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Pathogenicity of Clostridium Perfringens and its Relationship with Gut Microbiota in Chickens

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Pathogenicity of *Clostridium perfringens* and its relationship with gut microbiota in chickens

By

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Necrotic enteritis (NE), a devastating enteric disease caused by *Clostridium perfringens* type A, contributes to the losses of 6 billion dollars worldwide per year and is currently being considered as a major global threat to the poultry industry. In past decades, it has been well-controlled by in-feed antimicrobial growth promoters (AGPs). The withdrawal of AGPs due to antibiotic-resistance concerns resulted in a spike in NE incidence and led to the re-emergence of NE in the modern broiler production system.

To unveil the association of toxin genes of *C. perfringens*, particularly for *netB*, with clinical NE, a self-designed qPCR primer set targeting *netB* was developed to qualify and quantify *netB* in NE-producing and non-NE-producing isolates. The *netB* was demonstrated to exist in the majority of *C. perfringens* type A isolates. The presence and the amount of *netB* were not significantly different between two types of isolate, indicating that those indicators are insufficient to predict an association with the pathogenicity of NE. The virulence of *netB* is suggested to be expressed or triggered under certain conditions, further promoting NE.

A side by side trial was implemented with different combinations of *netB*-positive *C. perfringens* (CP1) and two predisposing factors to assess their role in NE development. Both CP1 and predisposing factor(s) are required for consistent NE reproduction, and particularly, *Eimeria* exerts significant effects on NE induction. The use of CP1 without a predisposing factor failed to induce NE. The severity and incidence of NE were positively correlated with the number of predisposing factors given in the NE induction.

Analyzing gut microbiota in chickens challenged with CP1 and/or *Eimeria* by metagenomic sequencing, significant overgrowth of *Clostridium sensu stricto 1*, the genus contains *C. perfringens*, was associated with NE. *Eimeria* infection precedent to CP1 challenge had a synergistic effect on the overrepresentation. In addition to *C. perfringens*, the other member under *Clostridium sensu stricto 1* was found to participate in NE development. Given supplementary dose of 0.4 kg/ton in feed, lauric acid neither exerted the inhibitory effect against proliferation of *Clostridium sensu stricto 1* and *C. perfringens* nor reduced the incidence and severity of NE.

DEDICATION

I dedicate this dissertation to my parents, in-laws, my beloved wife, and cutie daughter. Without their love and support, I will never have been here and make it.

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CHAPTER I

INTRODUCTION

1.1 *Clostridium perfringens*

Clostridium perfringens (*C. perfringens*) is a Gram-positive, spore-forming anaerobe that is widely distributed in the environment, as well as a commensal in the intestines of humans and animals (Timbermont et al., 2009). It belongs to the Genus *Clostridium*, Family *Clostridiaceae*, Order *Clostridiales*, and Class *Clostridia* (Keto-Timonen et al., 2006) in the Phylum *Firmicutes* that is associated with gastrointestinal (GI) diseases (Uzal, 2016). The ability to form endospores supports this bacterium to survive adverse conditions for long periods of time, in turn, transmit the host (Novak et al., 2003). In addition, *C. perfringens* can produce at least 17 toxins and extracellular enzymes that have specific activities and roles in the disease process (Freedman et al., 2015; Rood, 1998; Uzal et al., 2014). Analysis of its genome revealed that *C. perfringens* lacks genes essential for 13 amino acid biosynthesis, but it acquires those nutrients from hosts through various actions of toxins and enzymes (Myers et al., 2006; Shimizu et al., 2002). Therefore, it is characterized as a versatile enteric pathogen that rapidly destroys tissues, uptakes nutrients and proliferates in the hosts (Shimizu et al., 2002; Songer, 1996).

1.2 Toxinotypes and toxins produced by *C. perfringens*

Among produced toxins, α (alpha), β (beta), ε (epsilon), and ι (iota) four toxins are designated as major toxins due to the toxinotyping classification system of *C. perfringens*, which classifies *C. perfringens* isolates into A, B, C, D, and E toxinotypes (Van Immerseel et al., 2004b). Other toxins are constantly termed minor toxins and nonetheless critical to the virulence of *C. perfringens* (Table 1.1). The toxin production varies in type from type and strain from strain. For the major toxins, type A only produces alpha toxin. However, type B generates alpha, beta, and epsilon toxins, type C has alpha and beta toxins, type D produces alpha and epsilon toxins, and type E possesses alpha and iota toxins. Regarding the minor toxins, all toxinotypes may carry the *cpe* and *cpb2* genes that encode enterotoxin (CPE) and the β2/beta 2 (CPB2) toxins, respectively (Gibert et al., 1997; Songer, 1996). In contrast, necrotic enteritis B-like (NetB) toxin is only found in type A strains (Keyburn et al., 2008; Keyburn et al., 2010). The production of toxin perfringens large (TpeL) toxin has been demonstrated in types A, B, and C (Coursodon et al., 2012; Li et al., 2013; Nakano et al., 2017).

Table 1.1 Toxinotypes and produced toxins of *C. perfringens*

Toxin	Gene	Toxinotype				
		A	B	C	D	E
Alpha	*cpa*	+	+	+	+	+
Beta	*cpb*	-	+	+	-	-
Epsilon	*etx*	-	+	-	+	-
Iota	*iap*	-	-	-	-	+
Enterotoxin	*cpe*	+/-	+/-	+/-	+/-	+/-
Beta2	*cpb2*	+/-	+/-	+/-	+/-	+/-
NetB	*netB*	+/-	-	-	-	-
TpeL	*tpeL*	+/-	+	+/-	-	-

1.3 Virulence factors of *C. perfringens*

The virulence of *C. perfringens* is mediated by its intimidating arsenal of toxins and degradative enzymes. (Petit et al., 1999; Prescott et al., 2016a; Uzal et al., 2014). Previous studies demonstrated the essentiality of alpha toxin (CPA) to the pathogenicity of gas gangrene caused by type A strain (Awad et al., 1995), necessity of beta toxin (CBP) to the development of enteric disease caused by type C (Sayeed et al., 2008), epsilon toxin (ETX) for type D isolates to promote enterotoxemia in sheep and goats (Uzal et al., 2014), and enterotoxin (CPE) to be the determinant virulence of human food poisoning and non-foodborne GI infection (Sarker et al., 1999). In the case of necrotic enteritis (NE), the role of toxins in the virulence of clinical NE remains controversial. In the past, CPA toxin was initially recognized as the key virulence factor. However, it lost the role in pathogenicity owing that a toxin null mutant constructed in NE strains retained full virulence during the experimental challenge (Keyburn et al., 2006). Afterward, the NetB toxin was demonstrated to contribute to the main virulence of *C. perfringens* in NE based on *in vivo* evidence that disease development was achieved with the participation of a wild-type strain with *netB* or a complemented *netB* strain, but not in null mutant strain (Keyburn et al., 2008). Despite CPB2 having not been associated with NE in broiler chickens (Crespo et al., 2007; Gholamiandekhordi et al., 2006), a study in the Netherlands suggested that *cpb2* might be involved in subclinical NE in laying hens (Allaart et al., 2012). Furthermore, TpeL toxin and perfrin were also demonstrated as important factors to enhance the virulence in NE (Chen and McClane, 2015; Coursodon et al., 2012; Timbermont et al., 2014).

The pathogenesis of a bacterial infection that regularly involves the participation of colonization, multiplication, nutrient acquisition, host evasion, damage, and transmission is dynamic and reasonably simultaneous (Prescott et al., 2016a). Therefore, factors that influence the process of disease development are also considered to have significant contributions to the virulence. Several studies suggested that bacitracin, chitinase, collagenolytic enzymes, and sialidase were associated with the virulence of *C. perfringens* and proposed that those act synergistically with toxins. Bacitracin generally inhibits the growth of closely related strains to promote single-strain dominance of pathogenic *C. perfringens* in diseased birds (Timbermont et al., 2014; Timbermont et al., 2011). Chitinases, a member of the glycoside hydrolases (GHs) family that are bacterial endoglycosidases hydrolyze the chitobiose core of N-linked glycoproteins, are involved in mucus colonization and degradation (Lepp et al., 2010; Prescott et al., 2016a). Collagenolytic enzymes cause the epithelial damage of the intestinal mucosa to initiate the pathological progress of NE (Olkowski et al., 2008). Sialidase catalyzes the sialic acid hydrolysis from the host cell membrane, exposing β-galactosyl determinant which acts in the adherence process among clostridia and the host mucosa, facilitating its attachment to the mucosa (Li and McClane, 2014; Llanco et al., 2015).

Since the contribution of *netB* to the virulence has been demonstrated to fulfill the molecular Koch's postulates (Keyburn et al., 2008), current studies continue to emphasize the importance of NetB toxin in the NE development. Nevertheless, several lines of evidence evoked the controversy that full virulence resulted from the expression of *netB*. Firstly, the discovery of *netB* was documented not merely in NE-producing *C. perfringens,* but also in *C. perfringens* isolates from healthy chickens, cattle (Martin and

Smyth, 2009) and human (Nakano et al., 2017). In addition, a proportion of *C. perfringens* strains isolated from healthy chickens showed the carriage of *netB* with some degree of *in vitro* NetB toxin production but did not induce NE (Abildgaard et al., 2010; Chalmers et al., 2008; Martin and Smyth, 2009). Moreover, a number of NE cases in broilers, which *C. perfringens* isolates were negative for the *netB* gene (Abildgaard et al., 2010; Bailey et al., 2015; Llanco et al., 2015; Martin and Smyth, 2009; Smyth and Martin, 2010). Lastly, use of *C. perfringens* harboring *netB* in the absence of other genes on the plasmid-encoded pathogenicity locus (NELoc-1) failed to restore full virulence of NE in the experimental model, suggesting that other genes present at NELoc-1 are required during NE pathogenesis in a role not related to NetB expression (Zhou et al., 2017). A number of genes with putative virulence-related functions were indicated, including adhesins, glycosidases, and a cyclic-di-GMP signaling system (Lepp et al., 2010). Those contradictory findings revealed that other factors may be involved in the NE development, not mainly depending on *netB*.

1.4 *C. perfringens*-associated illness in humans

C. perfringens type A is recognized as one of the major foodborne pathogens in humans (Petit et al., 1999). The pathogenicity are attributable to enterotoxin (CPE) encoded by *cpe*. The CPE-producing *C. perfringens* was estimated to cause 1 million illnesses each year in the United States (Batz et al., 2012; CDC 2017; Scallan et al., 2011). Beef, poultry, and pork are common sources of these infections (Grass et al., 2013) and the outbreaks are typically associated with improper cooling or inadequate reheating of contaminated meats (Schlundt 2015). However, the most important animal or environmental reservoirs for CPE-producing *C. perfringens* remains unknown (McClane,

2007). In addition to human food poisoning, CPE-producing *C. perfringens* also contributes to 5-15% of non-foodborne GI diseases in human, including antibiotic-associated diarrhea and sporadic diarrhea (Carman, 1997). CPE has been demonstrated essential for enterotoxicity in both foodborne and non-foodborne human illnesses (Sarker et al., 1999). Expression of CPE toxin occurs during sporulation of *C. perfringens* in the intestines (Navarro et al., 2018). The *cpe* encoding CPE was found able to locate either on a chromosome or on a plasmid (Freedman et al., 2016). The majority of food poisoning strains carry a chromosomal copy, whereas other non-foodborne strains harbor a plasmid-borne gene (Grant et al., 2008). A study investigating the *cpe* gene in 2,659 isolates from animals, humans, and foods demonstrated that less than 5% of isolates carry *cpe* gene (Daube et al., 1996). *C. perfringens* type A-associated enteric diseases are of important concern to the poultry industry due to significant economic losses and the potential risk of human food poisoning. Although the prevalence of *cpe* in *C. perfringens* type A from poultry is rare, there were several food poisoning reports epidemiologically traced back to chicken contaminated with *C. perfringens* type A (Hook et al., 1996; Schiemann, 1977). The source of contaminations was unknown. These outbreaks were correlated to deficiencies in hygiene and food preparation, but not directly linked to chicken-origin *cpe*-positive *C. perfringens*. Therefore, the screening of *cpe* in chicken-origin *C. perfringens* isolates is continuously highlighted to provide a fundamental database to evaluate the relationship of chicken-origin *C. perfringens* with food poisoning in humans.

1.5 Necrotic enteritis (NE)

NE is one of the important enteric diseases in broiler chickens, characterized by a mass of necrosis and inflammation of small intestines, a significant decline in growth performance, and an increase in mortality (Cooper et al., 2013; Martin and Smyth, 2009). It regularly results in significant levels of mortality in clinical phase and/or loss of productivity and poor feed conversion in subclinical manifestation. Up to 37% of commercial broiler flocks could be affected and it contributed to the losses of 6.25 cents per bird and 6 billion dollars to the global poultry industry (Van Der Sluis, 2000; Wade and Keyburn., 2015). In-feed antimicrobial growth promoters (AGPs) and anti-coccidial drugs, ionophores, were past effective approaches used to prevent and control *C. perfringens*-associated NE in poultry (Liu et al., 2010). The AGPs aimed at controlling the overgrowth of bacteria, whereas the ionophores prevented the damage to mucosa caused by coccidiosis which predisposes chickens to the NE. However, phasing out of those tools based on the rising concerns for the threat of spreading bacteria with antibiotic resistance from animals to humans had spiked incidence of the disease and promoted NE as re-emerging disease in the poultry industry (Casewell et al., 2003; Gaucher et al., 2015; Timbermont et al., 2011; Van Immerseel et al., 2009). Accordingly, the industry is seeking alternative interventions or strategies for the control of this economically significant disease.

1.5.1 Etiology and host range

C. perfringens types A (rarely type C) is the causative agent of NE. A higher amount of this bacterium is associated with the appearance of necrotic lesions in the jejunum of affected birds (Paiva and McElroy, 2014). Owing to the nature of its ubiquity

(Craven, 2000; Van Immerseel et al., 2004b), it is considered that the participation of other contributing factors are required for disease development. Typically, outbreaks of NE in poultry are sporadic (Cooper et al., 2016). Chickens aged from 2 weeks to 6 months are susceptible to NE, and this disease ordinarily affects broiler flocks raised on the litter between 2 to 6 weeks of age due to a window in the chick's anti-clostridial immunity (La Ragione and Woodward, 2003). The disease usually persists in a flock for 5 to 10 days (Merck Veterinary Manual, 1998). Mortality rates are between 2% and 10% in the acute phase; however, rates as high as 50% have been reported (Paiva and McElroy, 2014). Two forms of NE are noted in the field. Clinical or named as an acute form of NE has typical signs, including severe depression, reluctance to move, ruffled feathers, diarrhea, huddling, anorexia, and high mortality. The progress of clinical illness is often acute, with birds dying within 1 to 2 hours after the onset of clinical signs. However, the subclinical form of NE affects chickens without premonitory symptoms, leading to decreased feed conversion rates and poor growth performance which contributes to main economic losses to the poultry industry. NE has also been reported in 3 to 6-month-old commercial layers and in 12 to 16 week-old replacement pullets. In addition, a wide variety of avian species could be affected as well, including turkeys, ostriches, quails, capercaillies, geese, bluebirds, lorikeets, and crows (Cooper et al., 2013).

1.5.2 Epidemiology

The occurrence of NE varied in different temporal and spatial sequence. A cross-sectional survey of 857 broiler farms in the UK indicated a prevalence rate in the recently reared flock was at 12.3% (Hermans and Morgan, 2007). The disease can occur more

than once a year on any particular farm. Univariate analysis showed numerous factors associated with the occurrence of NE, such as the existence of other diseases, environments, husbandry, hygiene and biosecurity, feed and feeding, the use of preventative or curative treatments, and flock performance ($p \leq 0.05$) (Hermans and Morgan, 2007). Multilevel logistic regression demonstrated strong associations between NE and the occurrence of wet litter and coccidiosis (Hermans and Morgan, 2007). The use of disinfectant containing ammonia against coccidial oocysts was an independent risk factor. In addition, the positive association between NE and the use of plasterboard walls in poultry houses indicated an important role of cleaning and disinfection in the epidemiology of this disease (Hermans and Morgan, 2007). However, a subclinical form of NE regularly goes unobserved. It is difficult to depict the exact sense of the epidemiology of subclinical form.

1.5.3 Pathogenesis

The occurrence of clostridial enteritis is primarily correlated with the proliferation of *Clostridia* in the intestinal tract, which is followed by the incremental production of toxins (Theoret et al., 2016). In the case of NE, the disease starts when *C. perfringens* proliferates anarchically in the small intestine of chickens, producing toxins that cause necrosis (Cooper et al., 2016). During the initial disease development, *C. perfringens*-generated bacteriocins to inhibit the growth of other *C. perfringens* populations, particularly for isolates from healthy birds, allowing NE-producing strain to be dominant (Timbermont et al., 2014; Timbermont et al., 2011). Simultaneously, this strain harbors attachment factors to efficiently adhere to extracellular matrix molecules to perform the colonization. In addition, proteolytic enzymes produced by NE strain affect basement

membrane of the villous enterocytes to establish initial infection. By achieving single dominance, filling open niches, and securing invasion in the host, toxins secreted by *C. perfringens* exert their enterotoxicity in the small intestines (Bailey et al., 2015; Keyburn et al., 2008; Li et al., 2011).

1.5.4 Predisposing factors

The ubiquitous nature renders *C. perfringens* unable to develop the disease without other factors (Craven, 2000; Van Immerseel et al., 2004b). Experimentally, a variety of predisposing factors have been unveiled to promote the onset and development of NE by contributing to the colonization, overgrowth, and infection of *C. perfringens*, including parasites (*Eimeria* spp.), dietary factors (high level of protein or non-starch polysaccharides in diet), immunosuppressive diseases (Infectious Bursal Disease, Chicken Infectious Anemia, Marek's Disease), mycotoxin, host genetics, high stock density, and low temperature (Lee et al., 2011; Park et al., 2008; Prescott et al., 2016b; Thompson et al., 2006; Williams, 2005; Williams et al., 2003).

1.5.4.1 Coccidiosis

Coccidiosis is a parasitic disease in poultry caused by pathogenic *Eimeria* spp. These parasites colonize the small intestine and cause extensive damage to the intestinal mucosa as a consequence in their lifecycle (Williams, 2005). A preceding or co-infection with *Eimeria* targeting the small intestine is demonstrated as a major contributor to NE in chickens. They have multiple effects that promote NE development. For instance, physical damages to the gut epithelium facilitate the colonization and proliferation of *C. perfringens* (Van Immerseel et al., 2009; Williams, 2005; Williams et al., 2003). The

intestinal lumen then becomes rich with plasma proteins that serve as growth substrates for *C. perfringens* (Van Immerseel et al., 2004b). In addition, reduced digestibility due to extensive gastrointestinal damage substantially also increases nutrient availability for the growth of *C. perfringens*. Moreover, coccidiosis induces a T-cell-mediated inflammatory response that enhances mucin production, which is beneficial for the overgrowth of C. *perfringens* due to its ability to utilize mucus as a substrate (Collier et al., 2008).

1.5.4.2 Diets

Diets enriched with cereals (wheat, rye, barley, or oats) or high level of proteins such as fishmeal has been demonstrated as predisposing factors affecting the onset of NE. (Cooper and Songer, 2009; McDevitt et al., 2007; Williams, 2005). It was suggested that cereals (barley, rye, oats, and wheat) containing high levels of indigestible, water-soluble, non-starch polysaccharides (NSP) increased the viscosity of the digesta and hence slowed transit time in the gut (Annett et al., 2002; Langhout et al., 1999). NSP could also leave undigested nutrients available for microbial proliferation (Choct and Annison, 2007), change the mucosal architecture, and increased the number of intestinal goblet cells per crypt to elevate mucin production (Kleessen et al., 2003), thus promoting the overgrowth of *C. perfringens* (Shojadoost et al., 2012). High animal protein diets, especially those supplemented with fishmeal, provide high levels of available nutrients, such as specific amino acids that *C. perfringens* is unable to synthesize itself, subsequently improving its growth (Drew et al., 2004; Titball et al., 1999). Furthermore, the addition of fishmeal in the diet can increase intestinal pH, favoring the proliferation of *C. perfringens* (McDevitt et al., 2007), destabilize and alter the underlying gut microbial population, thus predisposing chickens to develop NE (Stanley et al., 2012; Stanley et al., 2014b).

1.5.4.3 Immunosuppression

Changes in the immune status of birds can increase the incidents of NE (Moore, 2016). Infectious bursal disease (IBD), chicken infectious anemia (CIA), and Marek's disease (MD) which have immunosuppressive effects in poultry (Hoerr, 2010) can increase the severity of NE and promote the development of NE in the field (Gholamiandehkordi et al., 2007; Stringfellow et al., 2009; Timbermont et al., 2009; Williams et al., 2003) . Other stresses on chickens such as high stocking density/overcrowding, heat or cold, environmental ammonia, and physiological stress also may cause immunosuppression that exacerbates NE (Hoerr, 2010; Tsiouris et al., 2015a, b).

