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Necrotic enteritis in chickens: a review of pathogenesis, immune responses and prevention, focusing on probiotics and vaccination

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Review

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Abstract

Necrotic enteritis (NE), caused by *Clostridium perfringens* (CP), is one of the most common of poultry diseases, causing huge economic losses to the poultry industry. This review provides an overview of the pathogenesis of NE in chickens and of the interaction of CP with the host immune system. The roles of management, nutrition, probiotics, and vaccination in reducing the incidence and severity of NE in poultry flocks are also discussed.

Introduction

Necrotic enteritis (NE) is an important intestinal disease of chickens which was previously known to be caused by *Clostridium perfringens* (CP) type A and to a lesser extent by type C strains (Songer, 1996). It is of note, however, that with identification of B-like necrotic enteritis toxin (NetB) as the major CP toxin, the NE-producing CP strains are currently classified as type G (Rood *et al.*, 2018). As an opportunistic pathogen, CP colonizes the intestinal tract of healthy chickens at a density of 10^2 colony forming units (CFU) per gram of intestinal content. The proliferation of CP in the gut is associated with production of various toxins, including α-toxin, NetB, TpeL, and perhaps other undefined toxins, which may in turn cause NE. There are several predisposing factors that enhance CP proliferation and toxin production, including unbalanced composition of the diet, immunosuppression, and intestinal damage caused by other diseases such as coccidiosis. NE is commonly seen in 2- to 5-week-old broiler chickens and the clinical form of the disease is characterized by a high mortality rate of up to 50%, with consequent economic losses to the poultry industry. The subclinical form of NE does not result in significant mortality and is only associated with mild damage to the intestine. However, this form may cause significant financial losses through impairing the ability of the intestine to absorb nutrients, ultimately resulting in a significant reduction in performance parameters (Gholamiandehkordi *et al.*, 2007; Shojadoost *et al.*, 2012).

Over the past several decades, following the discovery of NE by Parish in 1961 (Parish, 1961), several preventive measures have been attempted for disease prevention and control, with dietary inclusion of antibiotic growth promoters (AGPs) in poultry feed being the most widely used approach worldwide (Elwinger *et al.*, 1998). However, the ban on preventive use of in-feed antibiotics in the European Union countries (Levy, 2014), USA and Canada has the potential to impose substantial economic losses to the poultry industry due to the re-emergence of once well-controlled diseases, including NE (M'Sadeq *et al.*, 2015). As a result, a wide range of studies have been dedicated to understanding the pathogenesis of CP and development of novel methods for prevention of disease (Caly *et al.*, 2015; Prescott *et al.*, 2016; Smyth, 2016). Indeed, research in this area has led to the discovery of a novel pore-forming toxin called NetB (Keyburn *et al.*, 2008), as well as detection of specific genomic structures that play a role in the virulence potential of CP, indicating that NE is not merely a toxin-mediated disease (Lepp *et al.*, 2010). These breakthroughs have paved the path toward a better understanding of CP pathogenesis and developing effective control strategies against NE.

Many studies have been conducted to investigate the effect of antimicrobial alternatives such as probiotics, prebiotics, enzymes, essential oils, and organic acids, in addition to vaccination for prevention of NE in broiler chickens (Adhikari *et al.*, 2020). Yet, despite a tremendous amount of research on this topic, there is currently no vaccine or feed additive available that offers complete protection against NE; as a result, the annual global economic losses incurred by this disease are still estimated to be approximately $6 billion USD (Wade *et al.*, 2015). In this article, we review

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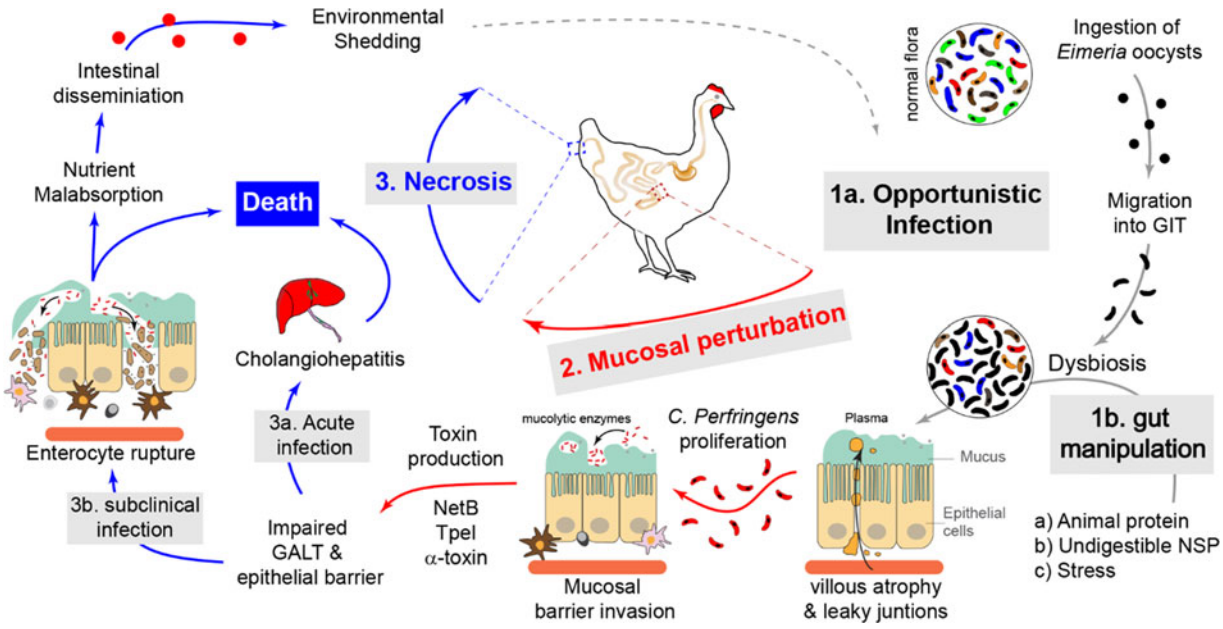

Fig. 1. Pathogenesis of *C. perfringens* infection in broiler chickens leads to NE. *C. perfringens* is an opportunistic pathogen that is normally found in a healthy gut flora of chickens. In a healthy gut, *C. perfringens* secretes low levels of enzymes and toxins that are subsequently neutralized by secretory antibodies (IgY and IgA). Mucosal and gut microbiota perturbation, caused by either feeding high animal protein or indigestible NSP diet (1b) and or ingestion of *Eimeria* oocysts which is associated with plasma leakage into the gut lumen, lead to *C. perfringens* growth and proliferation. The uncontrolled growth of *C. perfringens* is accompanied by secretion of a variety of mucolytic enzymes (sialidases, galactosidases, hexosaminidases and fucosidases) and pore-forming toxins such as NetB, allowing its penetration of the mucosa. Following penetration of the intestinal mucosa, *C. perfringens* secrete tissue-degrading toxins such as α-toxin and TpeL, which can lead to mild mucosal damage (subclinical NE) that is associated with nutrient malabsorption and dissemination of *C. perfringens* into the environment. In severe cases, the excessive secretion of these toxins may lead to extensive necrosis (clinical NE) and cholangiohepatitis that can eventually lead to death of chickens.

different aspects of NE with a focus on the pathogenesis and associated immune responses, in addition to highlighting the current strategies of NE prevention in chickens, including vaccines and antimicrobial alternatives.

Pathogenic mechanisms of *C. perfringens* in chickens

Pathogenesis of CP is very complex and depends on host, microbe, and management (including diet) factors (Fig. 1). Pathogenesis of NE is complicated due to the involvement of many factors, including colonization by CP via intestinal mucus degradation and subsequent penetration of mucosal surfaces; quorum sensing; and production of tissue-degrading toxins, such as NetB (Keyburn *et al.*, 2008), α-toxin and toxin C. perfringens large cytotoxin (TpeL) (Prescott *et al.*, 2016). Among the toxins produced by CP, the NetB toxin has long been thought to be the major virulence factor of CP. Following the initial identification of NetB, research efforts have provided a detailed understanding of the extra-chromosomal location of the gene encoding this toxin, uncovered its role in the pathogenicity of CP and explored the possibility of using it as a target antigen in vaccine strategies (Rood *et al.*, 2016). Genomic and transcriptomic analyses have provided new insights into pathogenicity. Novel findings surrounding pathogenic mechanisms have included toxin production, quorum sensing, attachment to host tissues, and production of other pathogenicity-related enzymes, in addition to new data related to subclinical CP infection.

