle, and F. Van Immerseel. (2014). Progress and problems in vaccination against necrotic enteritis in broiler chickens. Avian. Pathol. 43(4): 290-300. doi: 10.1080/03079457.2014.939942

Myers, G. S., D. A. Rasko, J. K. Cheung, J. Ravel, R. Seshadri, R. T. DeBoy, Q. Ren, J. Varga, M. M. Awad, and L. M. Brinkac. (2006). Skewed genomic variability in strains of the toxigenic bacterial pathogen, Clostridium perfringens. Genome Res. 16(8): 1031-1040.

Nakonieczna, A., C. J. Cooper, and R. Gryko. (2015). Bacteriophages and bacteriophage-derived endolysins as potential therapeutics to combat Gram-positive spore forming bacteria. J. Appl. Microbiol. 119(3): 620-631.

Olkowski, A., C. Wojnarowicz, M. Chirino-Trejo, B. Laarveld, and G. Sawicki. (2008). Sub-clinical necrotic enteritis in broiler chickens: novel etiological consideration based on ultra-structural and molecular changes in the intestinal tissue. Res. Vet. Sci. 85(3): 543-553.

Patel, S., and A. Goyal. (2012). The current trends and future perspectives of prebiotics research: a review. 3 Biotech. 2(2): 115-125.

Prescott, J. F., J. A. Smyth, B. Shojadoost, and A. Vince. (2016). Experimental reproduction of necrotic enteritis in chickens: a review. Avian. Pathol. 45(3): 317-322.

Rappuoli, R., S. Black, and P. H. Lambert. (2011). Vaccine discovery and translation of new vaccine technology. The Lancet. 378(9788): 360-368.

Rimoldi, G., F. Uzal, R. P. Chin, E. A. Palombo, M. Awad, D. Lyras, and H. Shivaprasad. (2015). Necrotic enteritis in chickens associated with Clostridium sordellii. Avian. Dis. 59(3): 447-451.

Rood, J. I., A. L. Keyburn, and R. J. Moore. (2016). NetB and necrotic enteritis: the hole movable story. Avian. Pathol(just-accepted): 1-20.

Savva, C. G., S. P. F. da Costa, M. Bokori-Brown, C. E. Naylor, A. R. Cole, D. S. Moss, R. W. Titball, and A. K. Basak. (2013). Molecular architecture and functional analysis of NetB, a pore-forming toxin from Clostridium perfringens. J. Biol. Chem. 288(5): 3512-3522.

Schoster, A., B. Kokotovic, A. Permin, P. Pedersen, F. Dal Bello, and L. Guardabassi. (2013). In vitro inhibition of Clostridium difficile and Clostridium perfringens by commercial probiotic strains. Anaerobe. 20: 36-41.

Shimizu, T., K. Ohtani, H. Hirakawa, K. Ohshima, A. Yamashita, T. Shiba, N. Ogasawara, M. Hattori, S. Kuhara, and H. Hayashi. (2002). Complete genome sequence of Clostridium perfringens, an anaerobic flesh-eater. Proc. Natl. Acad. Sci. 99(2): 996-1001.

Shojadoost, B., A. R. Vince, and J. F. Prescott. (2012). The successful experimental induction of necrotic enteritis in chickens by Clostridium perfringens: a critical review. Vet. Res. 43(1): 1.

Smyth, J. A. (2016). Pathology and diagnosis of necrotic enteritis: is it clear-cut? Avian. Pathol(just-accepted): 1-25.

Swayne, D. E., J. R. Glisson, L. R. McDougald, L. K. Nolan, D. L. Suarez, and V. L. Nair. (2013). Diseases of Poultry. 13th ed. John Wiley and Sons; New Jersey, USA.

Thompson, D., V. Parreira, R. Kulkarni, and J. Prescott. (2006). Live attenuated vaccine-based control of

necrotic enteritis of broiler chickens. Vet. Microbiol. 113(1): 25-34.

Timbermont, L., A. Lanckriet, J. Dewulf, N. Nollet, K. Schwarzer, F. Haesebrouck, R. Ducatelle, and F. Van Immerseel. (2010). Control of Clostridium perfringens-induced necrotic enteritis in broilers by target-released butyric acid, fatty acids and essential oils. Avian. Pathol. 39(2): 117-121.

Tsiouris, V. (2016). Poultry management: a useful tool for the control of necrotic enteritis in poultry. Avian. Pathol. 45(3): 323-325.

Tsiouris, V., I. Georgopoulou, C. Batzios, N. Pappaioannou, R. Ducatelle, and P. Fortomaris. (2014). Temporary feed restriction partially protects broilers from necrotic enteritis. Avian. Pathol. 43(2): 139-145.

Tsiouris, V., I. Georgopoulou, C. Batzios, N. Pappaioannou, R. Ducatelle, and P. Fortomaris. (2015). High stocking density as a predisposing factor for necrotic enteritis in broiler chicks. Avian. Pathol. 44(2): 59-66.

Umar, S., M. Younus, M. Shahzad, K. Aqil, R. Qayyum, A. Mushtaq, M. A. A. Shah, and M. T. Munir. (2016). Role of wheat based diet on the pathology of necrotic enteritis in Turkeys. Scientifica. 2016.

Unnikrishnan, M., R. Rappuoli, and D. Serruto. (2012). Recombinant bacterial vaccines. Curr. Opin. Immunol. 24(3): 337-342.

Uzal, F. A., J. C. Freedman, A. Shrestha, J. R. Theoret, J. Garcia, M. M. Awad, V. Adams, R. J. Moore, J. I. Rood, and B. A. McClane. (2014). Towards an understanding of the role of Clostridium perfringens toxins in human and animal disease. Future. Microbiol. 9(3): 361-377.

Van Immerseel, F., U. Lyhs, K. Pedersen, and J. Prescott. (2016). Recent breakthroughs have unveiled the many knowledge gaps in Clostridium perfringens-associated necrotic enteritis in chickens: the First International Conference on Necrotic Enteritis in Poultry. Avian. Pathol(just-accepted): 1-5.

Van Waeyenberghe, L., M. De Gussem, J. Verbeke, I. Dewaele, and J. De Gussem. (2016). Timing of predisposing factors is important in necrotic enteritis models. Avian. Pathol. 45(3): 370-375.

Volozhantsev, N. V., V. V. Verevkin, V. A. Bannov, V. M. Krasilnikova, V. P. Myakinina, E. L. Zhilenkov, E. A. Svetoch, N. J. Stern, B. B. Oakley, and B. S. Seal. (2011). The genome sequence and proteome of bacteriophage ΦCPV1 virulent for Clostridium perfringens. Virus Res. 155(2): 433-439.

Wade, B., and A. Keyburn. (2015). The true cost of necrotic enteritis. World Poult. 31(7): p16-17.

Wu, S.-B., D. Stanley, N. Rodgers, R. A. Swick, and R. J. Moore. (2014). Two necrotic enteritis predisposing factors, dietary fishmeal and Eimeria infection, induce large changes in the caecal microbiota of broiler chickens. Vet. Microbiol. 169(3): 188-197.

Yan, X.-X., C. J. Porter, S. P. Hardy, D. Steer, A. I. Smith, N. S. Quinsey, V. Hughes, J. K. Cheung, A. L. Keyburn, and M. Kaldhusdal. (2013). Structural and functional analysis of the pore-forming toxin NetB from Clostridium perfringens. mBio. 4(1): e00019-00013.