1.5.4.4 Mycotoxin

Mycotoxins are secondary fungal metabolites naturally produced by a wide range of fungi, primary molds in crops and feeds. Aflatoxins (AF), zearalenone (ZEN), ochratoxin A (OTA), fumonisins (FUM), trichothecenes such as deoxynivalenol (DON), and T-2 toxin are some of the mycotoxins that have the dramatic impact on the health and productivity of poultry (Murugesan et al., 2015). Recently, DON was shown as a predisposing factor for the development of NE, through damaging to the epithelial barrier and increasing intestinal nutrient availability for the proliferation of *C. perfringens* (Antonissen et al., 2014). Besides, another mycotoxin, FUM, produced by Fusarium fungi was demonstrated to modify the composition of the intestinal microbiota of the ileum and negatively affect the small intestinal length, ileal villus height, and crypt depth, predisposing chickens to NE (Antonissen et al., 2015). Although the immunosuppression

of mycotoxin was also suggested in affected chickens (Prescott et al., 2016b), the effect needs to be further investigated.

1.5.4.5 Other factors

Host genetics were reported to exert some influence on susceptibility to NE that certain breed of broilers appears to be more susceptible to NE in the field based on the observations of body weight loss, greater gut lesion severity, and higher NetB antibody levels (Jang et al., 2013). This difference of susceptibility may result from the difference in immune responses to *C. perfringens* (Kim et al., 2014). In addition, a retrospective study also showed the association of host genetics with a natural outbreak of NE in laying hens. Large differences between major histocompatibility complex (MHC) genotypes (genotype $B^{21}B^{21}$ being more resistant to NE compared to genotype $B^{13}B^{13}$) was noted in relation to mortality and hen-day egg production in NE-afflicted laying hen flocks (Siegel et al., 1993).

1.6 Gut microbiota and its role in enteric diseases

The chicken intestinal microbiota comprises various bacteria, methanogenic archaea, fungi, and viruses (Yeoman et al., 2012). Microbial colonization varies along the length of the gastrointestinal tract. Major taxa surveyed along the chicken gastrointestinal tract (GIT) showed microbial density was 10^7 and 10^8 CFU/g in the gizzard with the main species of bacteria being *Lactobacilli*. The bacterial density increased to between 10^8 and 10^9 in the small intestine with dominant bacteria that are *Lactobacilli*, *Clostridia*, *Escherichia* and *Enterococcus*. At the distal intestine, the density increased to 10^{10} to 10^{12} and bacterial populations are *Bacteroide*, *Fusibacteria*, *Coliforms*, *Lactobacilli*,

Clostridia, etc. (Gong et al., 2002; Qu et al., 2008; Saengkerdsub et al., 2007a; Saengkerdsub et al., 2007b). Among whole intestines, ceca are the site that has the most microbial diversity and abundance in the intestines (Gong et al., 2007).

Gut microbiota has functions on the nutrient exchange, modulation of the immune system, maintenance of the physiology of the digestive system, and the exclusion of pathogens (Clavijo and Florez, 2018; Pan and Yu, 2014). Chicken gut microbiota produces enzymes enabling the deconstruction of dietary polysaccharides (Beckmann et al., 2006), and producing short-chain fatty acids (SCFAs). SCFAs are the primary energy source of intestinal epithelia, essential to homeostasis of colonocytes and development of villus morphology, and important to colonic musculature and vasculature in the GIT (Yeoman et al., 2012). Germ-free birds have been found to possess shorter intestinal villi, shallower crypts, and lower load of bacteria when compared to conventionally-raised birds (Forder et al., 2007; Gabriel et al., 2006). Additionally, gut microbiota has been demonstrated to regulate intestinal gene expression (Yin et al., 2010) and T cell-mediated immunity (Mwangi et al., 2010) as well as to accelerate the maturation of the gut immune system (Crhanova et al., 2011). Healthy intestinal microbiota provides host resistance to infection through their involvement in the development of the host immunity and competitive exclusion to limit the colonization of pathogens (Rehman et al., 2007). Hence, disruptions of the microbial community were suggested to alter host susceptibility to infections and play a role in the development of enteric disease as well (Sekirov and Finlay, 2009).

1.7 Treatment and prevention of NE

1.7.1 Treatments

Treatment for NE greatly depends on antibiotics specifically designed to affect gram-positive bacteria such as bacitracin, penicillin, virginiamycin, and lincomycin to destroy clostridia and/or diminish bacterial populations in the intestines (Brennan et al., 2001a; Brennan et al., 2001b; Hamdy et al., 1983). Traditionally, the poultry industry used antimicrobial growth promoters (AGPs) in the feed to effectively treat and prevent NE (Liu et al., 2010). However, the emergence of antibiotic-resistant bacteria from animals and the potential threat of transmission to humans led to ban or limited use of AGPs (Marshall and Levy, 2011; Van Immerseel et al., 2004b). Due to the withdrawal of AGPs, NE has spiked the incidence and re-emerged in broiler production system (Casewell et al., 2003; Gaucher et al., 2015; Timbermont et al., 2011; Van Immerseel et al., 2009).

1.7.2 Measures to prevent and control NE

Recently, strategies for preventing NE are focusing on interventions that reduce exposure to risk factors, boost immunity, avoid or decrease damage to the intestinal mucosa, and inhibit the overgrowth of *C. perfringens* in the intestines (Brennan et al., 2001a). Since NE has been often a secondary infection in a flock with a coccidial infection, an effective control of coccidiosis is a reasonable way to significantly reduce the risk of NE outbreaks. Coccidiostatic drugs and anticoccidial vaccines are usually applied singly or together to limit the potential of coccidial exposure and provide coccidial immunity (Li et al., 2004; Williams, 2002), subsequently preventing the damage to the mucosa in the small intestines. Regarding induced immunity from NE

vaccine, an effective protection against the disease has not been developed as of yet (Alimolaei et al., 2018; Lillehoj et al., 2017; Mot et al., 2014; Thompson et al., 2006). However, recent pieces of evidence indicate that mucosal immune responses are crucial for the protection and multiple antigens are required for promoting the essential immunity (Jiang et al., 2015; Mot et al., 2014). To avoid mucosal damage and multiplication of *C. perfringens*, alternations to feed composition by removing NSP and high level of proteins have been shown some effects on NE reduction (Shawkat A. M'Sadeq, 2015). Addition of exogenous enzymes in wheat-based diets also can significantly decrease digesta viscosity in the small intestine, which in turn reduce the number of nutrients available to the bacterial growth (Choct et al., 1999).

Although several strategies have been developed to prevent and control NE, none of them provided the desirable or consistent protection against NE as AGPs did before. Hence, the poultry industry is still seeking for alternative supplements in the feed to replace AGPs. Probiotics, prebiotics, organic acids, various plant extracts such as essential oils, and feed enzymes were examined and demonstrated the different degree of effects on the reduction of NE (Caly et al., 2015; Dahiya et al., 2006).

1.7.2.1 Probiotics

Probiotics, defined as live microbial feed supplements, have been demonstrated to benefit chickens by improving immunity and intestinal morphology, stimulating the metabolism, producing molecules with antimicrobial activities (e.g. bacteriocins), inhibiting the adhesion of pathogens and the production of pathogenic toxins, or competing against pathogenic strains within the host (Caly et al., 2015; Pan and Yu, 2014). Different supplemented bacteria showed variable effects. For instance,

Lactobacillus agilis and *Lactobacillus salivarus* have the ability to stimulate the butyrate-producing microbiota and to re-establish the balance of microbiota (Meimandipour et al., 2009). Bacteria of the genera *Bifidobacterium* and *Lactobacillus* help to reduce adhesion of pathogenic microorganisms such as *Salmonella*, *Enterobacter sakasaki*, and *Clostridum difficile* (Collado et al., 2005; Servin and Coconnier, 2003). However, the effectiveness of probiotics is controversial with positive effects in some studies while intangible results in others (Clavijo and Florez, 2018). Accordingly, more studies are suggested to evaluate the significance of these applications on NE reduction.

1.7.2.2 Prebiotics

Prebiotics are non-digestible feed ingredients that selectively affect the host by stimulating growth or activity of beneficial flora in the intestine (Gibson and Roberfroid, 1995). The aim of using prebiotic is to stimulate acidolactic and bifidogenic bacteria in GIT. Their functions include attachment to pathogens to prevent colonization, the increment of luminal osmosis, being substrates for fermentation, indirect stimulation of macrophages and production of SCFAs, and immune modulation (Patel and Goyal, 2012). Several studies in chickens showed pieces of evidence that fructo-oligosaccharides (FOS) and manno-oligosaccharides (MOS) had positive effects on inhibition of *Salmonella* and *E. coli* (Chambers and Gong, 2011; Stanley et al., 2014a). In the case of the NE, dietary supplementation with MOS decreased the abundance *of C. perfringens* in the small intestine (Kim et al., 2011) and ceca (Yang et al., 2008) as well as reduced disease mortality and severity (Mohamed and S Abdel Hafez, 2011). Nonetheless, a previous study demonstrated that neither addition of FOS or MOS in feed significantly

affected weight gain, feed conversion rate, and NE mortality in 6-week-old broilers (L. Hofacre et al., 2003).

1.7.2.3 Organic acids

Organic acids are extensively used in feed due to their preservative effects on fungal and microbial inhibitions, (Kum et al., 2010). In addition, they also exert the antibacterial activity for a long time (Nieman, 1954). These acids used as additives in feed enhance growth, feed utilization, and the conversion rate for broilers (Hassan et al., 2010). Among them, lauric acid, a member of medium-chain fatty acids (MCFAs), was found to have strong *in vitro* antimicrobial activity against gram-positive organisms (Hermans et al., 2012; Van Immerseel et al., 2004a; Zeiger et al., 2017) and *C. perfringens* (Skrivanova et al., 2005; Timbermont et al., 2010). Butyrate, a short-chain fatty acid (SCFA), does not inhibit *C. perfringens* in birds (Jerzsele et al., 2012) but could promote the growth of villus (Kien et al., 2007) and is beneficial for the prevention or regeneration of epithelial damages (Timbermont et al., 2010). Organic acids have not been shown to effectively protect chickens from NE as they protect against *E. coli* (Izat et al., 1990) and *Campylobacter* (Chaveerach et al., 2004). A recent study demonstrated that the combination of butyrate and lauric acid in feed gave the best protection against the induction of NE lesions, indicating these additives can be used to control NE in broiler chickens (Timbermont et al., 2010).

1.7.2.4 Plant extracts and essential oils

Essential oils are secondary compounds extracted from plants. They are documented to improve intestinal health by enhancing digestions, modifying digestive

secretions, and supporting the histology of the intestine (Diaz-Sanchez et al., 2015). Besides, essential oils are particularly characterized as substances that engage in antimicrobial activities. Carvacrol and thymol obtained from oregano as well as eugenol from clove plants have been demonstrated to have an inhibitory effect against a wide range of pathogenic bacteria (Dorman and Deans, 2000). For NE, thymol, cinnamaldehyde, and eucalyptol were shown to have antimicrobial activity against *C. perfringens* (Candan et al., 2003; Juneja and Friedman, 2007; Mitsch et al., 2004). Some blends of essential oil even could reduce colonization and proliferation of *C. perfringens* (Mitsch et al., 2004). In addition, essential oils have also been reported to have an anticoccidial effect, thus may potentially have protective effects on NE (Giannenas et al., 2003). However, the effectiveness of essential oils still varies (Cross et al., 2007; Gonzalez-Gil et al., 2014) mainly because of the difference of their active components from different methods of extraction, geographical origin, plant genotype, and storage time (Clavijo and Florez, 2018). Further research is required to verify its effects on NE prevention and control.

1.7.2.5 Feed enzymes

The objective of adding enzymes in the feed is to improve digestion that could potentially reduce the risk of NE. Supplementation of xylanase into wheat-based diets has been demonstrated to decrease digesta viscosity and fermentation, increase nutrient digestion and digesta passage, and reduce the number of nutrients available to the microflora (Choct et al., 1999; Guo et al., 2014). In addition, xylanase could alleviate the impairment of intestinal mucosal barrier induced by *C. perfringens* challenge (Liu et al., 2012). The inclusion of β-mannanase in the feed was shown to reduce the severity of NE

in broiler chickens, but the effect was not superior to bacitracin or salinomycin treatment to limit the severity of lesions (Jackson et al., 2003). The addition of exogenous lysozyme significantly reduced the concentration of *C. perfringen*s in the ileum and the intestinal lesion scores, suggesting that the lysozyme could decrease *C. perfringens* colonization and subsequently reduce the NE incidence (Liu et al., 2010). However, supplementation of pentosanase in a wheat-based diet did not affect the level of NE-induced mortality (Riddell and Kong, 1992). Furthermore, the addition of protease and xylanase in the diets of broiler chickens did not reveal significant mitigation effect on NE-affected birds (Barekatain et al., 2013). These contradictory results indicate the supplementation of exogenous enzymes alone cannot provide sufficient protection. It should be incorporated with other interventions to exert the synergistic effect against NE.

1.8 Summary

NE, not resembling other clostridial diseases, is a complex disease that involves the participation of the pathogen, contributory factors, and host responses. Its economic significance drives the poultry industry to seek for solutions to control the disease. Nevertheless, the multifactorial nature of its pathogenesis makes the prevention and control of NE a real challenge. Although advances in NE research have greatly contributed to identifying predisposing factors and preventative resources, the ultimate goal of effectively preventing and treating NE without the aid of AGPs has not been achieved. To date, application of strategies such as the use of vaccines, prebiotics, probiotics, or other feed additives demonstrated a variable degree of protection against NE without consistent efficacy. Therefore, continuing works on the understanding of

pathogenesis and interaction of contributory factors with *C. perfringens* are required for developing practical measures to prevent and control this disease.

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CHAPTER II

CHARACTERIZATION OF TOXIN GENES AND QUANTITATIVE ANALYSIS OF *NETB* IN NECROTIC ENTERITIS-PRODUCING AND NON-NE-PRODUCING *CLOSTRIDIUM PERFRINGENS* ISOLATED FROM CHICKENS

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Abstract

Necrotic enteritis (NE) in chickens, a *Clostridium perfringens* (*C. perfringens*) infection, has re-emerged due to the removal of antibiotic growth promoters in feeds in recent years, thus contributing to significant economic losses for the industry. Toxins produced by *C. perfringens* in conjunction with predisposing factors are responsible for the onset and development of NE. Recently, several lines of evidence indicated the potential role of plasmid-encoded toxins in the virulence of NE, particularly necrotic enteritis B-like (NetB) toxin. However, the association of NetB, beta2 toxin (CPB2), and *C. perfringens* large cytotoxin (TpeL) in clinical NE isolates are not well-established. Therefore, we characterized the toxinotype and the presence of *netB*, *cpb2*, and *tpeL* genes in 15 NE-producing and 15 non-NE-producing *C. perfringens* isolates using conventional PCR and quantified *netB* among those isolates by quantitative PCR (qPCR). All isolates were characterized as toxinotype A and were negative for *cpe*, which is associated with human food poisoning. The *netB* was detected in 6.7% and 70% of NE-producing isolates by PCR and qPCR, respectively. In 15 non-NE-producing isolates, *netB* was not detected by conventional PCR, but was detected in 60% of isolates by qPCR. The presence of and the copy number of *netB* were not significantly different between NE- and non-NE-producing isolates ($p > 0.05$). No difference was observed between NE- and non-NE-producing isolates in the presence of *cpb2* or *tpeL* ($p > 0.05$). These results suggest that the presence of *netB*, *cpb2*, and *tpeL*, as well as the copy number of *netB* in *C. perfringens* is not correlated with clinical NE. In addition, we suggest that qPCR, but not conventional PCR, be used to detect *netB*.

2.1 Introduction

Necrotic enteritis (NE) is an important enteric disease in broiler chickens (Cooper et al., 2013) characterized by necrosis and inflammation of the small intestine. NE is caused by *Clostridium perfringens*, an anaerobic, endospore-forming, and a Gram-positive bacterium, which results in significant levels of mortality in the clinical phase and loss of productivity and poor feed conversion in the subclinical form. Up to 37.3% of commercial broiler flocks could be affected by NE, contributing to an annual loss of 6.25 cents per bird and 6 billion dollars to the global poultry industry (Van Der Sluis, 2000; Wade and Keyburn., 2015). In the past, in-feed antimicrobial growth promoters (AGPs) were the most effective strategy used to control *C. perfringens*-associated NE in poultry (Liu et al., 2010). However, the phase-out of AGPs due to concerns regarding the spread of antibiotic resistance has resulted in a spike in NE incidence, and hence considered as a re-emerging disease by the poultry industry (Casewell et al., 2003; Gaucher et al., 2015; Timbermont et al., 2011; Van Immerseel et al., 2009).

C. perfringens is classified into A, B, C, D, and E toxinotypes based on the production of four major toxins, alpha (α), beta (β), epsilon (ε), and iota (ι) toxins (Van Immerseel et al., 2004). NetB, a pore-forming toxin from NE strains *of C. perfringens,* has limited protein sequence identity to the beta-toxin of *C. perfringens*, which causes mucosal necrosis of the small intestine in humans and animals. It plays a major role in the virulence of NE based on an *in vivo* study demonstrating that the wild-type and complemented *netB C. perfringens* strain produced characteristic lesions in the small intestine, but a null mutant could not in contrast, a *cpa* mutant strain that did not produce CPA toxin, still produced NE in an experimental challenge study (Keyburn et al., 2006).

Although the expression of *netB* fulfilled the molecular Koch's postulate, several lines of evidence dispute the importance of *netB* on the pathogenesis. A proportion of *C. perfringens* strains isolated from healthy chickens possess *netB* with some degree of *in vitro* NetB toxin production, but these chickens do not develop NE after challenges with *netB*-positive isolates (Abildgaard et al., 2010; Chalmers et al., 2008; Martin and Smyth, 2009). Low carriage rates of *netB* in *C. perfringens* isolates from NE-affected chicken farms have been noted (Bailey et al., 2015), and isolates without *netB* also produce NE (Abildgaard et al., 2010; Bailey et al., 2015; Llanco et al., 2015; Martin and Smyth, 2009; Smyth and Martin, 2010). In addition, use of *netB* positive *C. perfringens* alone, without predisposing factors, failed to consistently reproduce NE in the challenge model (Zhou et al., 2017). These findings indicate that the pathogenesis of NE is highly complex and other factors should be involved and crucial in the disease development. Meanwhile, a number of studies demonstrated that predisposing factors were required for NE development (Moore, 2016; Shojadoost et al., 2012; Williams, 2005), and with a combination of predisposing factors, degradative enzymes, and toxins, *C. perfringens* could conceivably trigger NE, resulting in intestinal tissue destruction, bacterial colonization, and enterotoxicity (Bailey et al., 2015; Keyburn et al., 2008; Li et al., 2011). Besides, isolations of *netB*-positive *C. perfringens* from healthy birds further provide the evidence that a simple *C. perfringens* infection without other components is not sufficient to induce NE. Therefore, it is worth examining the possibility that *netB* may originally exist in all *C. perfringens*, not exclusively in NE-producing isolates.

Last but not least, Nakano et al. recently found that *netB* and *tpeL* were also present in *C. perfringens* strains isolated from healthy humans by PCR assay (Nakano et

al., 2017), pointing out the possibility that the presence of those genes may not correlate with the virulence of NE. Based upon these findings, we hypothesized that *C. perfringens* isolates from NE and non-NE chickens may harbor *netB* and other virulence genes regardless of the origin of the isolates. Traditionally, analysis of toxin genes in *C. perfringens* depends on a conventional or multiplex PCR, but quantitative real-time PCR (qPCR) is recognized as the superior technique with higher sensitivity and quantitative ability. Therefore, the aim of this study was to investigate the relevant toxin genes (*cpa*, *cpb*, *etx*, *iap*, *cpe, cpb2*, *tpeL*, and *netB*) in NE-producing and non-NE-producing *C. perfringens* by PCR and to quantify the virulence gene, *netB,* by qPCR. This study provides a better understanding of the association of *netB* with clinical NE and also demonstrates a sensitive qPCR assay to detect the presence of toxin gene.