Comparative genomic and transcriptomic analyses of pathogenicity-related genes of C. perfringens

Transcriptomic analysis of virulence genes in CP

Analyses of genome content and gene expression allow for mechanisms of CP pathogenicity to be assessed from a broader viewpoint. For instance, transcriptomic analysis of a NetB positive CP strain has been used to assess relative differences in gene expression under *in vitro* and *in vivo* conditions (Parreira *et al.*, 2016). In a study by Parreira *et al.* (2016), significant differences in global gene expression were observed in CP subjected to different environmental conditions. When considering virulence genes specifically, it was found that the expression of these genes was the lowest in bacteria grown in a nutrient-poor medium, similar to those observed in CP recovered from chickens. More importantly, the notable decrease in the expression of virulence genes was found to coincide with decreased expression of regulatory *VirR/VirS* genes; however, expression of *VirT*, an alternate regulator of *VirR/VirS* regulated genes, was upregulated. This research highlighted the potential regulatory mechanisms employed by CP in environments of different nutrient availability.

Genomic analysis of virulence genes in CP

Genomic analysis of CP isolates allows for the identification of pathogenicity-related genes in CP and enables comparison between pathogenic and non-pathogenic strains. For instance, whole genome sequence analysis of CP isolated from a NE outbreak in chickens revealed the presence of genes related to toxin production, virulence elements, antibiotic resistance, and inserts of bacteriophage origin (Li *et al.*, 2017*a*). Further, comparative analyses of the genomes of different strains of CP have identified differences among them (Ronco *et al.*, 2017; Lacey *et al.*, 2018*a*; Kiu *et al.*, 2019). In this regard, sequenced genomes from 30 chicken and turkey CP isolates revealed key differences in the presence of pathogenicity genes; NE-causing bacteria in chickens were found to contain NE pathogenicity loci (NELoc) 1, 2 and 3, *netB* and a collagen adhesin gene (*cnaA*), whereas in turkeys, only NELoc-2 was consistently observed along with limited

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representation of *netB* and *cnaA* (Ronco *et al*., 2017). Despite identifying NetB in all NE-causing isolates, the results of the study by Ronco *et al*. (2017) suggest significant differences in pathogenic mechanisms between turkeys and chickens. This was further supported by identification of *cnaD*, a proposed collagen adhesin gene found in all isolates from diseased turkeys but only in a 39% of isolates from diseased chickens. Among strains of CP, some differences exist at the chromosomal level, although large-scale sequencing of isolates of CP from multiple host species demonstrated that pathogenicity was more closely linked to extra-chromosomal genes located on plasmids (Lacey *et al*., 2018*a*). In a similar study, comparing healthy and NE-affected-broiler CP isolates (Kiu *et al*., 2019), *netB* was consistently observed in NE isolates, along with collagen adhesin genes and *tpeL*, the latter of which codes for a separate toxin, resulting in proposal of the existence of an extra-virulent lineage of bacteria. Overall, recent genomic and transcriptomic analyses of CP have provided further insight into the genes that in addition to *netB,* contribute to pathogenicity in chickens.

Toxin-mediated pathogenic mechanisms

The pathogenic mechanisms employed by CP toxins were recently summarized (Navarro *et al*., 2018). In chickens, primary pathogenic mechanisms are attributed to the presence of NetB toxin, yet research has suggested roles for other toxins. Rehman *et al*. have reported that α-toxin dysregulates the function of gastrointestinal epithelial cell membranes (Rehman *et al*., 2006, 2009). More recently, CP α-toxin has been shown to induce inflammatory responses in chicken intestinal epithelial cells (Guo *et al*., 2015). Nonetheless, in chickens, the association of NE and the presence of the NetB toxin in CP bacteria is well characterized. There has been considerable controversy whether other toxins that are produced by CP could also be mechanistically responsible for producing or enhancing NE disease in chickens; for example, production of the TpeL toxin has been suggested to enhance the virulence of NetB positive strains (Prescott *et al*., 2016). However, no such effect was observed in a subsequent study (Yang *et al*., 2018). To study this relationship further, recent work assessed 22 CP strains that all lacked the *netB* gene, while some harbored *tpeL* (Llanco *et al*., 2017). Specific *in vitro* pathogenicity analyses were performed, which showed that the presence of TpeL is associated with cellular adherence, but not necessarily with invasive processes. In a separate surveillance study, 19 *netB*-positive strains of CP were isolated from NE-afflicted broilers and TpeL carriage was observed in five of the strains which were also shown to produce the toxin protein (Gu *et al*., 2019). These strains were shown to be virulent in broilers, including one specific strain that decreased bird growth rate significantly. The study by Gu *et al*. (2019) highlighted the importance of these toxins in the occurrence of NE, and suggested the use of TpeL- and NetB-positive strains of CP for challenge studies. However, despite the apparent importance of NetB and TpeL toxins with NE, a recent qPCR analysis of CP isolates from NE-afflicted and healthy chickens identified no significant differences in the presence or copy number of the *netB* gene (Yang *et al*., 2018). Additionally, TpeL was only found in NetB-positive strains and was not associated with other NE-causing strains. These recent data cast doubt on the nature of the involvement of TpeL and NetB toxins in the pathogenesis of NE caused by CP, suggesting a multifactorial model of disease, although they do not negate NetB as a virulence factor. These results coincide with recent research examining the effects of NELoc-1-encoding genes, including *netB,* on the pathogenicity of CP in chickens (Zhou *et al*., 2017). Spontaneous loss of the plasmid that contains NELoc-1 after serial *in vitro* subcultures allowed Zhou *et al*. (2017) to restore only NetB in these bacteria, to explore mechanisms of pathogenicity. Restoration of NetB alone recovered *in vitro* cytotoxicity during infection of chicken hepatoma cells; however, lesion scores after *in vivo* chicken infection were not completely restored compared to the wild-type NELoc-1-containing bacteria. Finally, recent research has shown that transfer of *netB*-containing plasmids, from one strain of CP bacteria to another strain that does not contain *netB*, can occur in the chicken gastrointestinal tract, leading to enhanced virulence in the new strain (Lacey *et al*., 2017).

Non-toxin-mediated pathogenic mechanisms

Beyond toxin-mediated mechanisms, other proteins produced by CP have been shown to impact its virulence and pathogenicity. Previous work of Kulkarni *et al*. (2007) identified certain immunogenic CP proteins that were shown to induce robust antibody responses and immunity to NE in chickens. These proteins also included certain housekeeping enzymes such as glyceraldehyde-3-phosphate dehydrogenase (GPD), pyruvate: ferredoxin oxidoreductase (PFOR) and fructose 1,6-bisphosphate aldolase (FBA). The fact that these proteins are involved in NE protection (Kulkarni *et al*., 2008, 2010) highlighted the likely involvement of such housekeeping or energy metabolism proteins in the pathogenesis of NE. Along similar lines, one factor that is important in NE pathogenicity is the ability of CP to adhere to the intestinal mucosal surface. Binding capability to collagen and gelatin has been explored in disease- and non-disease-causing strains of CP, demonstrating that the former showed significantly greater binding capacity (Wade *et al*., 2015). This was attributed to the presence of a putative collagen adhesion gene, *cnaA*, in disease-causing strains, which was thought to be responsible for binding of CP to certain types of collagens in addition to gelatin. These observations were supported by further studies demonstrating that targeted mutation of *cnaA*, in two separate strains of CP, significantly decreases binding to collagens and gelatin, abolishing the ability for one strain to colonize while inhibiting colonization for the other (Wade *et al*., 2016). In addition to adherence, degradation of mucus in the chicken gastrointestinal tract has also been proposed as a mechanism of colonization and pathogenicity employed by CP. Mucus degradation was explored by MacMillan *et al*. (2019), where they showed that CP metabolizes specific monosaccharides that are present in mucin glycans present in the gastrointestinal tract of poultry, providing the bacteria a nutrient source and the ability to penetrate initial physical innate defenses. Similar work has been done, examining two zinc metalloproteases, ZmpA and ZmpB, which share some nucleic acid sequence homology but currently lack known substrates (Wade *et al*., 2020). *ZmpB* is chromosomally located, while *zmpA* is located on a plasmid within NELoc-1; in 83 isolates of CP, *zmpB* was identified in every isolate, while *zmpA* was identified in 34 of 36 isolates from NE-afflicted chickens. Despite the apparent connection between ZmpA and pathogenesis, targeted mutation of both genes resulted in mutant bacteria that were significantly less able to cause intestinal disease, and the effects of mutating both were not significantly different from the effects of single mutations. This accumulation of recent research is showing that pathogenesis of CP-induced NE is a complex process that likely extends beyond production of the NetB toxin.

Other potential determinants of pathogenesis include gene expression regulatory mechanisms such as the VirR/VirS two-

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component system and quorum sensing systems, including LuxS and Agr-like systems. Experimental mutation of these regulatory elements revealed that the loss of VirR/VirS-mediated regulation or Agr-like-mediated quorum sensing significantly decreases *in vitro* bacterial cytotoxicity, which is not affected by mutational loss of the LuxS system (Yu *et al.*, 2017). Expression of *netB* was also significantly decreased in a similar pattern following experimental mutations. Finally, Yu *et al.* (2017) showed that *in vivo* pathogenicity is also significantly reduced in broilers administered bacteria harboring mutations in the Agr-like system, but pathogenicity was restored in bacteria containing a plasmid with Agr-like gene sequences.