2.2 Materials and methods

2.2.1 Isolation and identification of *C. perfringens* from NE-diseased and non-NE chickens

The jejunum was targeted and collected to isolate *C. perfringens* in this study. NE-diseased chicken was defined by observing clinical symptoms and characteristic lesions in the small intestine. On the contrary, non-NE chicken was grossly inspected and determined by the absence of NE lesions in the same site of the gut. Fifteen jejunal tissue samples of NE-diseased chickens were obtained from thirteen broiler farms. For non-NE counterpart, four out of fifteen samples were collected from a processing plant and five jejunums originated from chickens at each different houses within two separated farms. The remaining samples were collected from specific pathogen free (SPF) layers and broilers evenly in untreated control groups of two chicken trails. Afterwards, *C.*

perfringens were isolated by streaking scrapings of the jejunal mucus membrane on blood agar plates (Fisher Scientific, Pittsburgh, Pennsylvania, USA) followed by an overnight anaerobic incubation at 37°C. The anaerobic condition was achieved by using container system sachets (BD Biosciences, San Jose, California, USA) or anaerobic jar (Sigma, St. Louis, Missouri, USA) with anaerobic gas generators (Fisher Scientific). A single colony with double hemolytic zones was subsequently transferred to an egg yolk agar plate (HiMedia, Mumbai, Maharashtra, India) for an additional overnight anaerobic incubation at 37°C. Following incubation, a colony harboring a positive lecithinase reaction was selected and cultured on a highly selective tryptone sulfite neomycin (TSN) agar plate (Sigma) and incubated anaerobically at 46°C for an additional 18 to 20 hours. A black colony presumptively identified as *C. perfringens* was then transferred to 10 ml thioglycolate broth (Himedia) and anaerobically incubated at 37°C overnight for further identification, total DNA extraction, and stock storage. Each strain or isolate was confirmed as *C. perfringens* using RapID ANA II biochemical tests (Fisher Scientific) and PCR assays targeting the *16S rRNA* gene (Table 2.1).

Table 2.2 PCR and qPCR targeted genes for *C. perfringens*, according primers and amplicon lengths

Gene	GenBank accession number	Primers	Sequence (5′-3′)	T_m (°C)	Product (bp)	Reference
cpa	L43545	CPA5L CPA5R	AGTCTACGCTTGGGATGGAA TTTCCTGGGTTGTCCATTTC	55	900	(Fan et al., 2016)
cpb	X83275	CPBL CPBR	TCCTTTCTTGAGGGAGGATAAA TGAACCTCCTATTTTGTATCCCA	56	611	(Fan et al., 2016)
cpb2	L77965	CPB2L CPB2R	AGATTTTAAATATGATCCTAACC CAATACCCTTCACCAAATACTC	53	567	(Garmory et al., 2000)
etx	M95206	CPETXL CPETXR	TGGGAACTTCGATACAAGCA TTAACTCATCTCCCATAACTGCAC	56	396	(Fan et al., 2016)
iap	X73562	CPIL CPIR	AAACGCATTAAAGCTCACACC CTGCATAACCTGGAATGGCT	57	293	(Fan et al., 2016)
cpe	X81849	CPEL CPER	GGGGAACCCTCAGTAGTTTCA ACCAGCTGGATTTGAGTTTAATG	57	506	(Fan et al., 2016)
tpeL	EU848493	TPELF TPELR	ATATAGAGGCAAGCAGTGGAG GGAATACCACTTGATATACCTG	55	466	(Coursodon et al., 2012)
netB	GU433338	NETBL NETBR	TGATACCGCTTCACATAAAGGTTGG ATAAGTTTCAGGCCATTTCATTTTTCCG	61	169	*This study*
16S rRNA	Y12669	16SL 16SR	CATCATTCAACCAAAGGAGCAATCC CATTATCTTCCCCAAAGACAGAGC	60	262	*This study*

2.2.2 Bacterial DNA extraction

Total DNA was isolated from the overnight broth-brown culture inoculated with single colony of *C. perfringens* from plate by using an Ultraclean Microbial DNA Isolation Kit (Mobio, Germantown, Maryland, USA) following the manufacturer's instructions with some modifications. Briefly, the bacterial suspension was mixed with a lysis buffer and 20 µl of 20 mg/ml proteinase K (Fisher Scientific). The mixture was subsequently incubated at 65°C for 15 minutes to lyse the bacterial cell wall and to prevent DNase digestion of DNA. In order to avoid DNA shearing, the tube containing

the reaction mixture was secured on a flat pad and horizontally vortexed for 10 minutes. DNA was sequentially eluted with 30 µl 10 mM Tris-HCl (pH 8.0), separated on a 0.8% agarose gel (BD Biosciences) for the quality assurance, and stored at -20°C until use.

2.2.3 PCR amplification of *C. perfringens* toxin genes

Major toxin genes, including *cpa*, *cpb*, *etx*, and *iap*, as well as minor toxin genes, *cpe*, *netB*, *cpb2,* and *tpeL*, were detected by PCR with specific primers (Table 2.1). ATCC strains 13124, 3626, 12917, 51880, and 27324 were used as reference strains for toxinotyping (A, B, C, D, and E, respectively) and as positive controls for *cpb2* and *cpe*. Strain JP17 kindly provided by Dr. John F. Prescott was used as the positive control for *netB* and *tpeL*. PCR amplification was performed in a 20-µl volume containing 50 ng template DNA, 10× *Taq* buffer $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 0.2 mM dNTP mix, 1U *Taq* DNA polymerase (Fisher Scientific, Pittsburgh, Pennsylvania, USA), and 0.2 µM of each primer on a thermocycler (Applied Biosystems GeneAmp PCR System 9700). The protocol was as follows: 1 cycle of 95°C for 2 minutes; 35 cycles of 95°C for 1 minute, annealing temperature for 1 minute, and 72°C for 1 minute; and a final extension at 72°C for 10 minutes. For *netB*, the assay conditions were modified to: 1 cycle of 95°C for 3 minutes; 35 cycles of 95°C for 30 seconds, annealing temperature for 30 seconds, and 72°C for 30 seconds; a final extension cycle of 72°C for 5 minutes. The amplified products were analyzed on a 1.5% agarose gel and stained with SYBR Safe DNA Gel Stain (Fisher Scientific). PCR amplified products on the gels were extracted, purified, and sequenced to ensure 100% match of reference sequences.

2.2.4 Quantitative real-time PCR (qPCR)

To estimate relative abundance of *netB* in NE- and non-NE-producing *C. perfringens* isolates, 20 µl reaction mixture was analyzed using a QuantStudio 5 Real-Time PCR System (Fisher Scientific). The mixture contained 10 µl PowerUp SYBR Green Master Mix (Fisher Scientific), 0.5 µM *netB* primer (Table 2.1), and 100 ng template DNA. Three technical replicates along with corresponding positive and negative controls were included for each plate. The qPCR amplification cycles were as follows: 1 cycle of 95°C for 2 minutes; followed by 45 cycles of denaturation at 95°C for 5 seconds, annealing and extension at 60°C for 30 seconds, and dissociation steps at 95°C for 1 second; 60°C for 20 seconds; and 95°C for 1 second. To differentiate the specific *netB* amplicon from non-specific products, the DNA melting curve was performed to confirm the specific amplicon. Likewise, amplified products were separated on 1.5% agarose gels to confirm that the size was the same as the positive control.

2.2.5 Efficiency of the qPCR assay

The PCR product of *netB* was cloned into pGEM®-T Easy Vector Systems (Promega, Madison, Wisconsin, USA) to generate plasmid DNA containing specific sequence of *netB*. The amplification efficiency (AE) of the qPCR for *netB* was examined using a 10-fold serial dilution of the plasmid DNA ranged from 1 ng to 100 attogram (ag). Standard curve was established based on the threshold cycles (Ct) plotted against the $\log_{10}$ values of the gene copies (Log-copy). The linearity was observed with the equation: $y = -3.4571x + 38.397$ ($R^2 = 0.9999$; AE = 95%), where y is the threshold cycles and x is the amount of the targeted gene with $\log_{10}$ value (Figure 2.1).

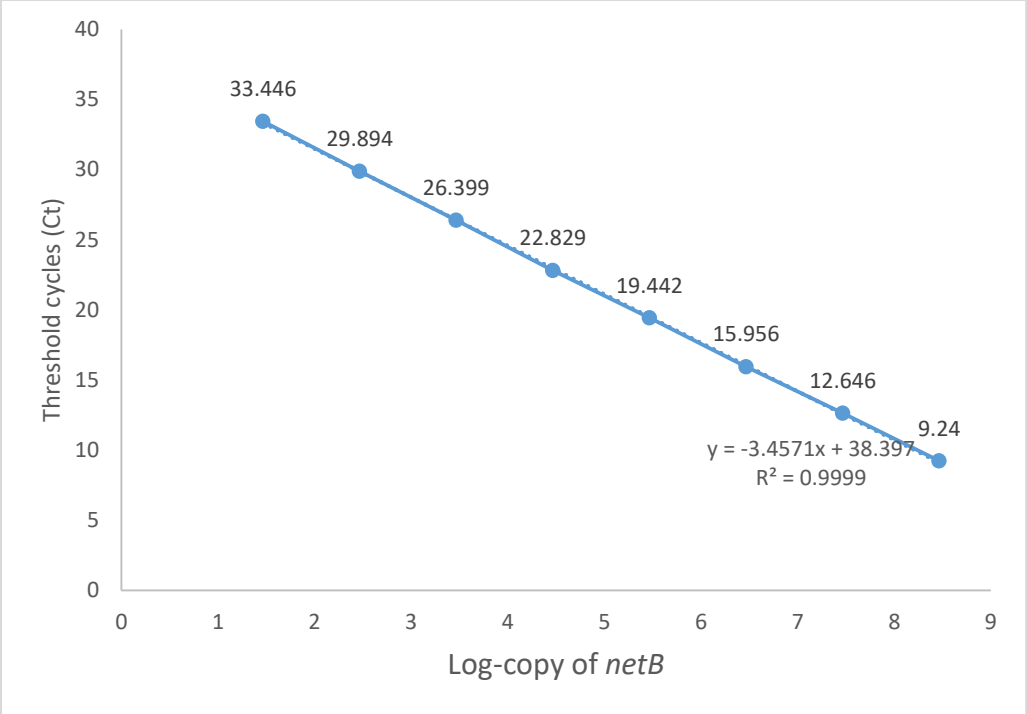

Figure 2.1 Standard curve of *netB* by qPCR.

It shows Ct-values of isolate samples plotted versus the log value of quantified *netB* copy numbers. The 10-fold serial dilutions (from 1 ng to 100 ag) were prepared ranging from 2.92×10^8 to 29.2 copies of *netB* gene. The linearity was observed with the equation: y = -3.4571x + 38.397 ($R^2 = 0.9999$; AE = 95%).

2.2.6 Statistical analysis

The average threshold cycle (Ct) from the positive replicate samples was used for calculating the copy number of *netB*, which was expressed as $\log_{10}$ copies/100 ng DNA. After log transformation of the copy number, the difference in copies between NE- and non-NE-producing *C. perfringens* was analyzed with the Wilcoxon Rank-Sum Test using SAS software version 9.4 (SAS Institute, Inc., Cary, North Carolina, USA). Fisher's exact test was used to compare the difference in frequency of *netB, cpb2*, and *tpeL* between NE- and non-NE-producing *C. perfringens* isolates. A level of *0.05* was considered statistically significant.

2.3 Results

2.3.1 Isolate identification

Thirty *C. perfringens* isolates were recovered from the jejunum of clinical NE and non-NE chickens in 15 broiler farms, one processing plant, and two experimental control groups (Table 2.2). All isolates were identified as *C. perfringens* through biochemical tests and *16S rRNA* gene sequence confirmation by PCR.

Table 2.3 Distribution of toxin genes and copy number of *netB* in NE- and non-NE-producing *Clostridium perfringens* isolates

Isolate	Origin	Source	Health status	Type	*cpa*	*cpb*	*etx*	*iap*	*cpe*	*cpb2*	*tpeL*	*netB*		
												PCR	qPCR	Copies/100 ng DNA
JP17	Reference	N/A	N/A	A	+	–	–	–	–	+	+	+	+	$>2.92\times10^8$
1N	Broiler	F1	NE	A	+	–	–	–	–	+	+	+	+	$>2.92\times10^8$
2N	Broiler	F2	NE	A	+	–	–	–	–	–	–	–	–	–
3N	Broiler	F3	NE	A	+	–	–	–	–	+	–	–	+	*7.26×10^3*
4N	Broiler	F4	NE	A	+	–	–	–	–	+	–	–	+	*4.19×10*
5N	Broiler	F5	NE	A	+	–	–	–	–	–	–	–	+	*$<2.92\times10$*
6N	Broiler	F6	NE	A	+	–	–	–	–	–	–	–	+	*1.35×10^3*
7N	Broiler	F7	NE	A	+	–	–	–	–	+	+	+	+	$>2.92\times10^8$
8N	Broiler	F8	NE	A	+	–	–	–	–	+	–	–	+	*$<2.92\times10$*
9N	Broiler	F9	NE	A	+	–	–	–	–	+	–	–	+	1.76×10^3
10N	Broiler	F10	NE	A	+	–	–	–	–	+	–	–	+	$<2.92\times10$
11N	Broiler	F11	NE	A	+	–	–	–	–	+	–	–	+	$<2.92\times10$
12N	Broiler	F12	NE	A	+	–	–	–	–	+	–	–	+	$<2.92\times10$
13N	Broiler	F13; H2	NE	A	+	–	–	–	–	+	–	–	–	–
14N	Broiler	F13; H2	NE	A	+	–	–	–	–	–	–	–	–	–
15N	Broiler	F13; H2	NE	A	+	–	–	–	–	+	–	–	–	–
1C	Broiler	P1	non-NE	A	+	–	–	–	–	+	–	–	+	3.11×10
2C	Broiler	P1	non-NE	A	+	–	–	–	–	+	–	–	+	7.49×10
3C	Broiler	P1	non-NE	A	+	–	–	–	–	+	–	–	–	–
4C	Broiler	P1	non-NE	A	+	–	–	–	–	+	–	–	–	–
5C	Broiler	F14; H1	non-NE	A	+	–	–	–	–	+	–	–	–	–
6C	Broiler	F14; H2	non-NE	A	+	–	–	–	–	+	–	–	+	2.96×10
7C	Broiler	F14; H3	non-NE	A	+	–	–	–	–	+	–	–	+	4.40×10
8C	Broiler	F15; H1	non-NE	A	+	–	–	–	–	+	–	–	+	$<2.92\times10$
9C	Broiler	F15; H2	non-NE	A	+	–	–	–	–	+	–	–	+	$<2.92\times10$
10C	Layer	EC1	non-NE	A	+	–	–	–	–	–	–	–	–	–
11C	Layer	EC1	non-NE	A	+	–	–	–	–	–	–	–	+	*7.23×10*
12C	Layer	EC1	non-NE	A	+	–	–	–	–	–	–	–	+	*$<2.92\times10$*
13C	Broiler	EC2	non-NE	A	+	–	–	–	–	–	–	–	–	–
14C	Broiler	EC2	non-NE	A	+	–	–	–	–	–	–	–	+	6.45×10
15C	Broiler	EC2	non-NE	A	+	–	–	–	–	–	–	–	–	–

Abbreviations: F, farm; H: house; P, processing plant; EC, experimental control; NE, necrotic enteritis; non-NE, chickens not affected by NE or healthy.
The copies of netB was calculated by the equation: $y = -3.4571x + 38.397$ ($R^2 = 0.9999$; AE = 95%).
No significant difference of mean log-copies of netB between NE and non-NE group (Wilcoxon Rank-Sum Test; $p > 0.05$).

2.3.2 Toxinotyping and detection of toxin genes by PCR

All thirty *C. perfringens* isolates were characterized as toxinotype A (Table 2.2). None of these isolates carried *cpb*, *etx*, *iap*, or *cpe*. Of the thirty isolates, twenty (66.7%) harbored *cpb2* (11 in the NE group and 9 in the non-NE group), two possessed *netB* (6.7%), and two had *tpeL* (6.7%). The prevalence of *cpb2*, *netB* and *tpeL* in NE-producing isolates was higher than that in the non-NE producing isolates. However, no statistical significance was noted. The simultaneous carriage of *netB* and *tpeL* was observed in two isolates.

2.3.3 qPCR

The carriage rate of *netB* in NE-producing *C. perfringens* was 73% (11/15) compared with 60% (9/15) in the non-NE-producing isolates (Table 2.3). The NE-producing *C. perfringens* carried a higher copy number of *netB* than the non-NE-producing isolates; however, there was no statistically significant difference ($p > 0.05$). Copies of *netB* in NE-producing isolates varied among different farms. Six NE-producing isolates carried lower *netB* copy number than non-NE producing isolates.

Table 2.4 Comparative results of *netB* carriage by PCR and qPCR in NE- and non-NE-producing *Clostridium perfringens* isolates

		Status	
		NE	non-NE
Conventional PCR	*netB* +	2	0
	netB −	13	15
	Subtotal	15	15
qPCR	*netB* +	11	9
	netB −	4	6
	Subtotal	15	15

Abbreviations: NE, necrotic enteritis; non-NE, chickens not affected by NE or healthy. No significant difference of prevalence detected by qPCR between NE and non-NE group (Fisher's exact test; $p > 0.05$).

2.3.4 Comparison of the *netB* detection by conventional PCR and qPCR

The detection rate of *netB* in total *C. perfringens* isolates was 6.7% (2/30) by PCR compared to 66.7% (20/30) by qPCR. In detail, the *netB* gene was recognized in 13.3% (2/15) and 73.3% (11/15) of NE-producing isolates by PCR and qPCR, respectively. However, *netB* was not found in non-NE producing isolates by PCR, whereas 60% of these isolates (9/15) were positive for *netB* by qPCR (Table 2.3).

2.4 Discussion

C. perfringens isolates recovered from NE-producing chickens in this study were all toxinotype A, but were negative for *cpe*, which is consistent with previous studies (Keyburn et al., 2006; Van Immerseel et al., 2009). The pathogenesis of NE has been the subject of considerable interest for over a decade in order to develop effective prevention and mitigation strategies. The NetB toxin, encoded by *netB*, was shown to be an important virulence factor through several experimental challenge studies and epidemiological surveys (Johansson et al., 2010; Keyburn et al., 2010a; Martin and Smyth, 2009). However, a recent study found that *netB* alone, in the absence of other NELoc-1 genes, was unable to restore full virulence in the challenge model performed by Zhou et al. (Zhou et al., 2017). This finding suggested that other genes present in the same pathogenicity locus, NELoc-1, and their encoded products were required for regulation of *netB* and for full virulence. Furthermore, global gene expression of *netB* harbored by *C. perfringens* was demonstrated to be highly modulated by environmental conditions (Parreira et al., 2016). These results indicate that *netB* may exist in each *C. perfringens* isolate, and the disease development depends on certain regulatory mechanisms to either activate or suppress genes. In other words, *C. perfringens* isolated from NE and non-NE chickens may all possess *netB*, but it may only be expressed under certain conditions, promoting the development of NE. Thus, the carriage and quantity of *netB* in NE- and non-NE-producing *C. perfringens* isolates were re-assessed in this study. The low detection rate of *netB* by PCR (6.7%) was consistent with that of a previous study (4.1%) (Bailey et al., 2015), indicating that NetB was not an important virulence factor, or at least not as important as originally thought. Nevertheless, qPCR data

demonstrated that the prevalence of *netB* was high in both NE and non-NE isolates (73% versus 60%, respectively), showing that *netB* is present in the majority of *C. perfringens* regardless of NE production status. No significant difference of prevalence was observed between these two groups regardless of the detection method was used. The *netB* gene could be detected at the amount of 10-20 copies by qPCR, whereas high copy numbers (~10^8) were required for a positive identification by PCR in this study. Abildgaard et al. (Abildgaard et al., 2010) noted the potential significance of NetB toxin in virulence; however, the presence or absence of *netB* in *C. perfringens* itself is insufficient to predict the pathogenicity. Regarding the quantity of *netB*, NE-producing *C. perfringens* isolates possessed higher copy numbers than non-NE isolates on average. Among eleven NE-producing *C. perfringens* isolates, two had abundant copies of *netB*, while the remaining nine carried relatively low copies of *netB* and yet still established clinical NE, suggesting that the quantity of *netB* in *C. perfringens* is not a reliable indicator of pathogenicity as well.

NE-producing *C. perfringens* strains/isolates typically carry two to five highly conserved, low-copy number plasmids (Lepp et al., 2010). The *netB*, *cpb2,* and *tpeL* toxin genes are encoded in pathogenicity loci on different large plasmids (Bannam et al., 2011; Lepp et al., 2013; Lepp et al., 2010). As *C. perfringens* ordinarily relies on plasmid-encoded toxins to produce NE in chickens (Li et al., 2013), the prevalence of *cpb2* and *tpeL*, in addition to *netB*, were selected for this investigation. Several epidemiological studies suggest that *cpb2*-positive *C. perfringens* isolates are highly associated with enteric diseases in domestic animals (Waters et al., 2005). In the present study, the high carriage rate of *cpb2* in NE and non-NE isolates was observed by PCR; this result was

consistent with other findings (Crespo et al., 2007; Gholamiandekhordi et al., 2006; Park et al., 2015; Siragusa et al., 2006). We also found that *cpb2* was not differently distributed between isolates from NE-producing and healthy chickens, which disputes that there is an association between *cpb2* and NE, as previously reported by Crespo et al. (Crespo et al., 2007).