There is currently evidence demonstrating that pathogenesis of CP extends beyond the presence of NetB and includes other pathogenic proteins and enzymes which are under genetic regulation. Further understanding of these pathogenic mechanisms will allow for the design of novel prophylactic and therapeutic approaches for chickens.

Immunity against *C. perfringens*

Immunity against CP is mediated by a concerted interaction between innate and adaptive host mechanisms. The following summarizes our current understanding of these mechanisms.

Host innate responses

The intestinal epithelial cell barrier is well equipped for microbial sensing by means of pattern recognition receptors (PRRs), including Toll-like receptors (TLR) (Keestra *et al.*, 2013). Most of the immunological processes that take place in the intestinal mucosa against CP are initiated by secreted products of cells of the epithelium and underlying lamina propria (Guo *et al.*, 2015). Structural components of CP or its secreted toxins interact with epithelial cells leading to pro-inflammatory responses which contribute to disease progression. Lu *et al.* (2009), have demonstrated significant increases in ileal expression of immune response genes in broiler chickens in response to CP infection, including expression of TLR1, myeloid differentiation primary response 88 (MyD88), tumor necrosis factor receptor-associated factor 6 (TRAF6), TIR-domain-containing adapter-inducing interferon-β (TRIF), interleukin (IL)-8 and interferon regulatory factor 3 (IRF3). Expression of TLR2, TLR4, TLR7 and TLR15 was also increased in the spleen. Thus, it is clear CP induces both mucosal and systemic responses to infection (Lu *et al.*, 2009). Intracellular signaling mediated by TLR1, TLR2 and TLR4 can trigger expression of antimicrobial peptides (cathelicidins and β-defensins) (Cuperus *et al.*, 2013; Wang *et al.*, 2020). Defensins are host defense peptides (HDPs) that are involved in host innate immunity (Hancock *et al.*, 2016). Avian β-defensins (avBD) are produced by epithelial cells of the gastrointestinal tract and play a critical role in gut defense mechanisms against a broad spectrum of enteric pathogens by disrupting microbial cell membranes, inducing cell death (van Dijk *et al.*, 2008; Sugimura *et al.*, 2013). Hong *et al.* have examined expression of avBD 1−14 in intestines and spleens of broiler chicks infected experimentally with CP. CP induced distinct patterns of expression of β-defensins, with different avBD being expressed in spleen and intestinal tissues (Hong *et al.*, 2012). Significant differences in patterns of expression were noted between chicks from two commercial broiler lines, suggesting that genetic differences may contribute to variation in β-defensin responses. It has been demonstrated *in vitro* that a recombinant avBD6 is highly effective, in a time- and concentration-dependent manner, against CP by limiting its growth (van Dijk *et al.*, 2007). Importantly, there is no evidence to date demonstrating that expression of intestinal β-defensins provides an effective anti-CP activity *in vivo*. Indeed, necrosis of intestinal tissue progresses despite expression of avBD.

Modulation of TLR signaling pathways has been investigated as a means of reducing morbidity due to CP. Diets formulated with yeast extract containing mannan-oligosaccharides (TLR2 ligands), however, have had limited impact on bird body weight gains, performance, and gut morphology of CP-infected chickens (Yitbarek *et al.*, 2012; Alizadeh *et al.*, 2016). An *in vitro* experiment has demonstrated that the pro-inflammatory effects of CP mediated through the TLR4 signaling pathway can be blocked by treatment with *Saccharomyces boulardi*, a non-pathogenic probiotic yeast (Wang *et al.*, 2020). Confirmation from *in vivo* studies is needed.

Chicken professional antigen presenting cells (APCs), such as macrophages and dendritic cells, play an important role in the development of adaptive immunity (De Geus and Vervelde, 2013). There is very little information on the type and function of APCs involved in the initiation of immune responses against CP. While direct functions are not clear, detection of interleukin (IL)-1β, IL-6, IL-8 and IL-12 transcripts indicate a likely rapid host response and a role for APCs in supporting activation of B and T cells (Yitbarek *et al.*, 2012; Fasina and Lillehoj, 2019). In addition to pro-inflammatory properties of the chemokine, IL-8, it plays a role in the recruitment and activation of granulocytes and macrophages, with subsequent nitric oxide (NO) production (Guo *et al.*, 2015) and translocation of major histocompatibility complex (MHC) class II receptors to the cell surface (Li *et al.*, 2010). It has been reported that *in vitro* stimulation of chicken embryonic fibroblast cells (CEFs) with CP can lead to NO production in a TLR4-dependent manner (Zhang *et al.*, 2017*a*). Limiting TLR4 induction in macrophage cell lines can reduce CP-mediated inducible nitric oxide synthase expression (Guo *et al.*, 2015) and corresponding NO production (Wang *et al.*, 2020). Localized intestinal macrophage activation can increase the permeability of the endothelium leading to serum loss into the intestinal lumen. Thus, innate responses that are essential for defense against many pathogens may, in the case of CP, exacerbate the disease process.

Adaptive immune responses

The mechanism for B-cell activation is not well defined in chickens. B-cell activation and antibody production is mediated by a combination of cytokines and cognate antigen (Davani *et al.*, 2014). Infection with CP leads to a significant increase in intestinal IL-4 and IL-10 transcripts (Collier *et al.*, 2008), key cytokines involved in B-cell activation. In addition, CP infection leads to induction of transforming growth factor (TGF)-β (Fasina and Lillehoj, 2019) and IL-10 (Yitbarek *et al.*, 2012) cytokine transcripts in the small intestine, traditionally associated with inducing an immunosuppressive milieu. In combination with specific cognate antigens, these cytokines (IL-10, TGF-β, IL-4) can induce antigen-specific B-cell differentiation (Davani *et al.*, 2014). While these mechanisms have not been fully explored in CP-infected chickens, detection of antigen-specific IgY antibodies in vaccinated breeder hens demonstrates class switching (IgM to IgY antibody isotype) as well as the presence of plasma B cells (Keyburn *et al.*, 2013*a*). Therefore, passive immunity in progeny

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chicks from vaccinated hens is mediated by maternal IgY antibodies that can effectively neutralize toxins secreted by pathogenic CP. In addition, IL-4, IL-10 and IL-23 initiate cellular repair processes, limit inflammation and promote B-cell activation (Degen *et al*., 2005; Fasina and Lillehoj, 2019). These results provide a framework whereby host response against infection with CP elicits a regulatory environment to limit tissue damage and increase antibody production while attempting to decrease bacteria tissue penetration.

Recent studies of immunity to CP have led to the discovery and characterization of antigens within its bacterial immunome (Kulkarni *et al*., 2008; Keyburn *et al*., 2013*a*). Vaccine candidate antigens were identified by screening IgY and IgA antibodies from immunized and challenged broiler chickens against CP cellular and secretory antigens. PFOR, α-toxin, FBA and hypothetical protein (HP) were selected for further investigation (Kulkarni *et al*., 2010). Epitope mapping studies demonstrated a broad range of B-cell epitopes in PFOR, α-toxin, FBA and HP (Kulkarni *et al*., 2008, 2010). Two major segments of α-toxin (amino acid positions 96−122 and 183−212) were highly antigenic (Kulkarni *et al*., 2010) whereas the length of HP was demonstrated to be highly antigenic (Kulkarni *et al*., 2008). In addition, B-cell epitope mapping demonstrated a total of 94 peptides in PFOR showing potential for broad antigen responses (Kulkarni *et al*., 2008). Recombinant α-toxin, HP and PFOR administered intramuscularly induced significant protection in broiler chickens against challenge with CP (Kulkarni *et al*., 2007). In subsequent work, B-cell epitopes of HP and α-toxin were cloned separately into a *Salmonella enterica* vector, and chickens were vaccinated orally with the live vaccines. Both antigens induced significant protection against experimental challenge (Kulkarni *et al*., 2010).

Several groups have examined maternal vaccination to provide passive (maternal) antibodies against CP to their chicks (Kulkarni *et al*., 2010; Keyburn *et al*., 2013*b*). Keyburn *et al*. have investigated vaccines containing recombinant NetB protein and/or a CP toxoid preparation. Hens received the vaccine subcutaneously, and their progeny were challenged at 2 weeks post-hatch. Maternal antibodies from the hens mediated significant protection (Keyburn *et al*., 2013*a*). These studies highlight important practical considerations. Because NE is a major concern in rapidly growing young birds, vaccination schemes must provide protection at a young age and must be convenient for mass administration.