Regarding *tpeL*, our PCR data were in accordance with other findings (Bailey et al., 2015; Chalmers et al., 2008; Keyburn et al., 2010b) and revealed low frequencies of *tpeL* carriage in NE-producing isolates. The *tpeL* gene was found only in *netB*-positive isolates. These observations indicate that the putative TpeL toxin may not be essential for NE in chickens due to such a low carriage rate. However, one study demonstrated that several *tpeL*-positive *C. perfringens* in the absence of *netB* causes typical NE in the disease induction model (Llanco et al., 2015), proposing that the TpeL toxin or another toxin plays a role in NE pathogenesis. In another challenge experiment, inoculation of broilers with *tpeL*- and *netB*-positive strains was associated with greater severity of gross lesions compared with strains containing only *netB*, suggesting that *tpeL* potentiates the effect of other virulence attributes of NE strains (Coursodon et al., 2012). Collectively, these data indicate that the role of *tpeL* in pathogenesis remains inconclusive.

In summary, we demonstrated that a qPCR assay was a sensitive and reliable method for characterization and quantification of *netB* in *C. perfringens*. The results provide not only new insights into the prevalence of potential virulence toxin genes in *C. perfringens* populations from NE and non-NE chickens, but also a conclusion that the presence or absence of those genes as well as the quantity of *netB* are insufficient to predict an association with the virulence or pathogenicity. As NE is a multifactorial

disease, the understandings of gut microbiota, environmental conditions, and regulation of virulence genes and attributes are recommended and those are rationally required to elucidate the dynamic pathogenesis of *C. perfringens.*

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CHAPTER III

THE *NETB*-POSITIVE *CLOSTRIDIUM PERFRINGENS* IN THE EXPERIMENTAL INDUCTION OF NECROTIC ENTERITIS WITH AND WITHOUT PREDISPOSING FACTORS

(Submitted to Poultry Science)

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Abstract

The *netB*-positive *Clostridium perfringens* (*C. perfringens*) has been considered as the requisite to consistently induce necrotic enteritis (NE). However, use of a *netB*-positive strain did not guarantee consistent NE reproduction unless high protein diets or *Eimeria*, conceived as two major predisposing factors, was incorporated. To establish a refined model, the roles of dietary fishmeal inclusion, *Eimeria* inoculation, and *netB*-positive *C. perfringens* challenge in NE induction and the confounding effects of *Eimeria* infection on NE were examined. The results showed that the use of *netB*-positive *C. perfringens* without a predisposing factor failed to induce NE. Fishmeal incorporation promoted the occurrence of NE but did not significantly affect the incidence of the disease in conjunction with the challenge of *netB*-positive *C. perfringens*. However, the additional participation of *Eimeria* infection in the same induction procedure produced significantly higher numbers of NE cases and promoted more severe lesions in chickens ($p < 0.05$). Inoculation of *Eimeria* resulted in a significantly higher incidence of NE compared to the non-*Eimeria* treated group ($p < 0.05$). The results demonstrated that both *netB*-positive *C. perfringens* and predisposing factors were required for the reproduction of the disease. Mild to moderate coccidial infection (coccidial lesion score ≤ 2) was noted in NE cases in this model but severe coccidial infection did not correlate with the occurrence of NE, indicating mild coccidial infection may be beneficial for the development of NE. If multiple species infection of *Eimeria* precedes the challenge of *C. perfringens*, days 19 to 21 (1 to 3 days after the last clostridial challenge) was the time period favorable for observations of NE lesions. The time after this period may be subject to the bias of severity, incidence, or mortality of NE owing to the profound coccidial

lesions in the intestinal region. This study demonstrated that the co-infection with *netB*-positive *C. perfringens* and *Eimeria* species under fishmeal incorporation produced a desirable NE model, being of value in studying the effectiveness of novel feed additives and alternative mitigation strategies to prevent NE.

3.1 Introduction

Necrotic enteritis (NE), an enteric disease caused by *Clostridium perfringens* (*C. perfringens*) type A, contributes enormous economic losses to the poultry industry worldwide and is currently being considered as a major global threat (Timbermont et al., 2009; Van Der Sluis, 2000; Van Immerseel et al., 2009; Wade and Keyburn., 2015). In past decades, the use of in-feed antibiotics or antimicrobial growth promoters (AGPs) was the most effective approach to prevent and control this disease (Liu et al., 2010). However, due to the rising concerns about the spread of bacteria with antibiotic resistance from animals to humans, numerous countries and companies have consequently prohibited the use of AGPs. The withdrawal of AGPs has resulted in a spike in the incidence of NE and has led to the reemergence of this disease in the broiler production system (Casewell et al., 2003; Gaucher et al., 2015; Timbermont et al., 2011; Van Immerseel et al., 2009). The resurgence and dissemination of NE have promoted urgent demands for alternatives to mitigate this economically significant disease.

The occurrence of clostridial enteritis is primarily correlated with the proliferation of *Clostridia* in the intestinal tract, which is followed by the incremental production of toxins (Theoret et al., 2016). Toxins are recognized as the critical components that induce the lesions and clinical signs of clostridial enteritis (Awad et al., 1995; Keyburn et al., 2008; Sarker et al., 1999; Sayeed et al., 2008; Uzal et al., 2014). In chickens, the *netB*

gene, encoding necrotic enteritis B-like (NetB) toxin, has been recently shown to play a leading role in the virulence of NE (Keyburn et al., 2008), and several experimental studies demonstrated that a reliable NE induction model mainly relied on the strain of *C. perfringens* that is *netB*-positive (Cooper and Songer, 2010; Keyburn et al., 2010; Smyth and Martin, 2010; Timbermont et al., 2009). However, the isolation of *netB*-positive *C. perfringens* from healthy chickens (Martin and Smyth, 2009) indicates that an infection of this type of bacteria without other components is not sufficient to induce NE. Given the ubiquity of *C. perfringens*, the disease often fails to develop without other predisposing factors (Craven, 2000; Van Immerseel et al., 2004). Therefore, a great number of studies have demonstrated that disease induction of NE is highly complex, normally requiring more than just bacterial infection both experimentally and in the field. The process of disease induction is dynamic and involves many steps, including colonization, multiplication, acquisition of nutrients, evasion of the host defenses, infliction of damage, and transmission (Prescott et al., 2016a). This multifactorial feature of this disease contributes to difficulties in achieving consistent results in the experimental reproduction of NE.

Feed manipulation is the most widely applied predisposing factor implemented prior to NE induction. This involves making changes in feed ingredients in the period before *C. perfringens* challenge (Uzal et al., 2015). Chickens fed with wheat-based diets are shown more susceptible to NE than those fed with corn-based diets (Branton et al., 1987; Riddell and Kong, 1992). Similarly, a high level of fishmeal in the diet has been demonstrated to predispose chickens to or exacerbate the outbreak of NE (Drew et al., 2004; Gholamiandehkordi et al., 2007; Johnson and Pinedo, 1971; Truscott and Al-Sheikhly,

1977). High levels of non-starch polysaccharides (NSP) in wheat exert its effects by increasing the viscosity of the digesta and hence slowing transit time in the gut (Annett et al., 2002). NSP can also leave undigested nutrients available for microbial proliferation and interact with glycoproteins on the epithelial surface to increase mucin production (Kleessen et al., 2003), thus promoting the overgrowth of *C. perfringens* (Shojadoost et al., 2012). High animal protein diets, especially those based on fishmeal, provide high levels of available nutrients, such as specific amino acids that *C. perfringens* is unable to synthesize, subsequently improving bacterial growth (Drew et al., 2004; Titball et al., 1999). Moreover, a biogenic amine known as gizzerozine is present in fishmeal and can lead to erosions in the alimentary tract of birds (Tisljar et al., 2002). Lastly, the addition of fishmeal in the diet can destabilize and alter the underlying gut microbial population, thus predisposing chickens to develop NE (Stanley et al., 2012; Stanley et al., 2014b). Switching to a high fishmeal or protein diet during a *C. perfringens* challenge has been linked to successful NE induction (Keyburn et al., 2008; Wu et al., 2010).

Coccidial infection is another predisposing factor that has been extensively used to develop NE. *Eimeria* in the gut has multiple effects that promote the development of NE in chickens. First, *Eimeria* infection leads to physical damage to the gut epithelium and thus facilitates the colonization and proliferation of *C. perfringens* (Van Immerseel et al., 2009; Williams, 2005; Williams et al., 2003). At the same time, the damage contributes to ruptured epithelial cells leaking plasma proteins to the gut lumen, which acts as a rich nutrient source for *C. perfringens* growth (Van Immerseel et al., 2004). Moreover, coccidial infection also induces a host mucogenic response to produce mucus, providing another protein-rich nutrient source for the growth advantage of *C. perfringens* (Collier et

al., 2008; Forder et al., 2012). Lastly, an *Eimeria* infection may exert an immunological stress on chickens, increasing their susceptibility to *C. perfringens* infection (Uzal et al., 2015).

Although quite a number of predisposing factors have been unveiled and incorporated with the process of clostridial infection, the reproducibility of the clinical NE model is still variable under experimental conditions (Van Waeyenberghe et al., 2016). The success and severity of reproduced NE have been demonstrated to depend on the strain of *C. perfringens,* diet, the species of *Eimeria*, and the route, dose, timing, and frequency of the challenge (Prescott et al., 2016b). To establish a refined NE model that can assess the efficacy of novel feed additives and the contribution of the gut microbial profile to NE, the effects of two most widely used predisposing factors on disease induction by *C. perfringens* harboring the recognized virulent toxin gene were investigated. The influence of *Eimeria* species parasitizing different gut locations on the success of NE induction was also evaluated. The need for a consistent NE induction model prompted us to study the interaction between *net*-positive *C. perfringens,* wheat-fishmeal mixed diet, and multiple species of *Eimeria*; the resulting data provides practical operational guidelines for the experimental reproduction of NE.

3.2 Materials and Methods

3.2.1 Chickens and diets

A total of 147 male and female one-day-old unvaccinated broiler chicks (Cobb strain) from commercial hatcheries were used. Sixty-three chicks were assigned to trial 1 and 84 chicks were allotted to trial 2. Chicks were randomly grouped and placed in separate iron tanks with nets in the floor-pen facility and lined with fresh litter. Feed and water were provided *ad libitum*. No antibiotics and anticoccidials were added in feeds. For trial 1, Natural Chick Starter/Grower commercial feed (Co-op, Starkville, Mississippi) containing minimal 18% crude protein was applied throughout the entire experiment. In trial 2, a wheat-based diet was prepared based on the formula previously described by Branton (Branton et al., 1987). The diet was further mixed with fishmeal 60N (Seven Springs Farm, Check, Virginia) having minimal 60% crude protein at a ratio of 1:1 to form wheat-based diets containing 50% fishmeal (fishmeal diet). The wheat-based diets were fed to chickens for the first 7 days, and then the rations were replaced by fishmeal diets from day 8 to day 19. Afterward, wheat-based diets were resupplied until the end of the trial.

3.2.2 Experimental designs

A side-by-side experimental design was implemented with the different combination of *netB*-positive *C. perfringens* (CP1) and predisposing factors. The reproduction of NE was evaluated based on clinical signs and intestinal lesion scored 2 or more. In trial 1, no predisposing factor was applied for NE induction. Sixty-three broiler chicks were randomly divided into three groups and fed with non-medicated starter/grower feeds. A tenfold dose of a commercial coccidial vaccine was orally administered to the *Eimeria*-

treated group at 9 days of age to establish the coccidial infection. For the induction of NE, 3 ml of freshly prepared CP1 inoculum at $2.5x10^8$ colony-forming units (CFU)/ml was gavaged *per os* once per day starting on day 14 for 3 consecutive days by using an 18 G curved, round-tipped animal feeding needle with 2 mm tip diameter (Gavageneedle.com, Sherman Oaks, California). Subsequently, 7 chickens from each group were humanely euthanized by carbon dioxide and scored for NE or coccidial lesions at day 17, 19, and 23.

In trial 2, dietary and parasitic predisposing factors were included to evaluate their effects on the induction of NE with CP1. Eighty-four chicks were equally assigned to 4 groups in a completely randomized design that included a CP1-challenged group, a co-infection group with CP1 and *Eimeria* species, an *Eimeria*-treated group, and a control group. *Eimeria* was provided for *Eimeria*-treated groups using the same methodology described in trial 1 at day 10. The infection of *C. perfringens* was achieved by orally administrating 3 ml of CP1 inoculum starting at day 15 for 4 consecutive days at a frequency of 3 times a day. Chickens in each group were euthanized at day 19 and day 21. The information concerning experimental designs is listed in Table 3.1.

Chickens in both trials were clinically inspected on a daily basis. Whole small intestine (duodenum, jejunum, and ileum) and cecum were targeted for pathological examination of NE and coccidial lesions. One specialized veterinarian was in charge of necropsy and lesion scoring. Dead chickens were removed from trials after identifying the cause of death by necropsy and recording the corresponding information, such as pen, date, and lesion score. These trials were reviewed and approved by the Mississippi State University Institutional Animal Care and Use Committee (IACUC-16-439).

Table 3.1 Trial designs

Trial	N[1]	Group	Dietary factor[2]	Clostridial challenge	Coccidial challenge	Sampling time
1	21	CP1	-	d14, d15,d16	-	d17 (P1D)[3], d19 (P3D)[4], d23 (P7D)
	21	*Eimeria*[5]	-	-	d9	d17, d19, d23
	21	CTL[5]	-	-	-	d17, d19, d23
2	21	CP1	+	d15, d16, d17, d18	-	d19 (P1D), d21 (P3D)
	21	*Eimeria*[5]	+	-	d10	d19, d21
	21	CP1+*Eimeria*[5]	+	d15, d16, d17, d18	d10	d19 (P1D), d21 (P3D)
	21	CTL[5]	+	-	-	d19, d21

[1]*Number of chickens.*
[2]*Dietary factor: the wheat-based diet containing 50% fishmeal (Fishmeal diet).*
[3]*P1D: 1 day after the last challenge of CP1.*
[4]*P3D: 3 days after the last challenge of CP1.*
[5]*Eimeria: multi-species Eimeria; CTL: control group.*

3.2.3 Strains and preparation of inocula

In this study, multiple species of *Eimeria* were applied to predispose chickens to NE. The design was based on the evidence that natural infections by *Eimeri*a are generally due to a mixture of more than one species (Williams, 2003). A coccidial live vaccine, containing live oocysts of 4 *Eimeria* species, including *E. acervulina*, *E. maxima*, *E. maxima MFP*, *E. mivati*, and *E. tenella*, was selected to induce coccidiosis in chickens. One vaccine bottle contained 10,000 doses of oocysts in an unspecified proportion of *Eimeria* species. A tenfold dose of vaccine was prepared as the inoculum by dilution using autoclaved distilled and deionized water and then applied on *Eimeria*-treated and co-infection groups.

The *netB*-positive *C. perfringens* used in the trials was strain CP1, kindly provided by Dr. John F. Prescott (Ontario Agricultural College, University of Guelph, Canada). It is a clinical NE isolate identified as toxinotype A and *tpeL*-negative by PCR.

This strain has been demonstrated to favorably reproduce NE in a number of experimental models (Jiang et al., 2009; Thompson et al., 2006; Yu et al., 2017; Zhou et al., 2017). To prepare the inoculum, CP1 was streaked on blood agar plates (Fisher Scientific, Pittsburgh, Pennsylvania) followed by an overnight anaerobic incubation at 37°C. A single colony with double hemolytic zones was subsequently transferred to 3 ml fluid thioglycollate (FTG) medium (Himedia, Mumbai, Maharashtra, India) and anaerobically incubated at 37°C overnight. Thereafter, the bacterial suspension was transferred into fresh FTG broth at a ratio of 1:10, and the mixture was incubated at 37°C for specific time points (15 hours for trial 1; 15, 19, and 23 hours for trial 2) to acquire the fresh inocula applied in the two trials. The bacterial concentration of each inoculum (CFU/ml) was determined by plating 100 µl of the inoculum culture on Brain Heart Infusion agar (Sigma-Aldrich, St. Louis, *Missouri*) plates, followed by anaerobic incubation at 37°C for 16 hours and counting the numbers of colonies. The whole broth culture was used to induce NE based on previous evidence that clostridia with toxins in the supernatant produce more severe disease than cells only (Thompson et al., 2006).

3.2.4 Macroscopic lesion scoring

The lesion score in each intestinal tissue was a single-blinded evaluation. The scores of gross intestinal NE lesions (duodenum to ileum) were recorded in accordance with the criteria of Keyburn (Keyburn et al., 2006) and ranged from 0 (no gross lesions), 1 (congested intestinal mucosa), 2 (small focal necrosis or ulceration; one to five foci), 3 (focal necrosis or ulceration; 6 to 15 foci), and 4 (focal necrosis or ulceration; 16 or more foci). Chickens with lesion scores of 2 or more were identified as NE positive. After comparing scores of duodenum, jejunum, and ileum, the highest score was determined as

the final NE lesion score for each chicken. For coccidial infections, the small intestine and cecum were both examined and scored following the system described by Johnson and Reid (Johnson and Reid, 1970). This scoring system, ranging from one to four (0 for a normal appearance of the intestine), was used to assess the infection of *E. acervulina*, *E. maxima*, and *E. tenella*.

3.2.5 Statistical analysis

SAS software version 9.4 (SAS Institute, Inc., Cary, North Carolina, USA) was used for statistical analysis. Significant differences in NE lesion scores between groups were determined by Tukey test. Fisher's exact test was conducted to evaluate the differences of NE incidence levels between treatment and non-treatment groups with respect to fishmeal and *Eimeria* species. Statements of statistical significance were based on the level of $p < 0.05$.

3.3 Results

3.3.1 Induction of NE by *netB*-positive *C. perfringens* with and without predisposing factors

We examined the ability of CP1 to produce NE with and without dietary and parasitic predisposing factors in our model. In trial 1, which did not include any dietary predisposing factor of NE, chickens challenged with CP1 exhibited simple hyperemic or congested mucosa in the small intestines at each time sampling point (Figure 3.1A; Table 3.2). A higher number of chickens having a lesion score of 1 was noted in the CP1-challenged group compared to the control, but no NE cases (lesion score ≥ 2) were identified.

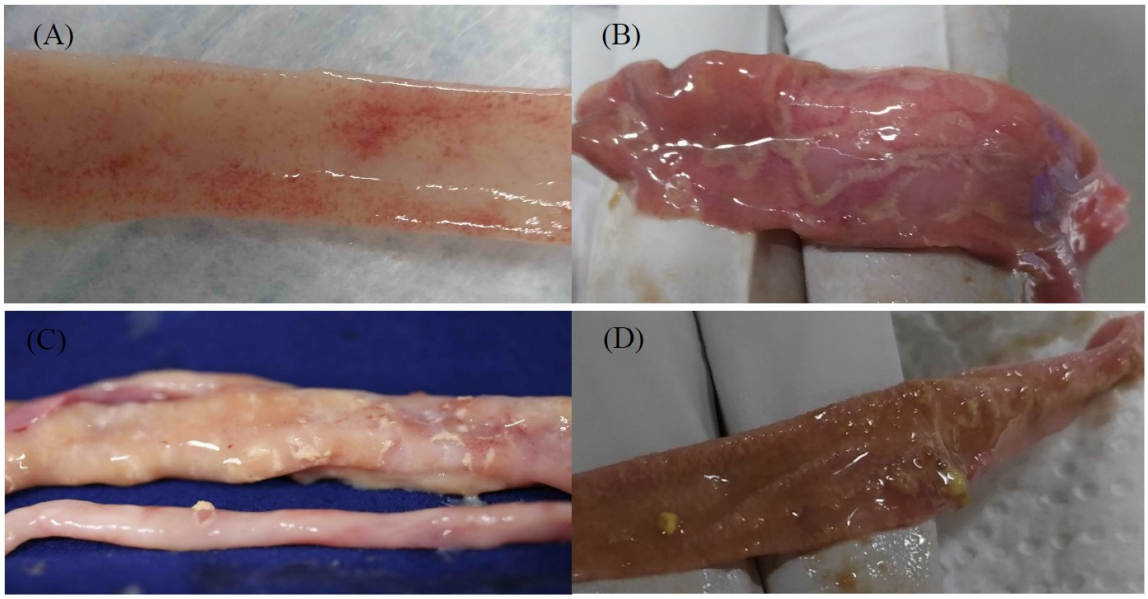

Figure 3.1 Macroscopic NE lesions produced by CP1 in challenged chickens.

(A) Hyperemia or congestion on jejunal mucus membrane (Score 1); (B) Multifocal necrosis on duodenal mucosa (score 3); (C) Multifocal necrosis on jejunal mucosa (score 3); (D) Confluent necrosis on the mucosa of the jejunum covered by pseudo-membranes (Score 4).

Table 3.2 The frequency of NE lesions by groups

Trial	Group	Dietary factor[1]	NE lesion score					Subtotal	NE case
			0	1	2	3	4		
1	CP1	-	1	20	0	0	0	21	0
	CTL[2]	-	12	9	0	0	0	21	0
2	CP1	+	0	17	1	1	0	19	2
	CP1+*Eimeria*[2]	+	0	14	0	4	1	19	5
	CTL[2]	+	6	15	0	0	0	21	0

[1]*Dietary factor: the wheat-based diet containing 50% fishmeal (Fishmeal diet).*
[2] *Eimeria: multi-species Eimeria; CTL: control group.*

In contrast, thickened intestinal walls, which were not observed in trial 1, were noticed in a higher portion of chickens in the control group that ingested fishmeal diets in the second trial (Table 3.2), showing that a fishmeal diet promoted apparent mucosal damage in the small intestine. Characteristic macroscopic lesions of NE were found in the CP1-challenged group as well as the CP1-*Eimeria* co-infected group. The affected chickens displayed variable degrees of clinical signs, including depression, reluctance to move, diarrhea and ruffled feathers. A total of two and five NE cases were reproduced in the CP1-challenged and co-infection groups, respectively. These cases demonstrated focal or multifocal to coalescing necrosis of the intestinal mucosa (Figure 3.1B, Figure 3.1C, and Figure 3.1D). Four out of five (80%) NE-affected chickens in the co-infection group displayed lesions in both duodenum and jejunum. The most severe lesions were found in the jejunum, between its proximal end and Meckel's diverticulum. No NE-caused mortalities were recorded in the present study. The statistical analysis shows that the CP1-challenged and co-infection groups both had significantly higher lesion scores than the control group (Tukey test; $p < 0.05$), but the co-infection group exhibited the highest difference (Tukey test; $p < 0.01$). Additionally, the co-infection group showed a

significantly higher incidence of NE compared to the CP1-challenged group ($p < 0.05$). These findings demonstrate that the co-infection of *netB*-positive *C. perfringens* and *Eimeria* species and feeding with fishmeal in this model successfully reproduced NE. Challenge with CP1 without the presence of predisposing factors was not sufficient to produce clinical symptoms and a case of NE. Through the addition of fishmeal, two NE cases with mild lesions were produced, and a significant difference in the incidence of NE between groups fed with and without fishmeal was noted. However, in conjunction with coccidial infection, five NE cases with severe lesions were specifically observed in the CP1-challenged group (Table 3.2). The dot density of each NE case and the mean lesion score with case number by groups are shown in Figure 3.2 and Table 3.3.

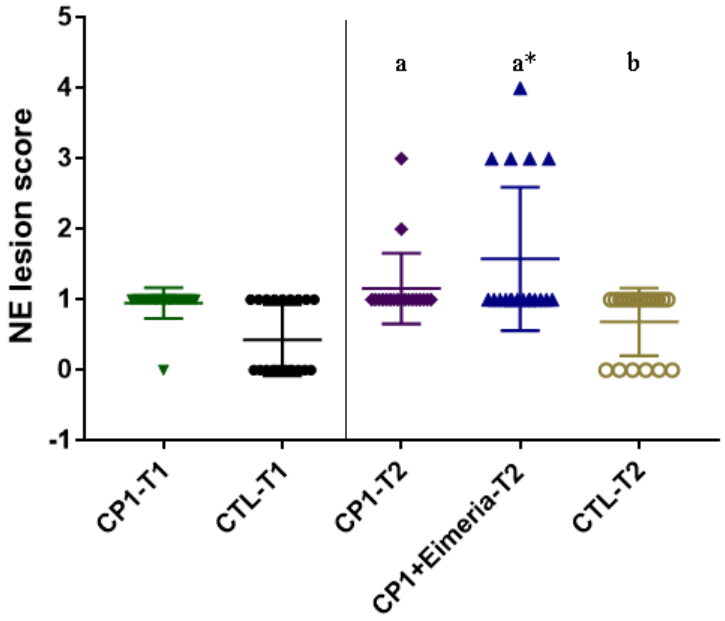

Figure 3.2 Dot density of NE case by groups in two experimental trials.

The data presents the mean NE lesion score ± SD with the frequency of NE. NE case was defined by lesion score reaching 2 or above. Dissimilar letters indicate a significant difference at a level of $\alpha=0.05$ (Tukey's test); * mean highly significant ($p < 0.01$).

Table 3.3 Severity and incidence of NE by groups in trials.

Trial	Group	Dietary factor[1]	Lesion score (NE case[2]/chickens in group)				
			Day 17	Day 19	Day 21	Day 23	Average
1	CP1	-	0.86 ±0.35[3] *(0/7)*	1.00 ± 0 *(0/7)*	-	1.00 ± 0 *(0/7)*	0.95 ± 0.21 *(0/21)*
	CTL[4]	-	0.29 ± 0.45 *(0/7)*	0.43 ± 0.49 *(0/7)*	-	0.57 ± 0.49 *(0/7)*	0.43 ± 0.49 *(0/21)*
2	CP1	+	-	1.11 ± 0.33 *(1/9)*	1.20 ± 0.63 *(1/10)*	-	1.16 ± 0.50[a5] *(2/19)*
	CP1+*Eimeria*	+	-	1.50 ± 1.08 *(2/10)*	1.67 ± 1.00 *(3/9)*	-	1.58 ± 1.0[a*] *(5/19)*
	CTL	+	-	0.44 ± 0.52; *(0/9)*	0.92 ± 0.29 *(0/12)*	-	0.71 ± 0.46[b] *(0/21)*

[1]*Dietary factor: the wheat-based diet containing 50% fishmeal (Fishmeal diet).*
[2]*NE case is defined by lesion score reaching 2 or above.*
[3]*Lesion score is shown in mean ± SD.*
[4]*CTL: control group.*
[5]*Dissimilar letters indicate a significant difference at a level of α=0.05 (Tukey's test); * mean highly significant ($p < 0.01$).*

3.3.2 Influence of the course of coccidial infection on NE induction and lesion observations

The lesion score system was also applied to assess the severity of coccidial development. It was evident that each species of *Eimeria* produced observable gross changes in the intestine in either singly *Eimeria*-challenged or co-infected with CP1 groups. The pathological gross lesions are shown in Figure 3.3 and the distribution of coccidial lesion scores by groups is summarized in Table 3.4. We observed that *E. acervulina* occupied a much greater area from duodenum to proximal jejunum. The severity of its infection spiked at day 19 and minimized at day 23 in trial 1. However, in trail 2 that incorporated fishmeal as a dietary factor, severe lesions (score ≥ 3) resulted from *E. acervulina* lesions observed at day 21. *E. maxima* commenced developing severe

lesion at day 21 as well. None of the macroscopic lesions of *E. maxima* was noted in the ileum in the two trials, indicating that this species caused slight or mild infestation to the ileum of chickens in this timeframe or under a tenfold dose of the coccidial vaccine. For the infection of *E. tenella,* bloody diarrhea was first observed at day 21. Severe diarrhea and lesions (score 3 to 4) in the cecum were recorded on day 23, leading to mortality for the afflicted chickens. At that time, mucosal discoloration to paleness was apparent, and either CP1- or other *Eimeria* species-caused lesions affecting small intestines had vanished.

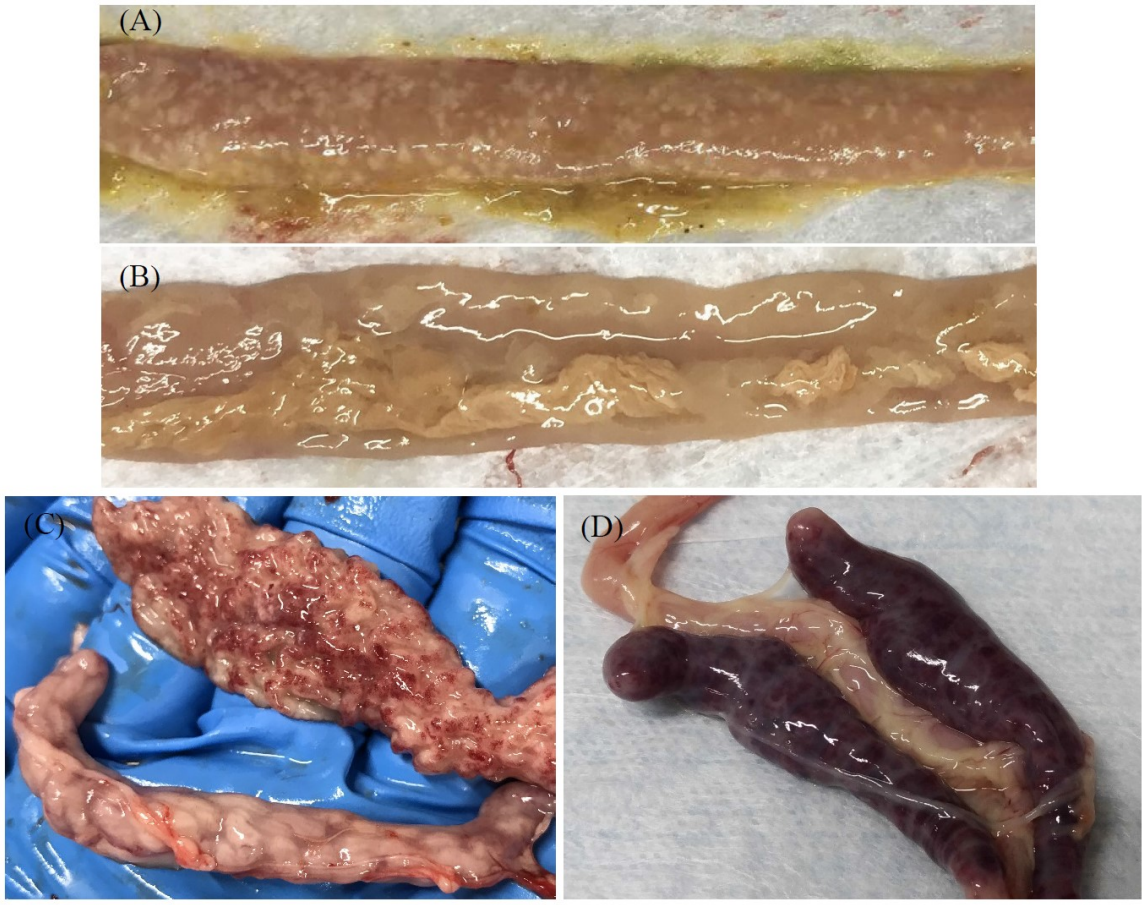

Figure 3.3 Coccidial infections by different species in trials.

(A) Characteristic lesions caused by *E. acervulina* in the duodenum. Numerous white plaques on the mucous membrane and mucosal discoloration were observed (Score 3); (B) Infection of *E. maxima* in the jejunum. Thickened intestinal wall with watery contents and light orange mucus (Score 2). (C) & (D) Gross intestinal lesions produced by *E. tenella* in the cecum. (C) Petechiae on the mucosal surfaces. The thickened cecal wall which contained fibrin and blood (Score 2); (D) Distended ceca filled with blood clots (Score 4).

Table 3.4 Distribution of coccidial lesion score by groups with sampling times

Trial	Site	Group	Species	Lesion score[2] 0	1	2	3	4	Total
1	Du[1]	*Eimeria*-D19	*E. acervulina*	0	0	0	0	7	7
	Du	*Eimeria*-D23	*E. acervulina*	0	6	0	0	0	6
2	Du	*Eimeria*-D19	*E. acervulina*	3	6	1	0	0	10
	Du	*Eimeria*-D21	*E. acervulina*	0	2	8	1	0	11
	Du	CP1+*Eimeria*-D19	*E. acervulina*	1	4	5	0	0	10
	Du	CP1+*Eimeria*-D21	*E. acervulina*	0	0	7	2	0	9
1	Je[1]	*Eimeria*-D19	*E. acervulina*	0	0	0	2	5	7
	Je	*Eimeria*-D23	*E. acervulina*	0	4	2	0	0	6
2	Je	*Eimeria*-D19	*E. maxima*	7	3	0	0	0	10
	Je	*Eimeria*-D21	*E. maxima*	0	3	4	4	0	11
	Je	CP1+*Eimeria*-D19	*E. maxima*	7	3	0	0	0	10
	Je	CP1+*Eimeria*-D21	*E. maxima*	0	4	4	1	0	9
1	Il[1]	*Eimeria*-D19	*E. maxima*	0	7	0	0	0	7
	Il	*Eimeria*-D23	*E. maxima*	0	6	0	0	0	6
2	Il	*Eimeria*-D19	*E. maxima*	10	0	0	0	0	10
	Il	*Eimeria*-D21	*E. maxima*	11	0	0	0	0	11
	Il	CP1+*Eimeria*-D19	*E. maxima*	10	0	0	0	0	10
	Il	CP1+*Eimeria*-D21	*E. maxima*	9	0	0	0	0	9
1	Ce[1]	*Eimeria*-D19	*E. tenella*	7	0	0	0	0	7
	Ce	*Eimeria*-D23	*E. tenella*	0	0	0	3	3	6
2	Ce	*Eimeria*-D19	*E. tenella*	0	9	1	0	0	10
	Ce	*Eimeria*-D21	*E. tenella*	1	5	5	0	0	11
	Ce	CP1+*Eimeria*-D19	*E. tenella*	0	10	0	0	0	10
	Ce	CP1+*Eimeria*-D21	*E. tenella*	0	9	0	0	0	9

[1]*Du: duodenum; Je: jejunum; Il: ileum; Ce: cecum.*
[2]*Lesion scores in duodenum, jejunum, ileum, and cecum were recorded according to predominant lesions caused by the according Eimeria species.*

The pathological progress of coccidiosis differed between inoculated species, and it affected the observation of lesions caused by NE. In the co-infection group, coccidial lesions resulting from *E. acervulina* or *E. maxima* together with NE lesions were noticed. On day 19 and 21, NE lesion could still be recognized. Mild to moderate coccidial infection (coccidial lesion score ≤ 2) was noted in NE cases, but severe coccidial infection did not correlate with the occurrence of NE. In the case of *E. tenella*, once the coccidial infection became predominant, for instance on day 23, the gross lesions in the intestine faded out except in the cecum. Therefore, day 19 to day 21 (or 1 to 3 days after the last clostridial challenge) was the suggested timeframe to observe for NE lesions in this model. After this period, assessing the severity, incidence, or mortality of NE became biased owing to the profound coccidial lesions in the intestinal region; this was especially evident if highly pathogenic species of *Eimeria*, such as *E. tenella*, were applied as a predisposing factor of NE.

3.4 Discussion

The influence of strains of *C. perfringens* is recognized as the key to establish a reliable NE induction model (Uzal et al., 2015). In particular, the strain producing the NetB toxin has been considered as the definitive cause of NE in chickens (Keyburn et al., 2008). Many studies have demonstrated that only a *netB*-positive strain can reproducibly induce consistent levels of NE (Cooper and Songer, 2010; Keyburn et al., 2010; Smyth and Martin, 2010; Timbermont et al., 2009). In studies that applied predisposing factors with *netB*-positive *C. perfringens* to induce the disease, the success of NE reproduction has seldom been attributed to the provision of high-protein diets (Cooper and Songer, 2010; Keyburn et al., 2010; Timbermont et al., 2009) and/or *Eimeria* species (Smyth and Martin, 2010; Timbermont et al., 2009). To the best of our knowledge, few studies have addressed the association of predisposing factors with *netB*-positive *C. perfringens* on NE reproduction. In the present study, we have demonstrated that the reproduction of NE *in vivo* by the use of a *netB*-positive strain alone did not induce NE. Only if a dietary factor or the combination of dietary and coccidial factors was used in conjunction with a *netB*-positive strain of *C. perfringens* did develop NE, and its severity and incidence increased. These findings reveal that both *netB*-positive *C. perfringens* and predisposing factors are required for the reproduction of the disease. This is in line with a recent study showing that predisposing factors play a determinant role in NE induction (Li et al., 2017). The authors surveyed the toxinotypes of *C. perfringens* from normal chickens, field NE cases, and a human gas gangrene strain (ATCC 13124) and then examined their virulence *in vivo* by using them to reproduce NE. The results showed that *netB*-positive *C. perfringens* could not produce NE, whereas the same group co-infected with *E. maxima*

did. Furthermore, they found that *netB*-negative *C. perfringens* from normal chickens and field NE cases, even *netB*-negative strain 13124 could reproduce NE in conjunction with *E. maxima* (Li et al., 2017). These findings provide further evidence that predisposing factors have the weightier influence on the induction of NE than *netB* in *C. perfringens.*

The observation that the simple infection of *C. perfringens* is not sufficient to produce disease led to the hypothesis that *C. perfringens* challenge on its own is not capable of causing any significant perturbations in the intestinal microbiota that are favorable for NE development (Stanley et al., 2014a). In this study, chickens in the CP1 challenge group of trial 1 without a dietary factor displayed some degree of mucosal damage in the small intestine; however, the existence of mild mucosal damage without contributing effects from other factors was not enough to create a gut environment favorable for NE development. This agrees with the finding that the proliferation of *C. perfringens* requires predisposing factors to either provide nutrients or create a favorable niche both in disease outbreaks in the field and in models for experimental induction of the disease (Shojadoost et al., 2012; Williams, 2005). On the other hand, co-infection with *Eimeria* demonstrated the highest potential to facilitate NE development and promote the severity of the disease in this study. Notably, fishmeal had less effect on NE induction in the presence of *netB*-positive *C. perfringens* compared to *Eimeria*. These results agree with a previous study in which *Eimeria,* preceding *netB*-positive *C. perfringens*, greatly increased the severity of NE induced in broiler chickens, but fishmeal addition played only a marginal role in the challenge model (Rodgers et al., 2015). Moreover, a metagenomic analysis of gut microbiota showed that fishmeal and *Eimeria* both generated significant changes in the microbial communities in the cecum,

but *Eimeria* had the stronger influence on intestinal microbiota, thus potentially promoting the induction of NE (Stanley et al., 2014b; Wu et al., 2010).

Although *Eimeria* species produce distinct lesions from those by NE, most vaccine studies have adopted the NE induction model with dietary factors instead of *Eimeria* to evaluate the effectiveness of a vaccine. This is due to the consideration that profound coccidial lesions would complicate gross NE scoring and immunological effects elicited by *Eimeria* might compromise the response of chickens to vaccination (Uzal et al., 2015). Nevertheless, coccidiosis is frequently found to precede or occur concurrently with field outbreaks of NE (Broussard et al., 1986; Gazdzinski and Julian, 1992; Long, 1973; Porter, 1998). Both diseases tend to act synergistically since NE development strongly depends on the intestinal damage resulting from coccidiosis (Van Immerseel et al., 2009; Williams, 2005; Williams et al., 2003). The co-infection NE model with *Eimeria* actually fits the field condition and could be reproduced in both the clinical form with mortality and the subclinical form of NE (Gholamiandehkordi et al., 2007; Wu et al., 2010). In addition, a number of *Eimeria* species had little influence on the induction of NE challenge model (Van Waeyenberghe et al., 2016). Therefore, it would be of value and significance using this model to study the efficacy of novel feed additives, management strategies, and other mitigations toward NE.

E. acervulina, *E. maxima,* and *E. tenella* are the three species that most regularly affect commercially reared chickens (Clark et al., 2016; Gyorke et al., 2013; McDougald, 1998). Depending on the magnitude of infection, *Eimeri*a can cause mild to severe lesions and significant pathology. During the life cycle, *E. acervulina* and *E. maxima* both can contribute physical damage to the jejunal mucosa (Chapman, 2014), where *C.*

perfringens initially colonizes and expands in numbers (Moore, 2016; Prescott et al., 2016b). Thus, these two species have been most widely applied in NE induction. Owing to concerns that profound coccidial lesions would complicate gross scoring of NE (Uzal et al., 2015), the influence of the course of coccidial infection on NE induction and lesion observations were evaluated. In the co-infection group, NE lesions occurred together with coccidial lesions from mild to moderate infection (coccidial lesion score ≤ 2) of *E. acervulina* and *E. maxima*. Although there were overlapping areas of lesions either in the duodenum, jejunum, or both, NE lesions could still be well-recognized. However, severe *Eimeria*-affected chickens (coccidial lesion score ≥ 3) did not produce NE, suggesting that heavy coccidial infection might conceal the gross lesions of NE or alter environmental conditions which compromise the NE development. The most severe lesions of *E. acervulina* were recorded at day 19, and the severity declined to a mild degree at day 23 in trial 1. However, severe lesions started to appear at day 21 in trial 2, indicating that fishmeal might have some degree of delay effect on the development of coccidial lesions for *E. acervulina*. Similar results were also observed in another trial using the same methodology as trial 2 to induce NE and coccidiosis (data not shown). In the case of the highly pathogenic *E. tenella*, which regularly causes extensive hemorrhage and death to chickens by the thorough destruction of intestinal villus (Chapman, 2014), hemorrhagic enteritis was noted in chickens during trial 1. It developed to the highest level of severity at day 23. In the whole small intestine, the paleness of the mucosal membrane that resulted from bloody diarrhea would indisputably impede the observation of NE lesions. Accordingly, days 19 to 21 were indicated as the optimal period for NE observation in our co-infection model. If long-term observation of NE progress is

required, the application of multiple species of *Eimeria* before the *C. perfringens* challenge is not recommended, especially in infections with coccidia developing in the lower intestine, such as *E. tenella.*

In conclusion, the co-infection model with *netB* positive *C. perfringens* and *Eimeria* combined with fishmeal feeding favorably reproduced NE in this study. In contrast, the use of a *netB* positive *C. perfringens* without other contributory factors did not induce NE. When more predisposing factors participated in the induction, the severity and incidence of NE were increased. In addition, *Eimeria* had significant influence on the induction of NE. In the past, the intensive focus on the *netB*-positive strain of *C. perfringens* in NE induction has failed to guarantee the consistent experimental reproduction of NE. The insights provided by the present study would be of value in establishing a refined model that delivers consistent results and can be applied to study the efficacy of novel feed additives and alternative mitigation strategies toward NE.