T helper (Th) cells can be viewed as an essential component in the early phase of CP pathogenesis (Collier *et al*., 2008). Most experimental models of NE induce a T-cell-mediated inflammatory response, leading to enhanced intestinal mucogenesis (Collier *et al*., 2008). Increased expression of pro-inflammatory IL-1β and decreased expression of anti-inflammatory TGF-β have been documented in the jejunum of broiler chicks 7 days post-challenge with CP (Fasina and Lillehoj, 2019). Expression of IL-13 by intestinal T cells enhances mucin production, providing a growth advantage to CP (Collier *et al*., 2008; Fasina and Lillehoj, 2019). In contrast, mucosal effector T cells are characterized by expression of cytokines such as IL-2, IL-17 and interferon (IFN)-γ that activate innate immune system cells and enhance antigen presentation by APCs (Brisbin *et al*., 2012; Taha-Abdelaziz *et al*., 2016). However, broiler chicks infected with CP are reported to have reduced IFN-γ and IL-2 transcripts and increased IL-10 and IL-17 transcripts in jejunal tissue by 7 days post-challenge (Fasina and Lillehoj, 2019). Therefore, microbiota-driven IL-17 expression could indicate the involvement of Th17 which play a critical role in mucosal inflammation, induction of antimicrobial peptides and enhancing mucosal repair (Walliser and Göbel, 2018). Along with these effects, it has also been observed that CP infections induce a reduction in IL-22, expressed by Th17 cells, which is critical for maintaining gut epithelial cell survival, proliferation and induction of antimicrobial peptides (Collier *et al*., 2008). These results demonstrate a potential role for γδ T cells as an essential primary mucosal barrier defense against CP infection and progression to NE. Decreased intestinal T-cell function in CP-infected chickens compared to non-infected chickens has been demonstrated *ex vivo*, based on a lack of response to mitogen stimulation (Li *et al*., 2010). Studies of T-cell responses to CP have been relatively few in number compared to studies of B cells, and more extensive investigations are needed.

Farm management practices and nutritional strategies

Farm management is considered one of the key factors that contribute to the incidence of CP in poultry flocks (Tsiouris, 2016). Accumulating evidence indicates that environmental stressors, such as heat and cold stress, vaccination, processing in the hatchery, transportation to the farm, wet litter, poor ventilation, and high stocking density, can disturb the homeostasis of the intestine and negatively impact the immune systems of chicks, thereby increasing the incidence and severity of NE in chickens (Hangalapura *et al*., 2004; Hirakawa *et al*., 2020).

High stocking density increases the risk of horizontal transmission of CP between chickens by spreading spores through air or direct contact, and it is often associated with a substantial accumulation of litter, which provides a supportive niche for CP sporulation (McDevitt *et al*., 2006; Guardia *et al*., 2011). Furthermore, it has been reported that high stocking density increases the CP-associated gut lesion scores and pH in the intestine as well as the CP counts in the caeca of chickens (Tsiouris *et al*., 2015). In addition to increasing the susceptibility of birds to pathogens, cold stress may also contribute to the pathogenesis of NE in chickens (Regnier and Kelley, 1981). Tsiouris *et al*. (2015) investigated the role of cold stress in the pathogenesis of NE in broiler chickens and found that exposure to cold stress increases the incidence of NE as well as the severity of lesions in chickens experimentally challenged with CP. Likewise, exposure of chickens to heat stress could also impair their growth, disrupt intestinal integrity, and suppress immune responses, thereby increasing the susceptibility of chickens to infections (Calefi *et al*., 2014). Chickens subjected to heat stress have been shown to have high intestinal lesion scores associated with CP infection in addition to the enhanced pH and viscosity of intestinal digesta (Tsiouris *et al*., 2018). The mechanisms underlying these effects have not been fully established. However, existing evidence indicates that exposure of birds to low or high temperature causes immunosuppression, making them more vulnerable to intestinal infections (Tsiouris *et al*., 2015, 2018). Other environmental factors, such as reduced ventilation, high humidity, and poor litter condition, can also significantly influence the immune systems of birds and predispose them to NE (Dunlop *et al*., 2016; Hofacre *et al*., 2018).

In addition to the role of environmental stressors in increasing susceptibility to NE, gut damage caused by parasitic diseases such as coccidiosis is one of the major risk factors for NE (Williams, 2005). Coccidiosis is a common parasitic intestinal disease caused

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in poultry by protozoan parasites of the genus *Eimeria* (Lillehoj and Lillehoj, 2000). Infection with *Eimeria* usually occurs when birds ingest viable oocysts from contaminated litter. Following ingestion of sporulated oocysts, sporozoites penetrate the epithelial lining of the intestine and undergo extensive asexual reproduction, causing disruption of the gut integrity, followed by hemorrhage, inflammation, and excessive mucus production; all of these manifestations provide conditions favorable for CP colonization in the gut (Allen and Fetterer, 2002; Williams, 2005). Therefore, controlling coccidiosis through vaccination programs and litter management would reduce the incidence of NE in poultry flocks (Bangoura *et al.*, 2014).

Biosecurity practices at the farm level are also very important for prevention of potential horizontal transmission of infections, including CP, within the flock or to other flocks (Tsiouris, 2016). Generally, farm biosecurity measures include disinfecting poultry houses, equipment, vehicles, fly screens, boot dips, and restricted entry to the barn with shower-in and shower-out facilities (Tsiouris, 2016). Dietary composition is also considered one of the critical factors that may contribute to the pathogenesis of NE in chickens (McDevitt *et al.*, 2006). High levels of non-starch polysaccharides (NSPs) in the diet have been shown to increase gastrointestinal viscosity and alter the gut microbiota composition, consequently leading to overgrowth of CP in the intestine (Jia *et al.*, 2009; Palliyeguru *et al.*, 2010).

A higher incidence of NE has been observed in chickens fed wheat- or barley-based diets (contain large amount of NSPs; i.e. arabinoxylans and ß-glucans) than those fed corn-based diets (Annett-Christianson, 2012). This is thought to be due to the role of NSPs in increasing the water-holding capacity and the viscosity of the digesta, resulting in a prolonged passage rate of gut contents and excessive mucus production that serve as a nutrient source for CP (Jia *et al.*, 2009; Annett-Christianson, 2012).

There is some evidence that the level and source of dietary protein can significantly impact severity of NE in chickens. High indigestible protein in the digestive tract serves as a protein source for CP growth and proliferation, thus subsequently for NE. For example, high levels of amino acids such as methionine and glycine in the diet have been shown to accelerate CP growth in the small intestine of chickens (Drew *et al.*, 2004; Xue *et al.*, 2017). Drew *et al.* (2004) compared the effects of animal-based protein (fishmeal) and plant-based protein (soybean meal) on intestinal populations of CP in broilers and found that birds fed fishmeal-based diets had higher numbers of CP in ileum and caeca compared to those fed soybean-based diets. These data provide an explanation for the common use of animal proteins, especially fishmeal, to experimentally produce NE in chickens.

Considering the contributing roles of various environmental stressors, poor biosecurity practices and unbalanced diet composition in induction of NE, optimization of farm management practices together with implementing nutritional as well as disease control strategies could reduce the prevalence of NE in poultry farms. NE can be controlled with in-feed AGP; however, with concerns about bacterial resistance to antibiotics and antibiotic residues in poultry products, there is increased interest in the use of alternatives, such as probiotics, prebiotics, essential oils and organic acids for the control of NE (M'Sadeq *et al.*, 2015). Among all potential alternatives, probiotics have gained significant attention because of their broad immunomodulatory and antimicrobial activities (Koenen *et al.*, 2004; Sornplang and Leelavatcharamas, 2010). Thus, in this review we focus on the protective role of probiotic bacteria against NE in chickens.

Probiotics

Probiotics are defined as 'live microorganisms which when administered in adequate amounts confer a health benefit to the host'. Probiotics exert their beneficial effects on chicken health though modulation of mucosal immune responses and intestinal microbiota, improvement of the integrity of the intestinal epithelial barrier, alteration of mucus secretion, competitive exclusion, and production of antimicrobial and immunomodulatory substances (Ng *et al.*, 2009; Bermudez-Brito *et al.*, 2012). The probiotic mechanisms of action in prevention and control of CP-induced NE are summarized in Fig. 2.

Effects of probiotics on intestinal immune response of NE-infected chickens

Immunomodulatory activities of probiotics have been reported in several studies (Haghighi *et al.*, 2005; Brisbin *et al.*, 2008, 2011; Bai *et al.*, 2013; Alizadeh *et al.*, 2020). Probiotics can stimulate immune responses through interaction with PRRs expressed by various immune system cells and epithelial cells (Plantinga *et al.*, 2011). This activation, however, does not lead to inflammation, but rather maintains intestinal homeostasis and keeps the immune system in a state of readiness to fight off opportunistic or invading pathogens (Yan and Polk, 2011). Additionally, probiotics may reduce intestinal inflammation in response to enteric pathogens and inhibit apoptosis of intestinal epithelial cells (Plaza-Díaz *et al.*, 2017; Azad *et al.*, 2018). In the context of NE, CP induces intestinal inflammation, causing disruption of the structure of the gut barrier and enhancement of gut permeability (Prescott *et al.*, 2016). The potential role of probiotics in ameliorating CP-induced inflammation has been studied in several clinical trials. Cao *et al.* (2012) demonstrated that oral administration of *Lactobacillus fermentum* in chickens challenged with CP significantly reduces the severity of gut inflammation caused by CP. This protective effect was associated with reduced expression of TLR2 and IFN-γ, and increased expression of IL-10 in the ileum of lactobacilli-treated birds compared to non-treated, CP-infected cohorts, indicating the role of probiotics in regulating intestinal mucosal immune response and in maintaining gut homeostasis during the course of NE (Cao *et al.*, 2012).

In an *in vitro* study, pre-treatment of CP-infected intestinal cells with two *Lactobacillus* species (*L. acidophilus* and *L. fermentum)* was shown to reduce CP-induced expression of the transcription factor nuclear factor kappa B (NF-kB), peptidoglycan receptors, TLR2 and nucleotide-binding oligomerization domain-containing protein 1 (NOD1) receptors (Guo *et al.*, 2017). In another study, Wang *et al.* (2017), evaluated the effect of *L. johnsonii* on intestinal mucosal immunity of chickens challenged with CP. Supplementation with lactobacilli mitigated immune-related adverse events associated with NE, by enhancing the production of immunoglobulins (IgG and IgM) and the proliferation of IgA^+ B cells and T-cell subsets ($CD3^+CD4^+$ and $CD3^+CD8^+$) in the ileum. In addition, *L. johnsonii* down-regulated the CP-induced mRNA expression of various cytokines, including IL-2, IL-8, IL-10, and IFN-γ, suggesting immunomodulatory activities of lactobacilli and their role in maintaining intestinal homeostasis following CP infection (Wang *et al.*, 2017).

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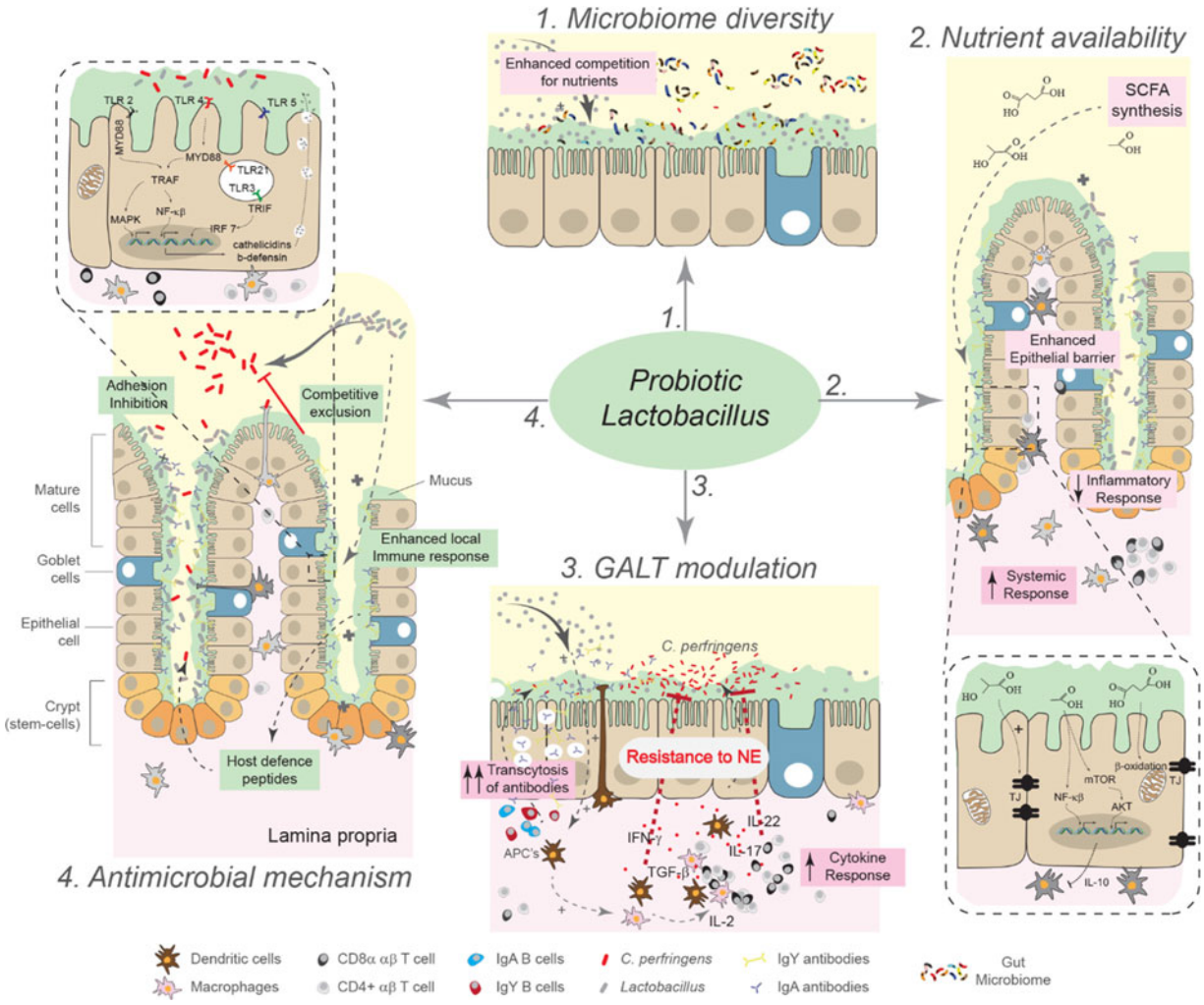

Fig. 2. Modulation of the gut microbiota by probiotic lactobacilli mitigates against CP-induced NE. The roles of probiotic lactobacilli in prevention and treatment of CP-induced NE are depicted in four different mechanisms. (1) Modulation of gut microbiota composition by enhancing microbial diversity and richness with specific microbial groups, such as microbial members phyla *Firmicutes* and *Bacteroidetes*. Probiotics also compete with gut microbial pathogens including CP for intestinal niches and nutrients. (2) Enhancing the production of SCFAs by gut microbes which in turn enhance local and systemic immune responses. Locally, SCFA induces mucin production and enhances epithelial cell integrity and the expression of an immunoregulatory cytokine, IL-10. Systemically, SCFA enhances secretion of chemotactic factors leading to gut infiltration of both innate (macrophages and γδ T cells) and adaptive immune system cells (αβ T cells and B cells), and, more importantly, an increased responsiveness to stimulation. (3) Modulation of immune responses in intestinal mucosa and gut-associated lymphoid tissue (GALT) by stimulating the tissue-resident B cells to produce secretory antibodies (IgA and IgY) that are released via transcytosis, and by stimulating tissue-resident macrophages that directly activate various T-cell subsets, making them functionally more responsive to pathogenic challenge. Specific *Lactobacillus* species can have distinct immunomodulatory effects, mainly by limiting colonic inflammation (e.g. reducing Th17, increasing Treg expression and shifting macrophages to the M2 subtype) or by enhancing antibacterial immunity (e.g. enhancing Th17, reducing Treg expression and MHC-1 expression). (4) Secretion of antimicrobial substances either through direct or indirect competition. Direct competition is recognized as either competitive exclusion (aggregation and production of bacteria-derived antibiotics) or limiting/inhibiting colonization. These functions are mediated by lowering luminal pH and access to their respective binding sites on epithelial cells. Alternatively, *Lactobacillus* stimulate the intestinal epithelial barrier through TLR (TLR 4 and TLR21) as well as tissue-resident immune cells which actively produce antimicrobial peptides (β-defensins and cathelicidins) that possess direct bactericidal activity against CP.

Effects of probiotics on intestinal barrier integrity

In chickens, intestinal barrier function is regulated by antimicrobial peptides and tight-junction proteins (TJPs) (Chelakkot *et al.*, 2018). This section briefly reviews current knowledge on the role of probiotics in strengthening intestinal barrier integrity.

CP infection of chickens induces the expression of β-defensin genes, which is indicative of the critical role of HDPs in controlling NE (Hong *et al.*, 2012). Lactobacilli can improve intestinal barrier function by up-regulating the expression of β-defensins without provoking inflammatory responses. Treatment of intestinal Caco-2 cells with *L. acidophilus*, *L. plantarum*, and *L. fermentum* has been shown to enhance the expression and secretion of human β-defensin 2 (Schlee *et al.*, 2008). Similar observations were made in chicken intestinal epithelial cells; treatment of these cells with *L. plantarum SJ*, *L. fermentum F6*, *L. rhamnosus* MLG_A, and *L. rhamnosus MB12* enhanced mRNA expression of AvBD9, with *L rhamnosus* MLG_A exhibiting stronger effects (Li *et al.*, 2012). In contrast, Akbari *et al.* demonstrated that the expression of antimicrobial peptides in cecal tonsils of chickens infected with *Salmonella enterica* was not altered following treatment with probiotics (*L. acidophilus,*

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Bifidobacterium bifidum, and *Enterococcus faecalis*) (Akbari *et al.*, 2008). These conflicting results might be explained by differences in *Lactobacillus* strains used in these studies, as well as the dose and frequency of administration. Furthermore, it should be noted that the latter study used only one concentration of these probiotics; it is unclear whether higher concentrations would affect the outcome and whether these lactobacilli would exert the same activity in CP-infected chickens. Overall, only a limited number of studies have evaluated the effects of probiotics on HDPs in chickens, and further research is needed to provide solid evidence of probiotic effects on intestinal antimicrobial peptides in CP-infected chickens.