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CHAPTER IV

ANALYSIS OF CONTRIBUTORY GUT MICROBIOTA AND LAURIC ACID AGAINST NECROTIC ENTERITIS IN *CLOSTRIDIUM PERFRINGENS* AND *EIMERIA* SIDE-BY-SIDE CHALLENGE MODEL

(Submitted to PLos One)

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Abstract

Gut microbiota has been demonstrated to be involved in intestinal nutrition, defense, and immunity, as well as to participate in disease progression. This study was to investigate gut microbiota changes in chickens challenged with *netB*-positive *Clostridium perfringens* strain 1 (CP1) and/or the predisposing *Eimeria* species (*Eimeria*). In addition, the effects of lauric acid, a medium-chain fatty acid (MCFA), on NE reduction and modulation of microbiota were evaluated. The results demonstrated that microbial communities in the jejunum were distinct from those in the cecum, and the microbial community change was more significant in the jejunum. The challenge of CP1 in conjunction with *Eimeria* significantly reduced species diversity in the jejunal microbiota, but cecal microbiota remained stable. In the jejunum, CP1 challenge increased the abundance of the genera of *Clostridium sensu stricto 1*, *Escherichia Shigella*, and *Weissella*, but significantly decreased the population of *Lactobacillus*. *Eimeria* infection on its own was unable to promote NE, demonstrating decrements of *Clostridium sensu stricto 1* and *Lactobacillus*. Co-infection with CP1 and *Eimeria* reproduced the majority of NE lesions with a significant increment of C*lostridium sensu stricto 1* and reduction in *Lactobacillus*. The changes of these two taxa increased following NE severity. Further analyses of metagenomeSeq, STAMP, and LEfSe showed significant overgrowth of *Clostridium sensu stricto 1* was associated with the NE and *Eimeria* infection precedent to *C. perfringens* challenge has a synergistic effect on the overrepresentation. In addition to *C. perfringens*, another member within *Clostridium sensu stricto 1* was found to participate in NE development. The supplementation of lauric acid did not reduce NE incidence and severity but decreased the relative abundance

of *Escherichia Shigella.* In conclusion, significant overgrowth of *Clostridium sensu stricto 1* in the jejunum is the major microbiota contributory to the NE. The controlling proliferation of this taxon in the jejunum should be the niche for developing effective strategies against NE.

4.1 Introduction

Necrotic enteritis (NE) as the result of proliferation of *Clostridium perfringens* (*C. perfringens*) type A and their associated toxins in the small intestine of chickens is a devastating enteric disease, characterized by sudden diarrhea, unexpected mortality, and mucosal necrosis (Cooper et al., 2013; Martin and Smyth, 2009). Up to 37% of commercial broiler flocks is estimated to be affected by this disease, and it has contributed to the losses of 6 billion dollars in the global poultry industry (Van Der Sluis, 2000; Wade and Keyburn., 2015). In recent decades, *C. perfringens*-associated NE in poultry has been well-controlled by in-feed antimicrobial growth promoters (AGPs) (Liu et al., 2010). However, the emergence of antibiotic-resistant bacteria from animals and the potential threat of transmission to humans has led to bans on using AGPs in many countries (Marshall and Levy, 2011; Van Immerseel et al., 2004b). Following the withdrawal of AGPs from poultry feed, NE has re-emerged as a significant disease to the poultry industry (Casewell et al., 2003; Gaucher et al., 2015; Timbermont et al., 2011; Van Immerseel et al., 2009).

Gut microbiota is one of the central defense components in the gastrointestinal tract against enteric pathogens, which works by modulating host responses to limit the colonization of pathogens (Rehman et al., 2007). Interactions between gut microbiota and the host could influence the intestinal morphology, physiology, and immunity (Pan and

Yu, 2014). Recently, gut microbiota has been demonstrated to regulate intestinal gene expression (Yin et al., 2010) and T cell-mediated immunity (Mwangi et al., 2010) as well as to accelerate the maturation of the gut immune system (Crhanova et al., 2011). Conversely, a growing number of studies have observed gut microbial shifts in enteric diseases, considering that gut microbiota plays a role in the progress of disease development. Similar results were also represented in NE induction models, proposing that the disturbance of gut microbiota interacts with the host, subsequently promoting the development of NE (Feng et al., 2010; Li et al., 2017b; Stanley et al., 2012b; Stanley et al., 2014). In the case of human necrotizing enterocolitis, an enteric disease in infants associated with *C. perfringens* (Dittmar et al., 2008), a recent study found that *Bacteroides dorei,* an opportunist pathogenic bacterium in anaerobic infections, was associated with an increased mortality of this disease (Heida et al., 2016). Furthermore, several studies have demonstrated that the increment of bacteria belonging to a genus of *Escherichia-Shigella* was associated with *C. perfringens* infection (Li et al., 2017b; Liu et al., 2010). This evidence raised the possibility that certain microbes or microbiota in the gut may contribute to the virulence or development of enteric disease in chickens, particularly for NE.

The removal of AGPs drove the poultry industry to search for an alternative in prevention to decrease the incidence of NE. Probiotics, prebiotics, organic acids, plant extracts, essential oils, and enzymes arose in response to this demand, but the efficacy of those on NE reduction was variable and inconsistent (Caly et al., 2015; Dahiya et al., 2006). However, a medium-chain fatty acid (MCFA), lauric acid, was found to have strong *in vitro* antimicrobial activity against gram-positive organisms (Hermans et al.,

2012; Van Immerseel et al., 2004a; Zeiger et al., 2017) and *C. perfringens* (Skrivanova et al., 2005; Timbermont et al., 2010). In an *in vivo* trial, lauric acid with butyric acid demonstrated the lowest incidence and severity of NE compared to other treatments (Timbermont et al., 2010). However, this promising result did not promote more applications of lauric acid against NE, and the interaction of lauric acid with gut microbiota was not even addressed. The evaluation of its modulation effect on NE reduction and gut microbiota simultaneously would be valuable in exploring specific microbial community contributory to the NE.

Although *C. perfringens* is the causative etiological agent of NE, it is evident that other predisposing factors are required for NE induction (Prescott et al., 2016b; Shojadoost et al., 2012; Timbermont et al., 2011; Wu et al., 2014). Even though gut microbiota has been suggested to be involved in the progress of NE development (Moore, 2016; Prescott et al., 2016a), the association between microbiota profile and NE development have not been well elucidated. Most studies intensively focused on changes of microbial communities in the ileum or in cecum where a higher quantity of microbes or/and more diverse microbial compositions was harbored; however, most results were inconclusive (Feng et al., 2010; Lin et al., 2017; Stanley et al., 2012b; Stanley et al., 2014; Xu et al., 2018). Inversely, microbiota in the jejunum, which serve as the primary site for colonization of *C. perfringens* and development of NE (Prescott et al., 2016a), was seldom evaluated. In the present study, we investigated gut microbiota targeting NE cases and in chickens with side-by-side treatments with the causative pathogen and parasitic predisposing factor, *Eimeria*, and expected to unveil the contributory microbe or microbiota to the NE. The effects of lauric acid on NE reduction and modulation of

microbiota were also examined to confer the alternative intervention to prevent and control NE.

4.2 Materials and Methods

4.2.1 Chicken, diet, and experimental design

A total of 50 male and female one-day-old unvaccinated broiler chicks (Cobb strain) were obtained from a commercial hatchery. The chicks were inspected on receiving to ensure their healthy status and randomly allotted to 5 groups, studying the NE incidence and gut microbiota after challenge of *netB*-positive *C. perfringens* (A group: CP1), co-infection with *netB*-positive *C. perfringens* and multi-species *Eimeria* (B group: CP1+*Eimeria*), addition of lauric acid to feed chickens co-infected with *netB*-positive *C. perfringens* and multi-species *Eimeria* (C group: CP1+*Eimeria*+LA), inoculation of multi-species *Eimeria* (D group: *Eimeria*), and no treatment (E group: CTL).

Chicks in groups were placed in separate temperature-controlled iron tanks with nets in the floor-pen facility and lined with fresh litter. Throughout the 19-day study period, wheat-based diets prepared based on the formula by Branton et al. (Branton et al., 1987) were offered for the first 7 days, and then the rations were replaced by fishmeal diets (wheat-based diets containing 50% fishmeal) obtained from the 1:1 mixture of wheat-based diet with fishmeal 60 N (Seven Springs Farm, Check, Virginia, USA), containing minimal 60% crude protein from days 8 until the end of the study. For the lauric acid supplementing group, 400 mg of lauric acid powder (Fisher Scientific, Pittsburgh, Pennsylvania, USA) was added into 1 kg of wheat-based diet or fishmeal diet to form the final ration for chickens from day 8 onward (Timbermont et al., 2010).

Co-infection with *netB*-positive *C. perfringens* (CP1) and multi-species *Eimeria* was applied to induce NE according to our previous studies. The success of reproducing

NE was determined by clinical signs and intestinal lesion scores reaching 2 or more. In brief, chickens in the co-infection group were given a single gavage of coccidial inoculum at day 10, followed by oral administration of 3 ml CP1 inoculum with average $2.5x10^8$ colony-forming units (CFU)/ml at day 15 for 4 consecutive days with a frequency of 3 times daily. For a single challenge of CP1 or *Eimeria*, the same methodology and time points were conducted as in the co-infection group. All chickens were inspected on a daily basis and humanely euthanized at day 19 by carbon dioxide. Dead chickens not resulting from NE were excluded from the trial after necropsy.

All procedures for the care, housing, and treatment of chickens were approved by the Institutional Animal Care and Use Committee at Mississippi State University (IACUC 16-439). This protocol complies with Guide for Care and Use of Laboratory Animals, Public Health Service Policy on Human Care and Use of Laboratory Animals and the U.S. Animal Welfare Act. Mississippi State University is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC). All chickens were acclimated for one week prior to any experimental procedures. Chickens had ad libitum access to water and feed and were monitored twice daily. The death was not the endpoint of this experiment. Chickens were humanely euthanized by CO_2 inhalation when they displayed respiratory distress, injuries, reluctant to move, or severe weight loss according to the approved protocol.

4.2.2 Challenge strain and inoculum preparation

Anticoccidial live vaccine containing live oocysts of *E. acervulina*, *E. maxima*, *E. maxima MFP*, *E. mivati*, and *E. tenella* was used as a disposing factor. The vaccine bottle contained 10,000 doses of oocysts in an unspecified proportion of *Eimeria* species. A

tenfold dose of vaccine was prepared then applied on *Eimeria*-treated and co-infection groups. *C. perfringens*, a clinical NE strain designated as CP1 obtained from Dr. John F. Prescott (Ontario Agricultural College, University of Guelph, Canada), was used to challenge chickens. This strain was characterized as *netB*-positive Type A and used to reproduce NE in a number of experiments (Jiang et al., 2009; Thompson et al., 2006; Yu et al., 2017; Zhou et al., 2017). CP1 was cultured on blood agar plates and incubated anaerobically at 37°C for overnight. A single colony was in turn transferred into 3 ml of fluid thioglycollate (FTG) medium (Himedia, Mumbai, Maharashtra, India) at 37°C for overnight. Thereafter, the bacterial suspension was inoculated into fresh FTG broth at a ratio of 1:10 and incubated at 37°C for 15, 19, and 23 hours, respectively. The whole broth cultures were used to induce NE based on the evidence that clostridia with toxins produce more severe disease than using cells alone (Thompson et al., 2006). The bacterial concentration (CFU/ml) of inoculum was calculated by plate counting using Brain Heart Infusion agar (Sigma-Aldrich, St. Louis, Missouri, USA), followed by anaerobic incubation at 37°C for 16 hours.

4.2.3 Sample collection and lesion scoring

Three chickens per group were randomly selected to collect fecal contents from the jejunum (AJ, BJ, CJ, DJ, and EJ) and cecum (AC, BC, CC, DC, and EC). Among three CP1-challenged groups (A, B, and C), chickens suffering NE (lesion score ≥ 2) were preferentially collected. Then, the remaining chickens were sampled randomly to reach a quantity of 3. One percent of 2-mercaptoethanol (Sigma-Aldrich) in PBS was used to wash fecal contents, and samples were immediately frozen at -80°C. The intestinal tissues (duodenum to ileum) were inspected for NE lesions and scored

following the criteria described by Keyburn (Keyburn et al., 2006), with a range of 0 (no gross lesions), 1 (congested intestinal mucosa), 2 (small focal necrosis or ulceration; one to five foci), 3 (focal necrosis or ulceration; 6 to 15 foci), and 4 (focal necrosis or ulceration; 16 or more foci). Chickens with lesion scores reaching 2 or higher were identified as NE cases, and the highest score in their small intestinal sections (duodenum, jejunum, and ileum) was recorded as the final score of NE.

4.2.4 DNA extraction

Total genomic DNA was isolated from approximately 250 mg of fecal contents using the MOBIO PowerFecal® DNA Isolation Kit (Mobio, Germantown, Maryland, USA) following the manufacturer's protocol with some modifications. After adding bead solution and lysis buffer, the mixture was heated in a water bath at 65°C for 30 minutes followed by 5 minutes of vortexing. The concentration and quality of harvested DNA were determined by NanoDrop™ One Microvolume UV-Vis Spectrophotometer (Fisher Scientific) and visualized on 0.8% agarose gel (BD Biosciences, San Jose, California, USA). Afterward, genomic DNA was stored at -20°C until further analysis.

4.2.5 16S rRNA library preparation and sequencing

The variable V3-V4 region of the 16S rRNA gene was PCR-amplified in 25-µl reaction mixtures, containing 12.5 µl Clontech Labs 3P CLONEAMP HIFI PCR PREMIX (Fisher Scientific), 1 µl of each 10-µm Illumina primer (forward primer-5'CCTACGGGNGGCWGCAG 3' and reverse primer-5' GACTACHVGGGTATCTAATCC 3') with standard adapter sequences, and 1 µl of DNA template. The PCR conditions started with an initial denaturation step at 95°C for 3

minutes, followed by 25 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds, and a final extension step at 72°C for 5 minutes on Applied Biosystems GeneAmp PCR System 9700 (Applied Biosystems Inc., Foster City, California, USA). The amplicons were cleaned up by Monarch® DNA Gel Extraction Kit (New England Biolabs, Ipswich, Massachusetts, USA). Subsequently, an index PCR was performed by using Nextera XT Index Kit (Illumina, San Diego, California, USA) to attach a unique 8-bp barcode sequence to the adapters. The applied 25-µl reaction was composed of 12.5 µl KAPA HiFi HotStart Ready Mix (Kapa Biosystems, Wilmington, Massachusetts, USA), 2.5µl of each index primer, and 1 µl of 16S rRNA amplicon and reaction conditions were as follows: 95°C for 3 minutes, 8 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds, and 72°C for 5 minutes on Mastercycler® pro (Eppendorf AG, Hamburg, Germany). The PCR products were purified using Agencourt AMPure XP beads (Beckman Coulter, Indianapolis, Indiana, USA), and the size and concentration were determined by Bioanalyzer with DNA 1000 chip (Agilent, Santa Clara, California, USA) and Qubit® 2.0 Fluorometer with Qubit™ dsDNA HS Assay Kit (Fisher Scientific). Those libraries were normalized and pooled to one tube with the final concentration of 10 pM. Samples were thereafter sequenced on the MiSeq® System using Illumina MiSeq Reagent Kit v3 (2×300 bp paired-end run).

4.2.6 Sequence processing and data analysis

Paired-end sequences were merged by means of fast length adjustment of short reads (FLASH) v1.2.11 (Magoc and Salzberg, 2011) after trimming of primer and adapter sequences. Reads were de-multiplexed and filtered by Quantitative Insights into Microbial Ecology (Qiime) software v1.9.1 (Caporaso et al., 2010), meeting the default

quality criteria and a threshold phred quality score of Q ≥ 20. Chimeric sequences were filtered out using the UCHIME algorithm (Edgar et al., 2011). The pick-up of operational taxonomic units (OTUs) was performed at 97% similarity by the UPARSE algorithm (Edgar, 2010) in USEARCH (Edgar, 2013). The OTUs were further subjected to the taxonomy-based analysis by RDP Classifier v2.11 with a cut-off of 80% (Wang et al., 2007) using the Silva v128 database. Differential abundance of OTU among treatments was evaluated by metagenomeSeq. The clustered OTUs and taxa information were used for diversity and statistical analyses by Qiime v1.9.1 and R package v.3.3.1 (http://www.R-project.org/). Differences of taxonomic profiles between groups were compared using Statistical Analysis Metagenomic Profiles (STAMP) software (Parks et al., 2014) v2.1.3 with Welch's t-test.

Furthermore, LEfSe (linear discriminant analysis effect size) from the LEfSe tool (http://huttenhower.sph.harvard.edu/lefse/), an algorithm for high-dimensional class comparisons between biological conditions, was used to determine the significant feature taxa between groups or intestinal location. It emphasizes statistical significance, biological consistency, and effect relevance and allows researchers to identify differentially abundant features that are also consistent with biologically meaningful categories (Segata et al., 2011). The Kruskal-Wallis rank sum test was included in LEfSe analysis to detect significantly different abundances and performed LDA scores to estimate the effect size (threshold: ≥ 4).

4.3 Results

4.3.1 NE reproduction and effects of lauric acid as an alternative prevention

Six of the NE cases were identified in three CP1-challenged groups (Table 4.1). They showed different degrees of characteristic gross lesions in small intestinal tissues. The most severe lesions were found in the jejunum, between its proximal end and Meckel's diverticulum. Under co-infection with CP1 and *Eimeria*, the incidence and severity of NE increased. No NE mortality was noticed. Statistically significant differences of lesion score (LS) were determined between three CP1-challenged groups (A, B, and C) and the control counterpart ($p \leq 0.05$). The co-infection groups (B and C) demonstrated a highly significant difference ($p \leq 0.01$). However, the supplementation of lauric acid did not reduce the incidence and severity which were similar to the NE positive control group.

Table 4.1 NE frequency and mean lesion score by groups

Group	Treatment	NE lesion score					Subtotal	Lesion score	NE[1] case[2]
		0	1	2	3	4			
A	CP1	0	9	1	0	0	10	1.11 ± 0.31^{a3}	1
B	CP1+*Eimeria*	0	8	0	1	1	10	$1.50 \pm 1.02^{a*3}$	2
C	CP1+*Eimeria*+LA[1]	0	7	1	1	1	10	$1.60 \pm 1.02^{a*3}$	3
D	*Eimeria*	-	-	-	-	-	10	-	0
E	CTL[1]	5	4	0	0	0	9	0.44 ± 0.50^{b3}	0

[1]LA lauric acid; NE necrotic enteritis; CTL: control group.
[2]NE case: lesion score reaching 2 or above.
*[3]Dissimilar letters indicate a significant difference at a level of α=0.05. * represents highly significant $p \leq 0.01$.*
One chick in CTL was misplaced in the other group during the trial and excluded.

4.3.2 Metadata and sequencing

A total of 11,191,102 sequence reads with an average length of 453 ±5 base pairs were obtained from 30 samples, including 15 jejunal samples (3 samples per group in AJ, BJ, CJ, DJ, and EJ) and 15 cecal samples (3 samples per group in AC, BC, CC, DC, and EC). The sequences were filtered and further clustered into OTU using a cut-off of 97% similarity. The estimate of Good's coverage reached 98% for all the jejunal and cecal samples. The rarefaction curve demonstrated that the sequencing depth was adequate to cover the bacterial diversity in the jejunal and cecal samples (Figure 4.1).