TJPs play a critical role in maintaining intestinal barrier functions in chickens by holding intestinal epithelial cells together, protecting the gut from pathogen invasion (Vermette *et al.*, 2018). Disruption of TJPs will, therefore, lead to increased epithelial permeability allowing translocation of luminal pathogens and their toxins to the submucosa and internal organs, resulting in endogenous infection, and eventually tissue damage (Chen *et al.*, 2006). It has been reported that CP endotoxins interact with structural components of epithelial TJPs, such as claudin and occludin, leading to increased tight junction permeability and diarrhea (Emami *et al.*, 2019). There is some evidence that probiotics can enhance tight junction stability and decrease membrane permeability to CP. Wu *et al.* (2019), investigated the effects of oral administration of *Enterococcus faecium* on intestinal integrity of chickens infected with CP. The results of this study revealed that while CP infection significantly decreased mRNA expression of TJPs, including CLDN-3 and ZO-1, and protein levels of ZO-1, and increased expression and protein levels of MLCK (a protein that increases paracellular permeability) in the jejunum, administration of *E. faecium* counteracted the adverse immunological effects of CP by up-regulating CLDN-1 mRNA transcript and protein levels in the jejunum of infected birds.

Alteration of mucin production and competitive exclusion

Mucus overlies the gut epithelium and functions as the first line of defense against pathogenic microorganisms (Pelaseyed *et al.*, 2014). Mucus mainly consists of mucin, a highly glycosylated and interlinked protein secreted by specialized epithelial goblet cells (Pelaseyed *et al.*, 2014). The mucus layer is the primary site for adherence and colonization by both commensal and pathogenic bacteria, including CP (Martens *et al.*, 2018). Thus, strategies that can enhance resistance to colonization by CP could potentially alleviate NE. CP secretes various toxins including mucin-degrading and pore-forming toxins that disrupt the intestinal mucosal barrier, ultimately leading to necrotic lesions in the gut (Prescott *et al.*, 2016). Lactobacilli and their metabolites may induce mucus production through regulation of intestinal mucin gene expression (Rosique *et al.*, 2019). Xu *et al.* (2020) demonstrated that dietary supplementation with *L. plantarum*, in laying hens infected with CP, significantly increased MUC2 gene expression in the ileum. Another study used *Bacillus subtilis* as a dietary supplement for broiler chickens and demonstrated a significant increase in the expression of intestinal MUC-2 mRNA, whereas supplementation with a multi-strain probiotic resulted in a significant increase in the number of goblet cells, with no detectable alteration of MUC-2 expression (Aliakbarpour *et al.*, 2012). Treatment of Caco-2 cells with *L. casei* has also been shown to significantly enhance mRNA expression and protein levels of MUC-2 (Mattar *et al.*, 2003). Taken together, these findings highlight the ability of probiotic bacteria in promoting mucus production, and suggest their potential use as prophylactic agents to potentiate mucosal resistance against CP or as therapeutic agents to restore mucosal barrier function following infection with CP.

In addition to their role in promoting mucin secretion, probiotic bacteria can adhere to and colonize the mucus layer of the small intestine, driven by non-specific physical binding or by specific surface adhesion proteins such as mucin binding proteins (produced mainly by LAB) (Boekhorst *et al.*, 2006). The adhesion abilities of probiotic bacteria enable them to compete with opportunistic enteric pathogens for ecological niches and nutrients, through a process referred to as competitive exclusion (CE) (Woo and Ahn, 2013). Recently, the anti-CP activity of *L. acidophilus* and *L. fermentum* has been investigated in CP-infected chicken intestinal epithelial cells. The results revealed that probiotic lactobacilli possess strong antagonistic activity against CP, demonstrated by a significant reduction of CP growth and α-toxin production as well as suppression of CP adhesion to intestinal epithelial cells, with *L. acidophilus* showing greater inhibitory effects (Guo *et al.*, 2017). In an *in vivo* study, La Ragione *et al.* (2004) demonstrated that oral inoculation of 1×10^9 CFU of *L. johnsonii* FI9785 to chicks 24 h prior to challenge with CP, significantly reduces CP colonization and shedding. The authors suggested that the beneficial effects observed in the study were a result of CE. However, the inconclusive nature of these results warrants more in-depth investigation to determine whether the observed effects are attributable to CE or related to other immunomodulatory effects associated with probiotics.

Antimicrobial activity of probiotics

One of the important mechanisms of action of probiotics is their ability to produce antimicrobial substances such as hydrogen peroxide, NO, and bacteriocins (Cotter *et al.*, 2013). Bacteriocins are a large family of ribosomally synthesized peptides that have antimicrobial activities against bacterial pathogens (Zacharof and Lovitt, 2012). These molecules have broad-spectrum activity and can target specific pathogens without exerting negative effects on commensal bacteria (Dobson *et al.*, 2012). Bacteriocins may directly eliminate pathogens or may function as signaling peptides that facilitate coordination of multicellular processes and synchronize group behavior within bacterial populations or may serve as colonizing peptides giving probiotics a competitive advantage over resident pathogens (Cotter *et al.*, 2013). Antagonistic activities of bacteriocin-producing probiotics against CP have been reported in different studies (Ben Lagha *et al.*, 2017). In an *in vitro* study, it was found that bacteriocin-producing *B. subtilis* PB6 exhibits significantly high inhibitory effects on the growth of various strains of CP (Teo and Tan, 2005). In another study, Grilli *et al.* (2009) demonstrated that bacteriocin produced by *Pediococcus pentosaceus* (pediocin A), exhibited potent antagonistic activities against CP and significantly improved growth performance of chickens infected with CP.

Production of short-chain fatty acids (SCFAs)

Another important mechanism by which probiotics contribute to pathogen clearance is through secretion of SCFAs, such as acetate, butyrate, and propionate (Sun and O'Riordan, 2013). In addition to their beneficial effects as a source of energy for intestinal

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epithelium, SCFAs lower the pH of the intestine, making the conditions unfavorable for growth and proliferation of entropathogens. There is also evidence that SCFAs influence bacterial populations in the ceca of broiler chickens (Van der Wielen *et al.*, 2000). Although fermentative bacteria, such as lactobacilli, are intrinsically resistance to low pH, a significant negative correlation has been observed between numbers of *Enterobacteriaceae* and the concentration of undissociated SCFAs in the cecum of chickens (Bearson *et al.*, 1997). It has been suggested that undissociated SCFAs may diffuse across bacterial membranes into bacterial cells and dissociate in the cytoplasm, leading to a decreased internal pH and finally cell death (Russell and Diez-Gonzalez, 1997). Recent studies have demonstrated that dietary inclusion of microencapsulated sodium butyrate significantly reduces the CP-induced intestinal lesions and improves body weight gain of chickens experimentally challenged with CP (Song *et al.*, 2017; Liu *et al.*, 2019).

Antioxidant activity of probiotics

The ability of lactobacilli to stimulate the antioxidant system of the host has been investigated both *in vivo* and *in vitro* (Mishra *et al.*, 2015). It is thought that probiotics exert their antioxidant activities through scavenging free radicals in the intestine (Kodali and Sen, 2008). In the context of NE, it has been reported that infection with CP significantly lowers the antioxidant capacity of birds by increasing the malondialdehyde (MDA) level (an indicator of lipid peroxidation) and decreasing the activity of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) in serum (Zhou *et al.*, 2016; Wang *et al.*, 2017). On the other hand, dietary supplementation of *L. johnsonii* to CP-challenged chickens enhances the antioxidant capacity of intestinal mucosa by reducing MDA levels and increasing the total antioxidation capacity (T-AOC), CAT, and SOD activities, all of which are vital for healthy intestinal function (Wang *et al.*, 2017). Similar observations have been made in another study demonstrating that *B. licheniformis* supplementation to broiler chickens challenged with CP significantly increases CAT and GSH-Px activities and reduces MDA levels in serum (Zhou *et al.*, 2016). In addition, the expression levels of genes related to fatty acid synthesis (acetyl-CoA carboxylase) and oxidation (carnitine palmitoyltransferase-1 and proliferator-activated receptor-α) were upregulated. The authors added that the positive impact of probiotics on chicken growth performance is mostly attributable to their role in improving antioxidant activities and lipid metabolism.