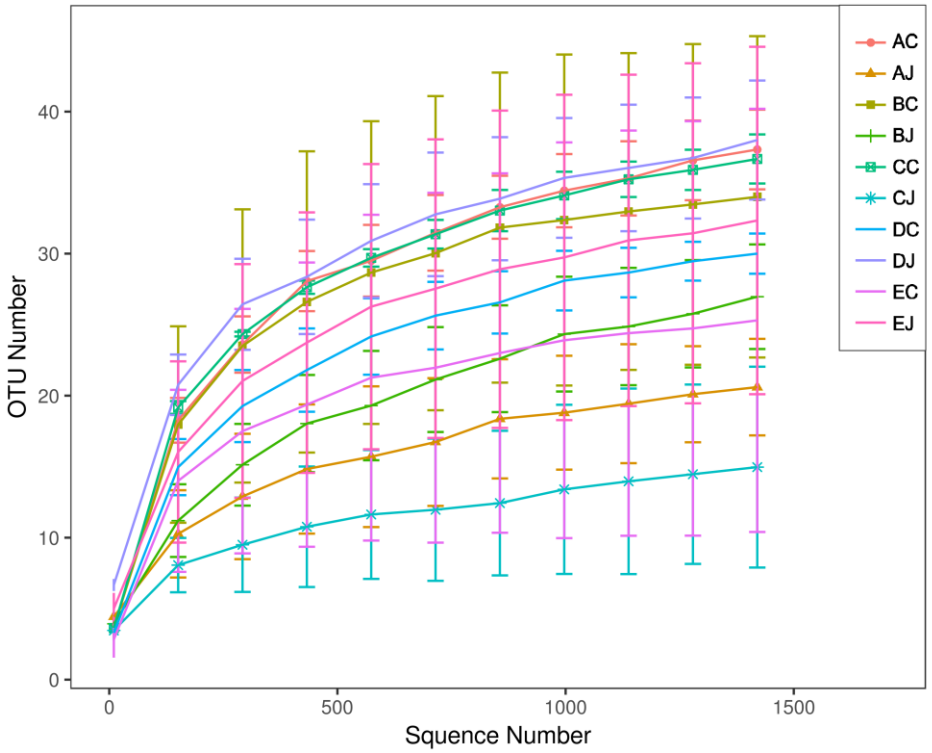


Figure 4.1 Rarefaction curve of OTUs in jejunal and cecal samples.

The curve demonstrated that all samples approximately reached asymptotes, indicating that deeper sequencing would only represent rare additional taxa.

4.3.3 Normal microbial composition in the jejunum and cecum

Firmicutes (92.1% of relative abundance) was the most dominant phylum in the jejunum, followed by *Cyanobacteria* (2.2%) and *Proteobacteria* (2.1%), *Bacteroidetes* (1.9%), and *Actinobacteria* (1.7%). On the contrary, the phylum of *Bacteroidetes* (75.5%) predominated in the cecum, followed by *Firmicutes* (19.8%) and *Proteobacteria* (4.7%) (Figure 4.2A). At the genus level, jejunal contents were dominated by *Lactobacillus* (41.2% of relative abundance) and *Clostridium sensu stricto 1* (39.1%), followed by other unclassified genus (8.7%), *Weissella* (3.6%), *Enterococcus* (1.9%), *Escherichia Shigella* (1.8%), and *Staphylococcus* (1.6%). *Bacteroides* (75.5%) was the most abundant genus in the cecum, followed by other unclassified genus (17.2%), *Escherichia Shigella* (3.1%), *Eisenbergiella* (1.7%), and *Anaerotruncus* (1.5%) (Figure 4.2B). The genera of *Lactobacillus*, *Clostridium sensu stricto 1*, *Weissella*, *Enterococcus*, *Staphylococcus*, and *Bifidobacterium* in the jejunum exhibited significant difference in abundance compared to those in the cecum. Cecal microbiota contained significantly higher abundances of *Bacteroides* and *Proteus* (Welch's t test, $p < 0.05$; Figure 4.3*)*.

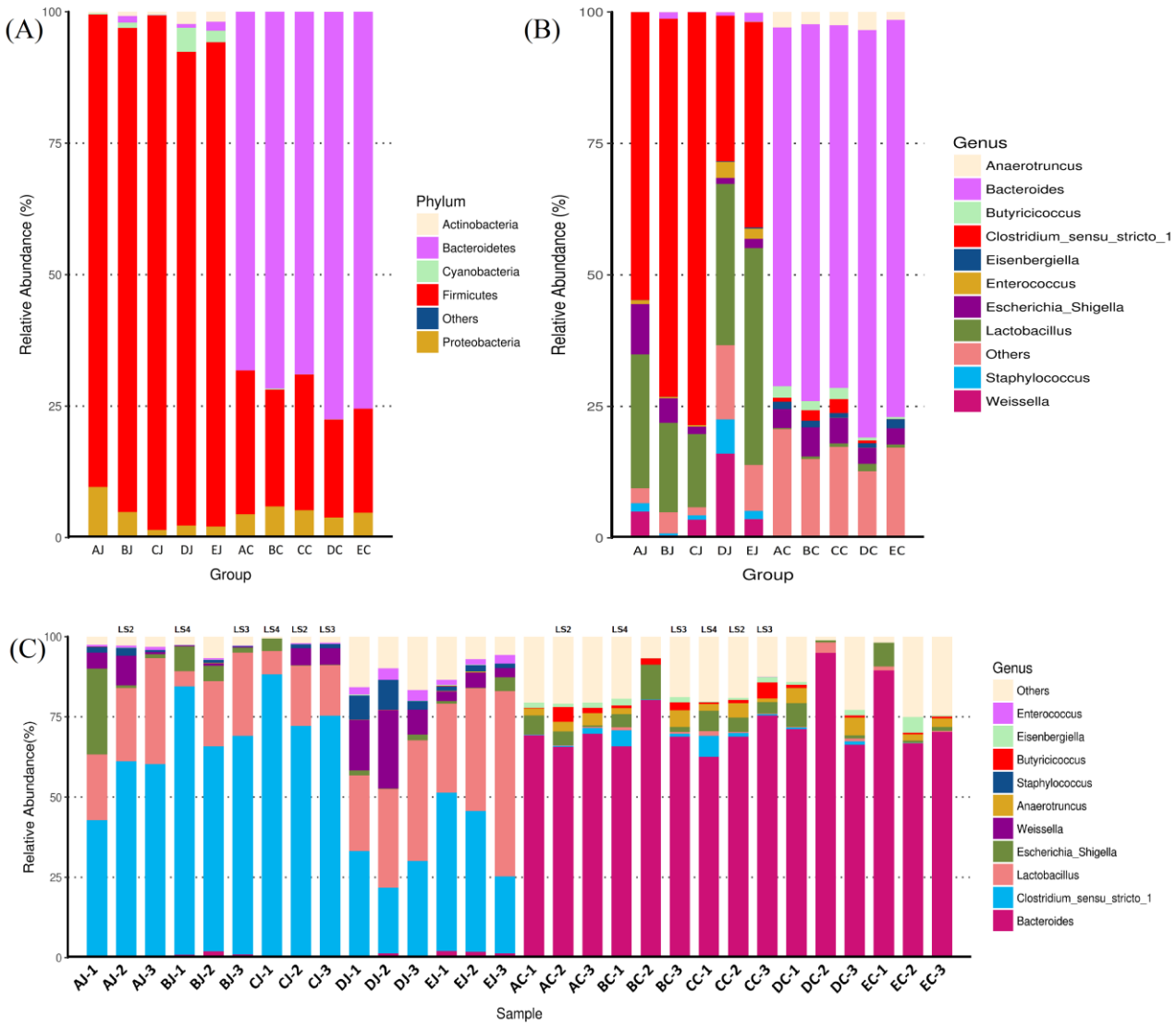


Figure 4.2 Microbial compositions in the jejunum and cecum with different treatments.

Each bar represents the average relative abundance of each bacterial taxon within a group. Microbial compositions in jejunal and cecal groups at the level of the phylum (A) and genus (B). Microbial compositions in jejunal and cecal samples at the genus level (C). LS stands for lesion score of NE. The top 5 and 10 abundant taxa are shown at the level of phylum and genus, respectively.

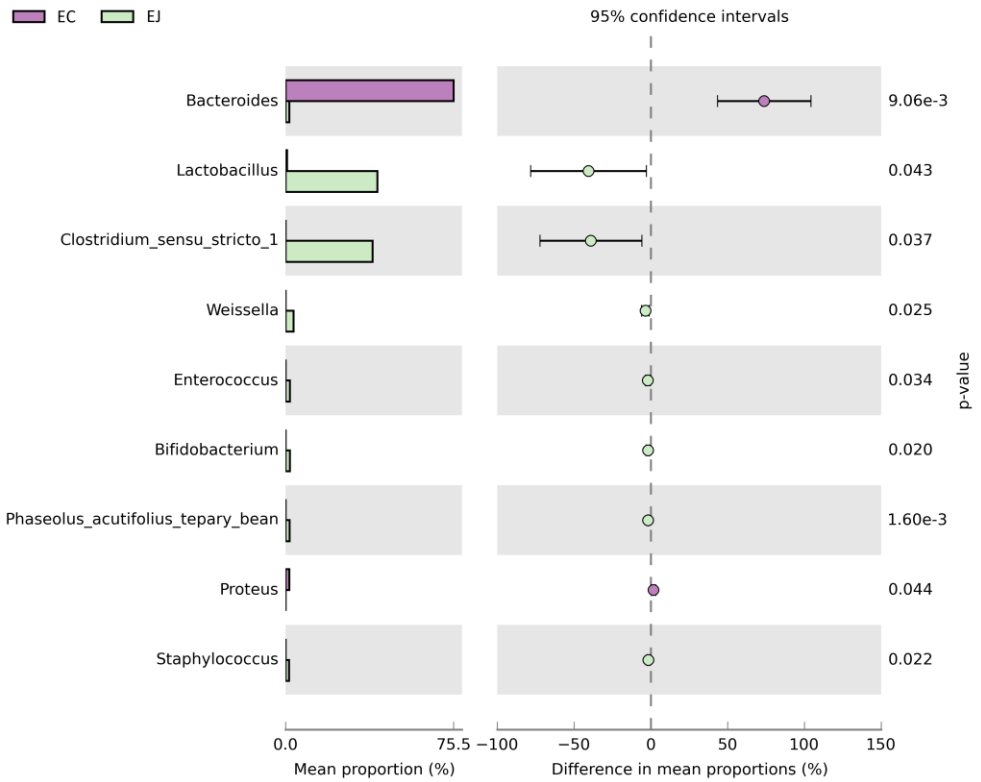


Figure 4.3 Differential abundant taxa at the genus level between jejunal and cecal microbiota in control chickens by STAMP with *Welch's t-test*.

The differential genus was assigned only to those presenting a minimum variation at a significant level ($p < 0.05$).

4.3.4 Changes of microbial communities in response to treatments

In the jejunum, the challenge of CP1 increased the relative abundance of the genera of *Clostridium sensu stricto 1* (54.75%), *Escherichia Shigella* (9.57%), and *Weissella* (4.99%) but significantly decreased the population of *Lactobacillus* (25.44%) (Figure 4.4). The inoculation of *Eimeria* to chickens significantly increased the relative abundance of *Weissella* (16.01%) and *Staphylococcus* (6.51%) but decreased the amount of *Lactobacillus* (30.66%) and *Clostridium sensu stricto 1* (27.69%). Co-infection with CP1 and *Eimeria* led to the significant increment of *Clostridium sensu stricto 1* (71.89%),

increased relative abundance of *Escherichia Shigella* (4.68%), but the decrements of *Lactobacillus* (16.99%), *Weissella* (0.44%) and *Staphylococcus* (0.40%). In the cecum, different treatments did not promote significant difference of taxa abundance between groups with an exception of *Eisenbergiella,* significantly increased in the co-infection group. However, the challenge of CP1 and co-infection of CP1 and *Eimeria* still promoted cecal increments of *Clostridium sensu stricto 1* (the relative abundance of this taxon in groups challenging with CP1, CP1 and *Eimeria,* and control was 0.75%, 1.99%, and 0.02%).

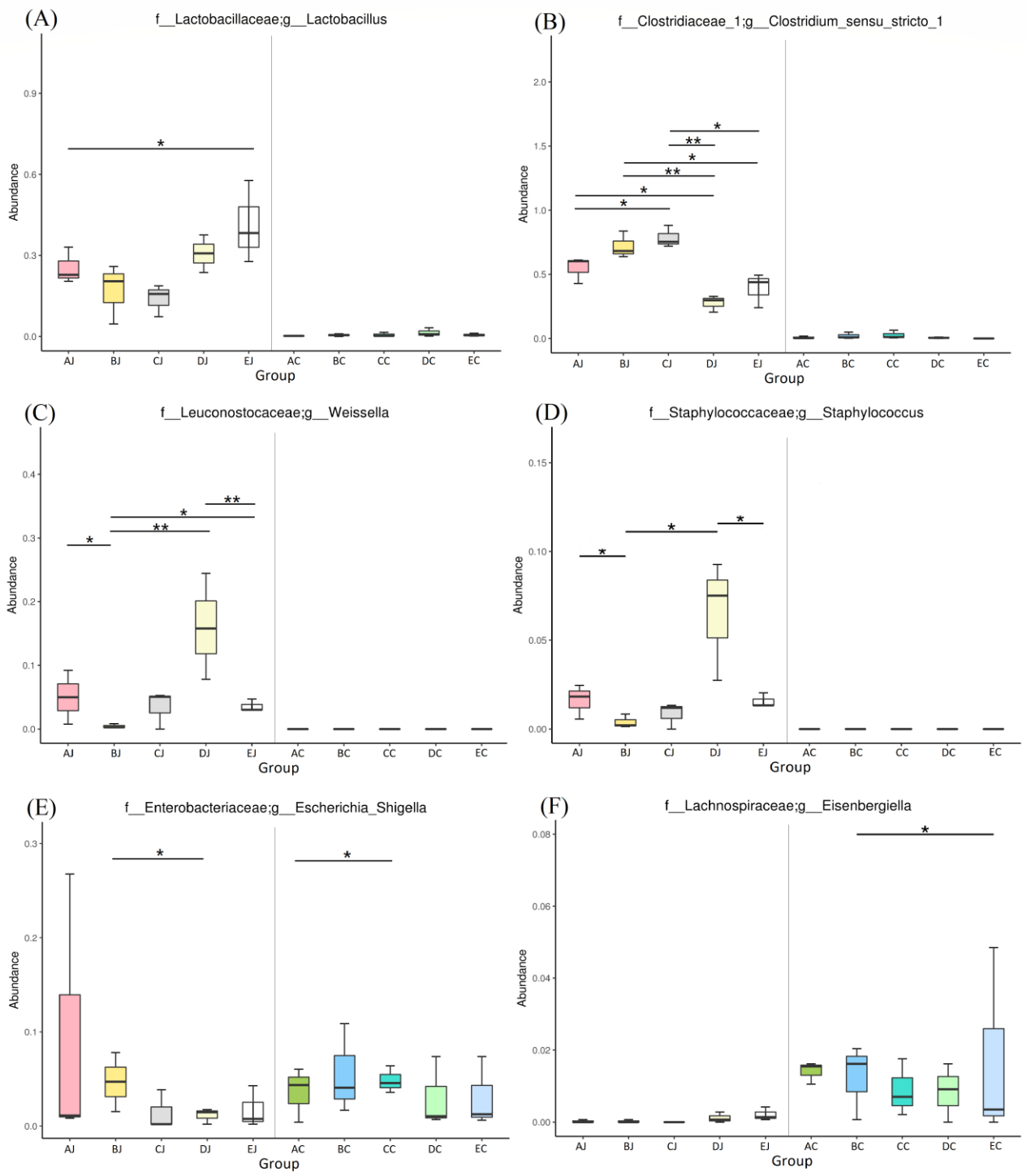


Figure 4.4 Differential abundance of genera between groups in the jejunum and cecum by metagenomeSeq.

(A) *Lactobacillus*; (B) *Clostridium sensu stricto 1*; (C) *Weissella*; (D) *Staphylococcus*; (E) *Escherichia shigella;* (F) *Eisenbergiella.* * $p \leq 0.05$ and ** $p \leq 0.01$.

4.3.5 Microbial diversities in response to treatments

In jejunal microbiota, the challenge of CP1 (AJ) and co-infection with CP1 and *Eimeria* (BJ) reduced species richness and evenness, but the infection of *Eimeria* (DJ) exerted counter results. The addition of lauric acid into the co-infected group (CJ) exacerbated the reduction observed in BJ group. However, no apparent effect was noted on cecal microbiota following above treatments (Figure 4.5 and Figure 4.6). Analysis of alpha diversity by Shannon index further demonstrated that the challenge of CP1 in conjunction with *Eimeria* infection significantly reduced species diversity in jejunal microbiota. The 16S rRNA gene survey by principal coordinate analysis (PCoA) and principal component analysis (PCA) showed a distinct separation of two community profiles between the jejunal and cecal microbiota. Cluster and heat map analyses exhibited distinct classifications and microbial compositions between the jejunum and cecum, coinciding with observations on PCoA and PCA (Figure 4.7). Additionally, the results of PCoA and PCA also depicted the differential diversity between the CP1-challenged (group AJ, BJ, and CJ), *Eimeria*-infected (DJ), and control (EJ) groups in the jejunal microbiota, showing that the challenge of CP1 shared similar microbial community structures with co-infection with CP1 and *Eimeria*. However, cecal groups with CP1 treatments did not display cluster phenomenon as jejunal groups displayed in PCoA. PCA with hierarchical clustering further reflected that *Clostridium sensu stricto 1* was contributory to the similarity of NE assemblage, and the genera of *Lactobacillus, Weissella,* and *Staphylococcus* contributed to discrepant community structures in *Eimeria*-treated and control groups in the jejunum. On the other hand, *Bacteroidetes* was

the main genus contributing to the distinct separation between jejunal and cecal groups (Figure 4.8).

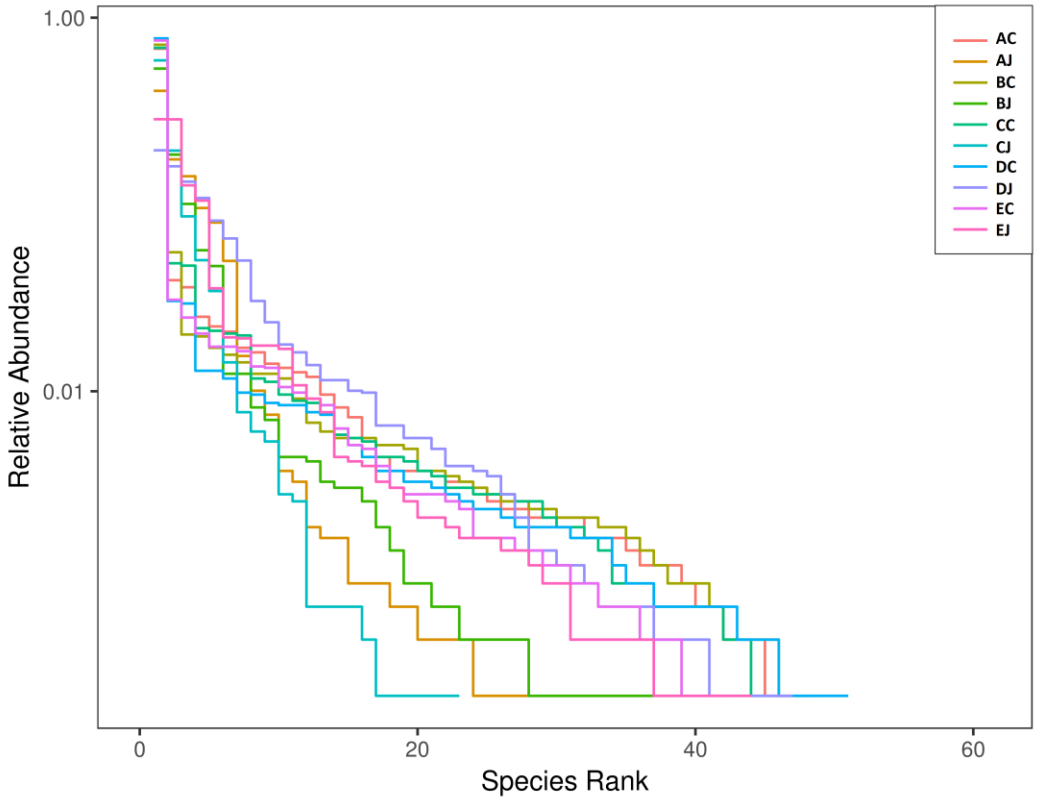


Figure 4.5 Rank abundance curve represented by groups.

The total number of species is presented in the maximum reading of each curve on the x-axis, while values on the y-axis indicate the relative abundance of each ranked species.