Effects of probiotics on gut microbiome

It has been reported that gut dysbiosis caused by CP may enhance host susceptibility to other bacterial infections leading to exacerbation of an already existing mucosal inflammation (Lacey *et al.*, 2018*b*). Probiotics play a critical role in maintaining intestinal homeostasis and restoring bacterial eubiosis in infected birds (Gagliardi *et al.*, 2018). During the course of NE, it has been shown that probiotics can be used to restore the composition of the gut microbiome and to ameliorate intestinal inflammation caused by CP (Lin *et al.*, 2017; Qing *et al.*, 2017). Lin *et al.* (2017) demonstrated that manipulation of the gut microbiota of chickens by probiotics such as *B. licheniformis* alleviates disturbances caused by CP on the cecal microbial community of chickens. In another study, CP infection significantly decreased diversity indices of ileal microbiota composition (ACE and Chao 1) by increasing the relative abundance of *Gammaproteobacteria* and decreasing the relative abundance of the phylum *Firmicutes* (Li *et al.*, 2017*b*). On the other hand, *L. acidophilus* treatment increased the diversity index of the ileal microbiota by enriching the members of phylum *Firmicutes* and reducing the relative abundance of phylum *Proteobacteria*. The authors suggested that *L. acidophilus* treatment modulates the relative abundance of certain bacterial species and restores the ileal microbiome disrupted by CP infection.

NE vaccines

Efforts have been made to develop an efficacious vaccine that can provide effective protection against NE. Since NE outbreaks may start as early as 2–3 weeks of age, early vaccination is of utmost importance for prevention of the disease. NE vaccines can be developed as live-attenuated vaccines, whole inactivated vaccines, subunit vaccines, toxoids, or recombinant vectored vaccines (Thompson *et al.*, 2006; Zhang *et al.*, 2017*b*). Toxoid vaccines containing inactivated CP toxins, such as α-toxin, NetB and TpeL could induce toxin-neutralizing antibodies, preventing damage to the gut mucosa (Keyburn *et al.*, 2013*a*). While a subunit vaccine comprises a pathogen-specific immunogenic antigen and an appropriate adjuvant to enhance its immunogenicity, a recombinant vector vaccine is made of a microbial vector carrying the antigen-encoding DNA sequences (Kulkarni *et al.*, 2008; Hegazy and Hensel, 2012). However, when making a recombinant or subunit vaccine, extra care should be taken to select the most immunogenic antigen that can induce a robust immune response. Therefore, molecular pathogenesis studies are necessary to identify the role of each antigen/toxin in pathogenesis as well as host responses to the infection. Other than α-toxin, NetB or TpeL, studies have shown that pathogenic CP have other immunogenic, protective antigens (Jiang *et al.*, 2009), which once identified could be used for developing an effective NE vaccine. NE vaccine studies are summarized in Table 1.

Live-attenuated vaccines

Thompson *et al.* (2006) have demonstrated differences in the ability of live-attenuated vaccines to protect against NE. Vaccinating chickens with a live virulent strain of CP protected against subsequent infection with a homologous strain, with a significant reduction in intestinal lesions, but vaccination with an avirulent CP strain did not protect against challenge with a virulent CP strain. In another study, two out of four attenuated α-toxin-negative mutants of a virulent CP strain conferred protection against experimental challenge, suggesting involvement of other immunogens (Thompson *et al.*, 2006). The variability in protection among different CP strains might suggest the existence of differences in antigenic composition and or genetic variation of the chromosome and plasmid content of CP strains (Lacey *et al.*, 2018*a*). Therefore, caution should be exercised in selection of CP strains for developing a live-attenuated vaccine.

Toxoid vaccines

For production of protein-based vaccines, the immunogenic protein components of CP have been investigated for NE vaccine development using different approaches. For instance, some investigators have explored the potential of the CP-secreted toxins

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Table 1. Overview of NE vaccines

Type of vaccine	Selected proteins/toxins	Time (days old)	Route	Efficacy	Researcher
Live; Virulent	–	5–12	Oral	Significantly reduced lesions	Thompson *et al.* (2006)
Live; non-virulent	–	5–12	Oral	Not effective	Thompson *et al.* (2006)
Subunit	α-Toxin	5, 10	SC[a]	Lesions reduced	Cooper *et al.* (2009)
Subunit	α-Toxin, GPD[b], PFOR[c], FBA[d], HP[e]	7, 14	IM[f]	α-Toxin, PFOR and HP were more effective	Kulkarni *et al.* (2007)
Toxoid	α-Toxoid and α-Toxin	7, 14	IM	Protected against severe challenge	Kulkarni *et al.* (2007)
Supernatant	–	3, 12	SC	Strain dependent	Lanckriet *et al.* (2010)
Subunit	Toxoids A, C and A + C	7, 21	SC	Reduction of chickens with lesions	Saleh *et al.* (2010)
Toxoid	NetB	3, 9, 15	SC	Partial protection	da Costa *et al.* (2013)
Toxoid	NetB	22, 24, 26 weeks old	SC	Reduced lesions in progeny	Keyburn, *et al.* (2013*a*)
Subunit	CnaA, FimA, FimB	7,14, 19	SC	Reduction of NE lesion severity	Lepp *et al.* (2010)
Toxoid	Toxoids types A and C	14, 18 weeks old	IM	High level of resistance to natural infection in progeny	Lovland *et al.* (2004)
Subunit	TpeL, Naglu[g], and Pgm[h]	7, 14, 21	IM	Significant protection against lower severity challenge	Jiang *et al.* (2009)
Subunit-chimeric	NetB, α-toxin and NAM[i]	7, 13, 21	SC, oral	Significantly lower lesions	Katalani *et al.* (2020)
Recombinant	*Salmonella* vectored: FBA, PFOR, HP	0, 14	oral	Significant decrease in lesions by FBA, HP	Kulkarni *et al.* (2008)
Recombinant	*Salmonella* vectored: α-Toxin, HP	1, 10	oral	Protection against more severe challenge by HP	Kulkarni *et al.* (2010)
Recombinant	*Salmonella* vectored: Nontoxin fragment of α-Toxin	3, 10	oral	Reduction of chickens showing lesions	Zekarias *et al.* (2008)

[a]SC: Subcutaneous.
[b]GPD: Glyceraldehyde-3-phosphate dehydrogenase.
[c]PFOR: Pyruvate: ferrodoxin oxidoreductase.
[d]FBA: Fructose 1,6-bisphosphate aldolase.
[e]HP: Hypothetical protein.
[f]IM: Intramuscular.
[g]Endo-beta-*N*-acetylglucosaminidase.
[h]Phosphoglyceromutase.
[i]Metallopeptidase.

in culture supernatants against NE. Despite the simplicity of their production, care should be taken to ensure that toxoid vaccines are produced correctly, as inactivation by formalin may negatively affect their immunogenicity (Mot *et al.*, 2013). In an *in vivo* study, subcutaneous vaccination of broiler chickens with a formalin-inactivated NetB toxoid or NetB genetic toxoid (W262A; a domain of netB that plays a role in regulating the binding of NetB to the cell membrane) provides partial protection against experimental NE. The results showed that both vaccines resulted in a significant decrease in the intestinal lesion scores and increased antibody responses to NetB (da Costa *et al.*, 2013).

In fact, the efficacy of toxoid vaccines appears to be highly strain-dependent, as it has been demonstrated that only one out of eight toxoid vaccines provides full protection against NE in chickens (Lanckriet *et al.*, 2010). When evaluated against CP type A and type C in broiler chickens, toxoid vaccines resulted in a significant increase in CP type A- and C-specific antibodies associated with a significant decrease in intestinal lesions (Saleh *et al.*, 2010). However, despite their protective ability against NE, vaccinating the birds subcutaneously at 7 and 21 days of age, and challenging them at day 35 of age (which is 2–3 weeks after the onset of naturally occurring outbreaks), raises concerns about the feasibility of this vaccine in commercial poultry farms.

As NE mostly occurs in the early weeks of life, the role of passively transferred immunity against NE following vaccination of parent stocks has been investigated. In view of this, Lovland *et al.* demonstrated that vaccination of parent stock with a toxoid vaccine, prepared from CP types A and C, confers protection against both types of CP in their progeny chicks (Lovland *et al.*, 2004). Following vaccination of broiler breeder hens with CP type A and type C toxoids adjuvanted with aluminum hydroxide, CP α-toxin-specific IgY antibodies were detected in sera of

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breeder hens and their progeny chicks. These passively immunized progeny chicks showed a high-level resistance against subsequent natural infection with both types of CP. In a NE disease model, CP type C induced a better protection, demonstrated by reduced intestinal and hepatic lesions (Lovland *et al.*, 2004). Despite their promising role in control of subclinical NE, the protective effects of these vaccines against the clinical form of NE were not evaluated in this study.

Subunit vaccines (toxin/other protein-based)

In another approach, genes encoding immunogenic proteins, such as toxins, can be cloned into plasmids that are then expressed in mammalian, bacteria, or insect cells, followed by identification and purification of the expressed proteins to produce a subunit protein-based vaccines (Nascimento and Leite, 2012). In the past, due to a lack of adequate understanding of the immunogenicity profiles of various protein targets in CP, attention was given to α-toxin as a potential vaccine candidate for NE (Kulkarni *et al.*, 2007). The immunogenicity and protective efficacy of recombinant α-toxin-based subunit vaccines have been extensively evaluated in NE challenge models in broiler chickens. Despite efforts to unveil the potential role of α-toxin as a vaccine antigen, there is still controversy regarding its effectiveness in inducing protection against NE in chickens. For instance, subcutaneous immunization of broiler chickens with a recombinant α-toxin-based subunit vaccine at 5 days of age, followed by a booster dose at 15 days of age, conferred a partial protection against experimental CP infection at 25 days of age, as assessed by α-toxin serum IgY levels and gut lesion scores (Cooper *et al.*, 2009). In another study, priming the birds with CP α-toxin toxoid at 7 and 14 days of age followed by vaccination with an active α-toxin at 21 days of age resulted in a higher level of protection than did either one alone (Kulkarni *et al.*, 2007). Attempts have been made to enhance the protective efficacy of α-toxin vaccines. For example, a previous study by Kulkarni *et al.* (2007) evaluated the effectiveness of a single or various combination of five different recombinant proteins including α-toxin, GPD, PFOR, FBA, and a HP, against oral virulent CP challenge in broiler chickens. While vaccination with each of these proteins resulted in a significant increase in serum IgY and protection against a mild challenge with CP, a combination of α-toxin, HP and PFOR offered better protection against more severe challenge as compared to other combinations.

Following the discovery of NetB as a major virulence factor in the pathogenesis of CP (Keyburn *et al.*, 2008), a significant amount of research was directed toward the use of NetB as a vaccine. Savva *et al.* designed different recombinant *netB* mutants and evaluated their toxicity, as compared to the pure NetB toxin, on a chicken hepatocellular carcinoma epithelial cell line (LMH). As the mutated proteins did not exhibit toxic effects on the cells, the authors hypothesized that these mutants could be used for NE vaccine development (Savva *et al.*, 2013).

Comparative evaluation of effectiveness of various clostridial immunogenic proteins, including α-toxin, NetB toxin, PFOR, and elongation factor-Tu (EF-Tu) adjuvanted with ISA 71 VG, against experimental NE challenge in broiler chickens, revealed that all of them afford comparable levels of protection against NE (Jang *et al.*, 2012). However, among these vaccines, a better weight gain was observed in chickens vaccinated with NetB toxin and PFO recombinant proteins. In another study, Keyburn *et al.* (2013*b*) evaluated the efficacy of purified NetB recombinant toxin (rNetB) alone or in combination with a formalin inactivated bacterin or a cell free CP supernatant toxoid against different challenge levels of virulent CP. The results showed that rNetB alone protects the birds against a mild challenge, while significant protection against moderate or heavy CP challenge was observed when rNetB was combined with either cell-free CP toxoid or bacterin. Similar to what has been observed for α-toxin (Cooper *et al.*, 2009), these results indicate that, although known as the major CP toxin, NetB alone does not induce an efficient, protective immune response against NE and a combination with other immunogens is required to potentiate its effect (Keyburn *et al.*, 2013*a*). Recently, *Escherichia coli* BL21 strain was used to express and purify a recombinant metallopeptidase (a CP virulence factor) to make an injectable vaccine against NE, which was shown to be protective in a CP challenge model (Katalani *et al.*, 2020). Further, tobacco plants were used to make an edible NE vaccine through the expression of a fusion protein containing NetB, α-toxin and metallopeptidase (Katalani *et al.*, 2020). Using this vaccine resulted in serum antibody response and partial protection in the CP-challenge model.

Taken together, these findings point to the variability in the immunogenicity and protective potential of CP antigens. Indeed, none of the studied CP antigens has shown ability to provide complete protection against severe CP challenge when administered alone. Nonetheless, a multivalent subunit CP vaccine consisting of hybrid antigens could conceivably result in a higher level of protection. Another point of consideration is that any parenteral route used for administration of inactivated, toxoid or recombinant vaccines will be less than ideal for mass administration on poultry farms. A more practical route and time of vaccination that results in an acceptable level of protection by 2–3 weeks of age is desirable.

Live vectored vaccines

Attenuated *Salmonella enterica* strains have been extensively utilized as vaccine vectors for genes encoding various CP toxins (Hegazy and Hensel, 2012). Although attenuation of *Salmonella* strains is critical to prevent the adverse effects of this bacterium on the host, care should be taken to avoid over attenuation as it may reduce the effectiveness of the vaccine. Moreover, the vector should be attenuated by two mutations to ensure it does not revert to virulence (Hegazy and Hensel, 2012). In a study by Zekarias *et al.*, oral administration of a recombinant attenuated *S. enterica* serovar Typhimurium vaccine (RASV), expressing the c-terminal part of α-toxin, followed by a parenteral boost vaccination with a recombinant PlcC protein (rPlcC), induced significantly high levels of α-toxin-neutralizing serum antibodies and serum IgG and bile IgA titers associated with a reduction in CP colonization and enteric pathology in chickens (Zekarias *et al.*, 2008). In another study, oral immunization of broiler chickens with a recombinant *Salmonella* vaccine expressing either a gene encoding FBA or HP has been shown to provide protection against NE challenge, and both were associated with higher serum and mucosal antibody responses. However, no such effects were observed for a PFOR vaccine (Kulkarni *et al.*, 2008). Following the identification of the B-cell epitopes of HP and α-toxin, the efficacy of an attenuated recombinant *Salmonella* vaccine expressing truncated HP (tHP) as well as α-toxin toxoid on NE was investigated in broiler chickens (Kulkarni *et al.*, 2010). Chickens vaccinated with α-toxoid were significantly protected against moderate challenge, while vaccination with tHR provided

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protection against both moderate and severe challenges. Further studies were conducted to assess and compare the effects of oral vaccination of broiler chickens with a RASV expressing genes encoding α-toxin and NetB (Jiang *et al.*, 2009). While vaccination with constructs expressing either toxin alone did not confer protection, concurrent vaccination with the two vaccines or vaccination with a vector expressing the two toxins resulted in protection against moderate to heavy challenges with CP. Additionally, in a more recent study, vaccination with the RASV system expressing either FBA (an enzyme known to be important for CP virulence) alone or a mixture of FBA, α-toxin, and NetB toxin provided better protection against NE than using either α-toxin or NetB toxin alone (Wilde *et al.*, 2019). In addition to using *Salmonella* strains as vaccine vectors, *Bacillus subtilis* and *Lactococcus lactis* bacteria have also shown considerable promise as potential vaccine vectors when used in mice against clostridial infections (Robinson *et al.*, 1997; Hoang *et al.*, 2008). The advantage of using *Bacillus* and lactic acid bacteria over *Salmonella* is that, in addition to being non-pathogenic, they can confer other benefits such as immunomodulatory effects (Sugiarto and Yu, 2004; Rhayat *et al.*, 2019), which could add an additional layer of protection against NE. Thus, experimental studies are needed to explore the possibility of using these vectors as vaccine carriers for CP antigens in chickens.

Route and time of vaccination

One of the most important points that needs to be taken into consideration when developing poultry vaccines is their suitability for mass application. Despite their considerable success in limiting NE in chickens, one of the shortcomings of toxoid vaccines is that they must be parenterally administered (Kulkarni *et al.*, 2007; Cooper *et al.*, 2009). On the other hand, in addition to their role in inducing local mucosal immune responses following oral administration, attenuated vector vaccines are suitable for mass immunization (Kulkarni *et al.*, 2008). Another important point is that the immune responses to vaccination should reach a protective level before 2–3 weeks of age, the time at which chickens are more vulnerable to NE. All these vaccines have shown comparable levels of protection, however, the delay in vaccination (1- or 2-week-old birds) together with the need for booster doses raises concern about the feasibility of these vaccines. The poultry industry is, therefore, seeking a vaccine which could be effective with just a single vaccination at a very early age, but attempts to immunize day-old broiler chickens have not been successful (Mot *et al.*, 2013). Another promising strategy is vaccination of parent stock. The idea is that if the breeder flocks are vaccinated, the maternally derived antibodies will be vertically transferred to their progeny chicks, thereby providing protection against NE in their early life. Keyburn *et al.*, demonstrated that vaccination of broiler breeder hens with purified recombinant NetB toxoid at 22, 24 and 26 weeks of age induced the production of significant levels of specific NetB-IgY antibodies in hens and egg yolks of the fertile eggs 4 weeks after the last vaccination and resulted in partial protection against NE in broilers at 14 and 21 days of age (Keyburn *et al.*, 2013*a*). It is, however, important to note that one of the limitations of parent stock vaccination is the gradual reduction of antibody titers in eggs, which are laid later in their production period together with the gradual decrease of the passive immunity in chickens as they age, which may indeed influence chicken resistance to late NE outbreaks. Therefore, more work is required to ascertain the effectiveness of this passive immunity transfer through breeder vaccination.

Conclusions

NE is a complex disease that is caused by an imbalance in the intricate relationship between CP and its host. Further understanding of the pathogenicity of CP and its interaction with the host immune system will allow the development of effective prevention and control interventions for NE. Optimization of nutritional and farm management practices in addition to the application of probiotics and new generation vaccines should be pursued as preventive strategies to control NE in broiler farms.

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