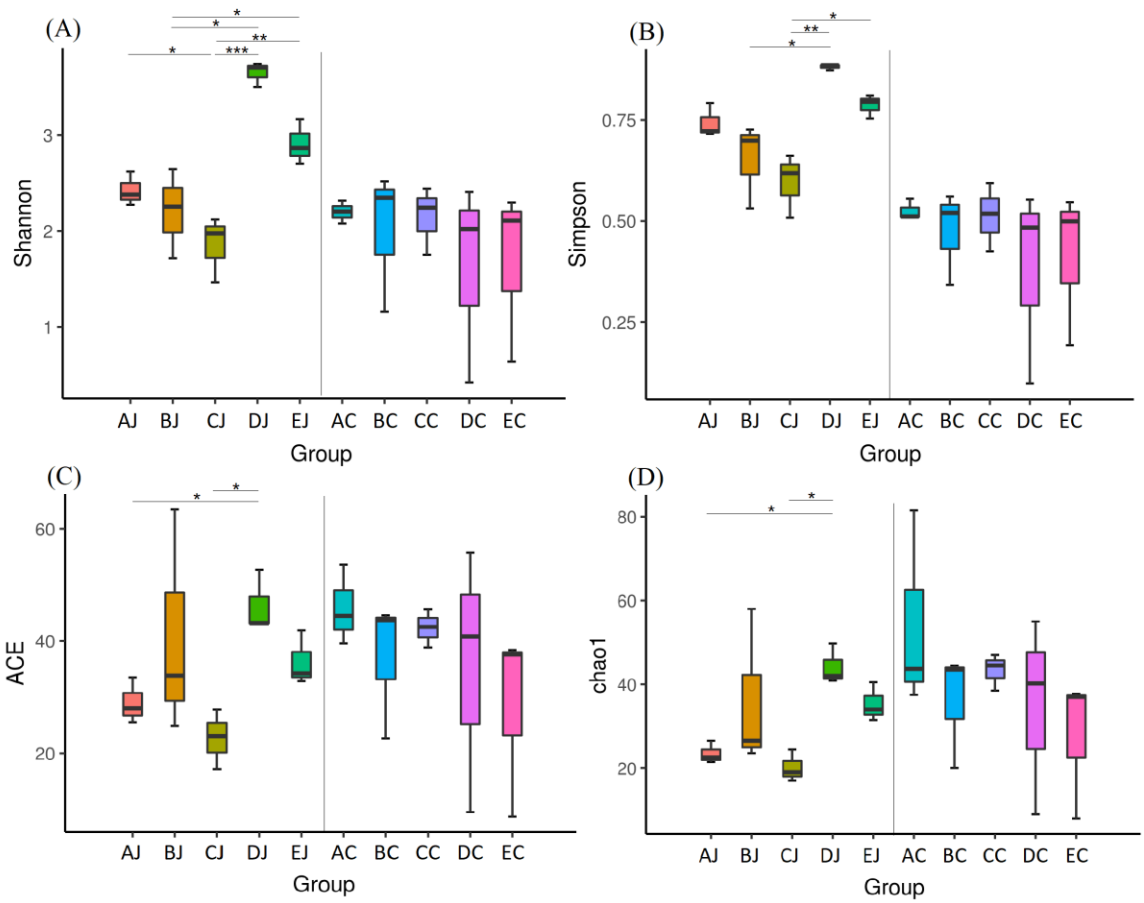
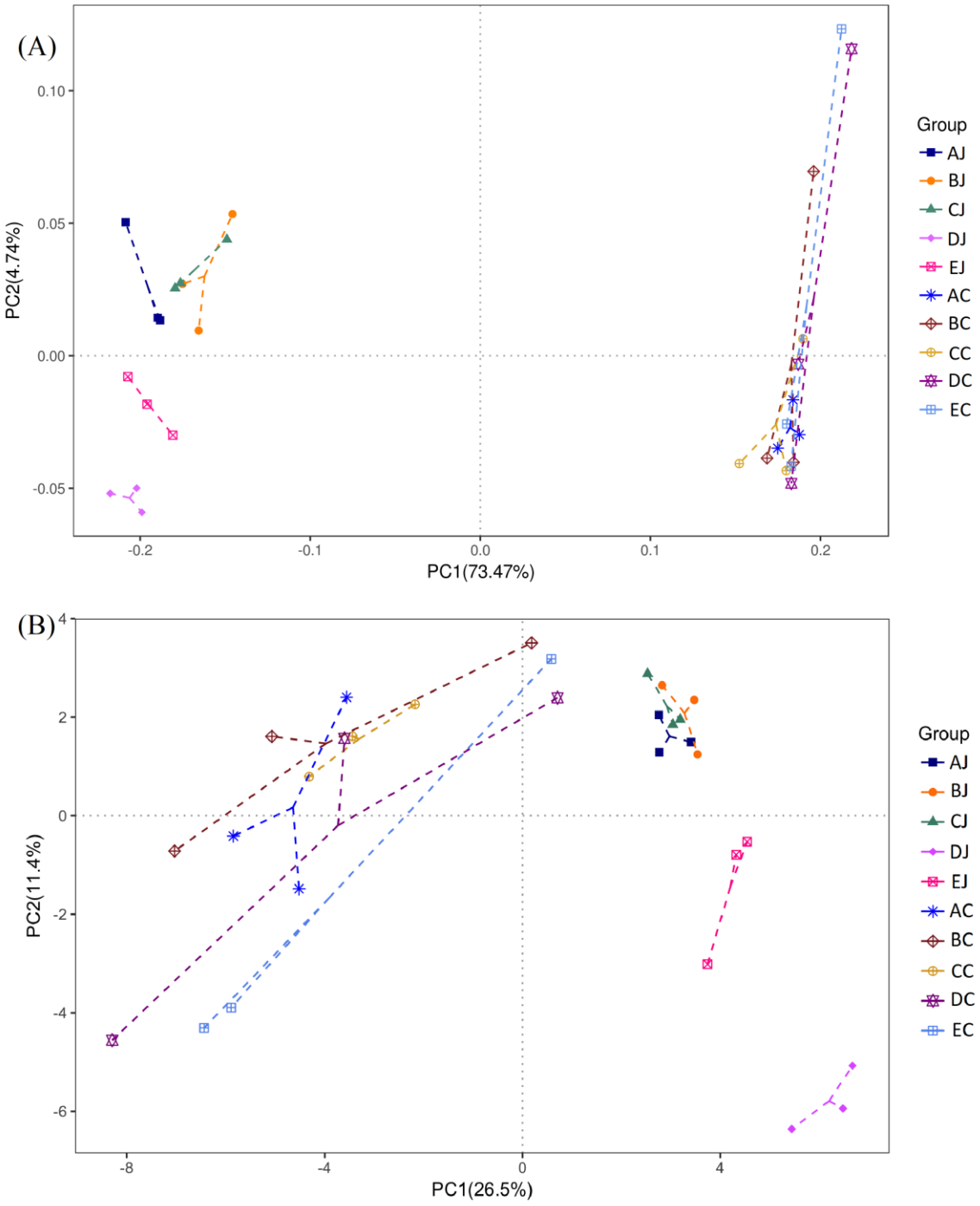


Figure 4.6 Alpha diversity analysis between groups in the jejunum and cecum.

(A) Shannon index, (B) Simpson index (C) Abundance-based Coverage Estimator (ACE) index, and (D) Chao1 index. Results are shown as mean ± SEM. Kruskal-Wallis test: * $p \leq 0.05$, ** $p \leq 0.01$, *and* *** $p \leq 0.001$.



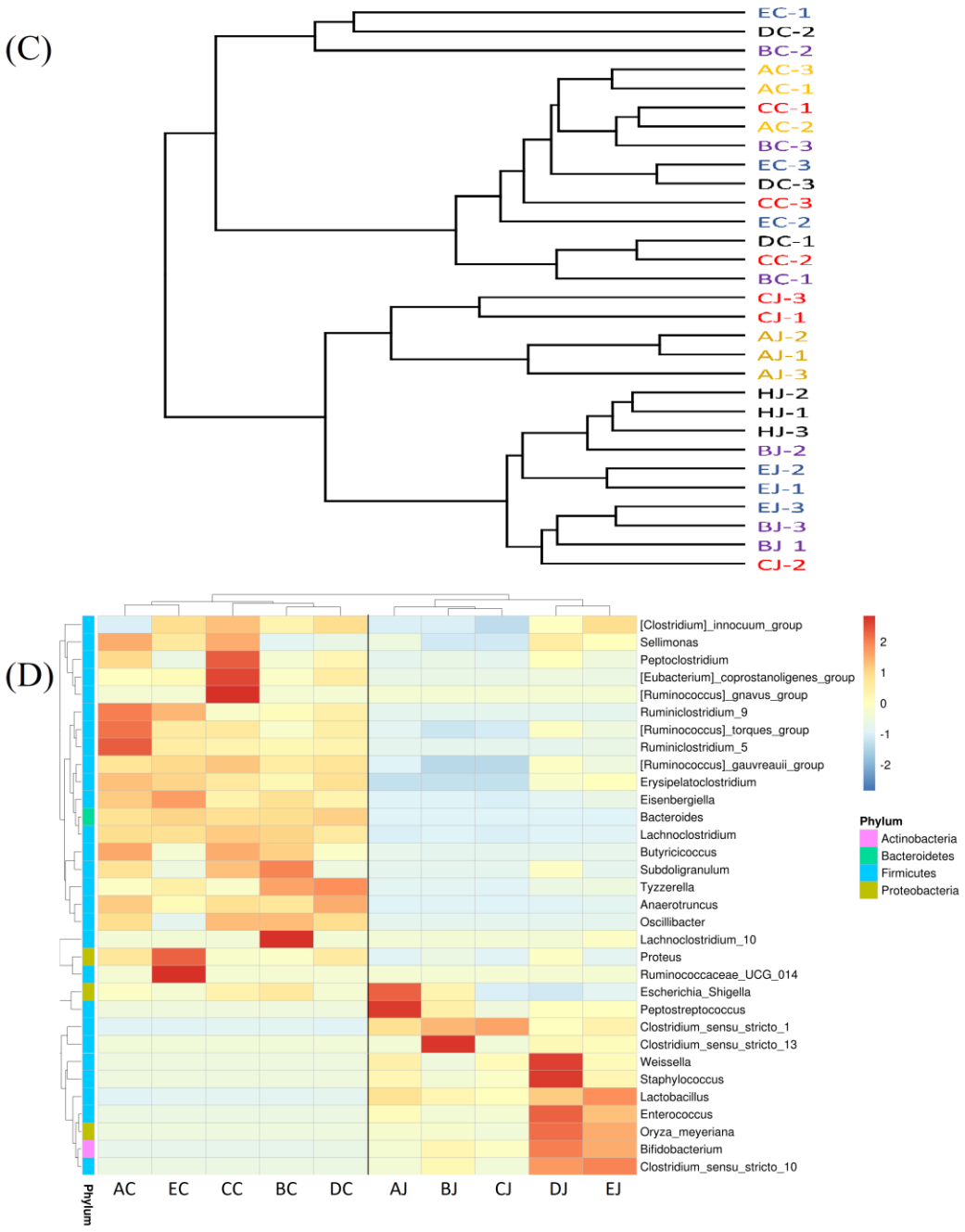


Figure 4.7 Comparison of microbial community profiles and similarities between jejunal and cecal groups with different treatments.

(A) Weighted unifrac principal coordinate analysis (PCoA); (B) principal component analysis (PCA); (C) cluster analysis by the unweighted paired-group method using arithmetic means (UPGMA) using unweighted unifrac distance; (D) heat map analysis at the level of genus.

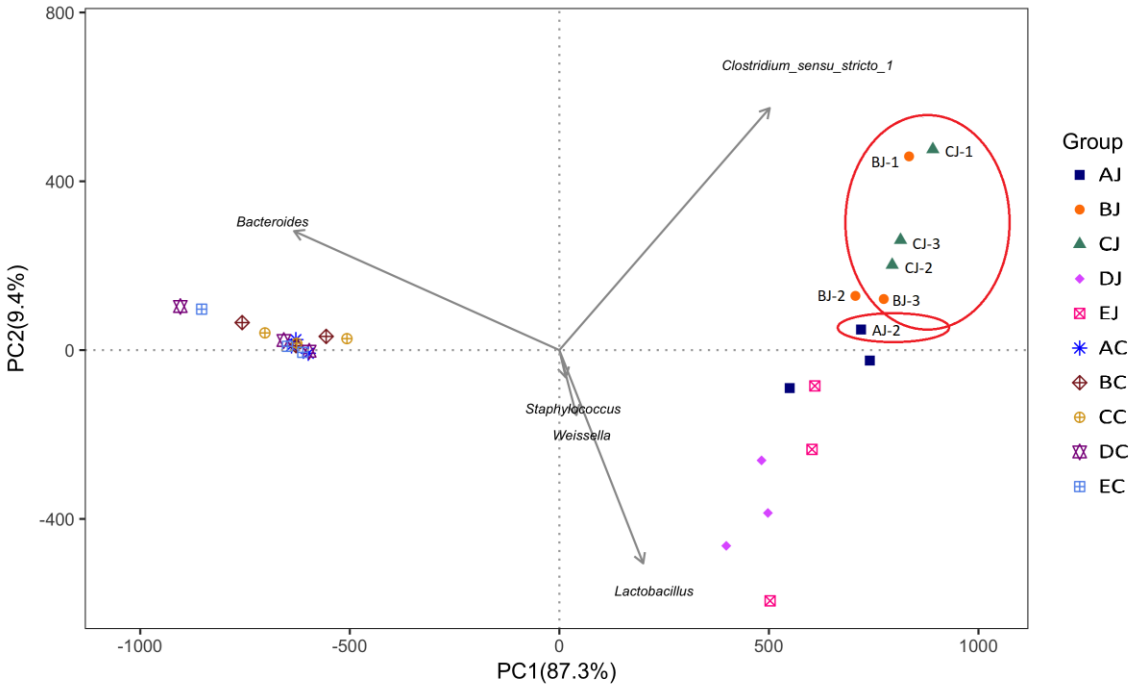


Figure 4.8 Contributory genera to NE assemblage and dissimilarity between groups by principal component analysis with hierarchical clustering.

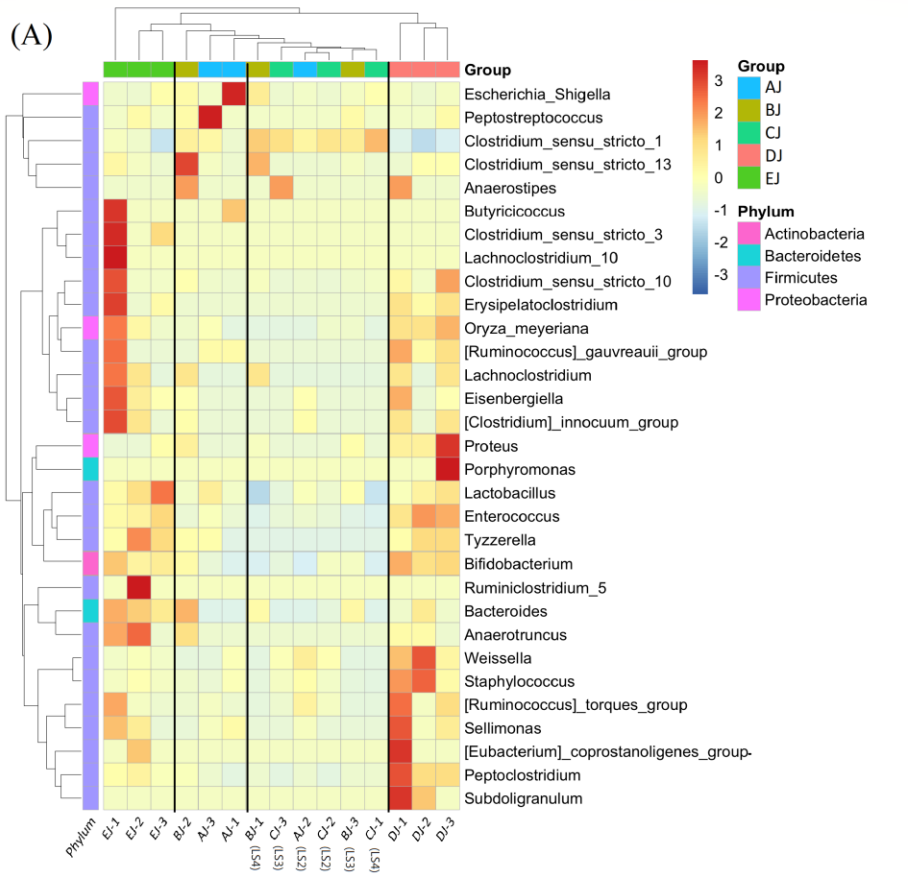
All NE cases were clustered as NE assemblage in the red circles.

4.3.6 Microbial community structure and taxa contributory to NE

Analysis of jejunal microbiota in NE cases revealed that *Clostridium sensu stricto 1*, to which causative *C. perfringens* belongs, was the most dominant genus, followed by *Lactobacillus, Weissella*, *Escherichia Shigella*, *Staphylococcus*, and others. Accompanying the elevation of NE severity, the relative abundance of *Clostridium sensu stricto 1* increased (relative abundance ≥ *75%* in LS4 compared to 50-75% in LS2 and LS3). Conversely, the population of *Lactobacillus* decreased while the lesion score was elevated. The relative amount of *Escherichia Shigella* was variable in NE cases,

presenting a higher abundance after CP1 challenge but low population following co-infection with CP1 and *Eimeria*. (Figure 4.2C).

Heat map analysis exhibited that NE cases harbored the similar microbial community profile. *Clostridium sensu stricto 1* and *C. perfringens* were consistently presented and abundant taxa in the jejunum (Figure 4.9). Opposite low abundance of *Lactobacillus* was noted. However, only the increment of *Clostridium sensu stricto 1* but not *C. perfringens* (data not shown) demonstrated significance on NE by metagenomeSeq (Figure 4.4B). Using Welch's t-test, jejunal groups further showed that CP1 in conjunction with *Eimeria* increased significantly *Clostridium sensu stricto 1* and *C. perfringens* when compared to the control (Figure 4.10 and Figure 4.11*; $p < 0.05$)*, whereas challenge of CP1 alone did not lead to significant increase of these taxa. Differential abundant taxa between different treatments in jejunum were further evaluated by LEfSe using the LDA score of 4. This threshold guarantees that the meaningful taxa are compared and eliminates most of rare taxa. LEfSe demonstrated similar results as the Welch's test that the challenge of CP1 unable to yield a significantly higher amount of *Clostridium sensu stricto 1* and *C. perfringens*. However, significant differences were displayed when CP1 co-infected with *Eimeria* (Figure 4.12). No differential taxon was found in cecal groups (AC, BC, CC, DC, and EC) while Welch's t-test and LEfSe were applied.



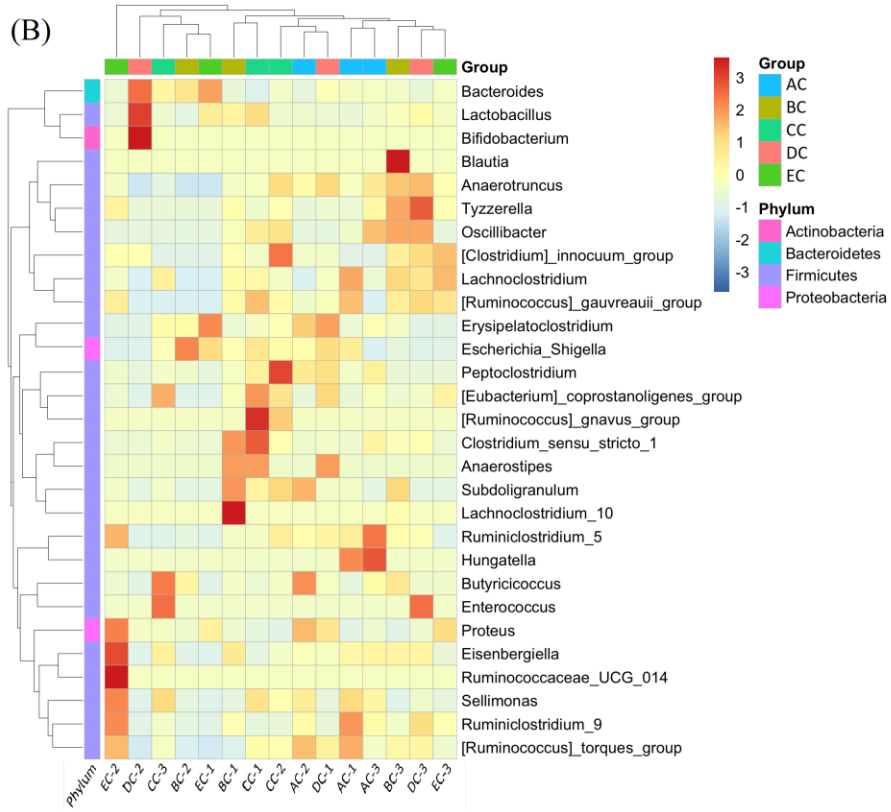


Figure 4.9 Heat map analysis of contributory taxa to NE.

The heat map is demonstrated at the level of genus in jejunal (A) and cecal (B) samples. Analysis of gut bacteria with the relative abundance of OTUS by z score (C) represents bacterial taxa information, including phylum, family, genus, and species. This analysis showed the increment of *Clostridium sensu stricto 1* and *C. perfringens*, as well as the decrement of *Lactobacillus* followed NE severity in the jejunum.

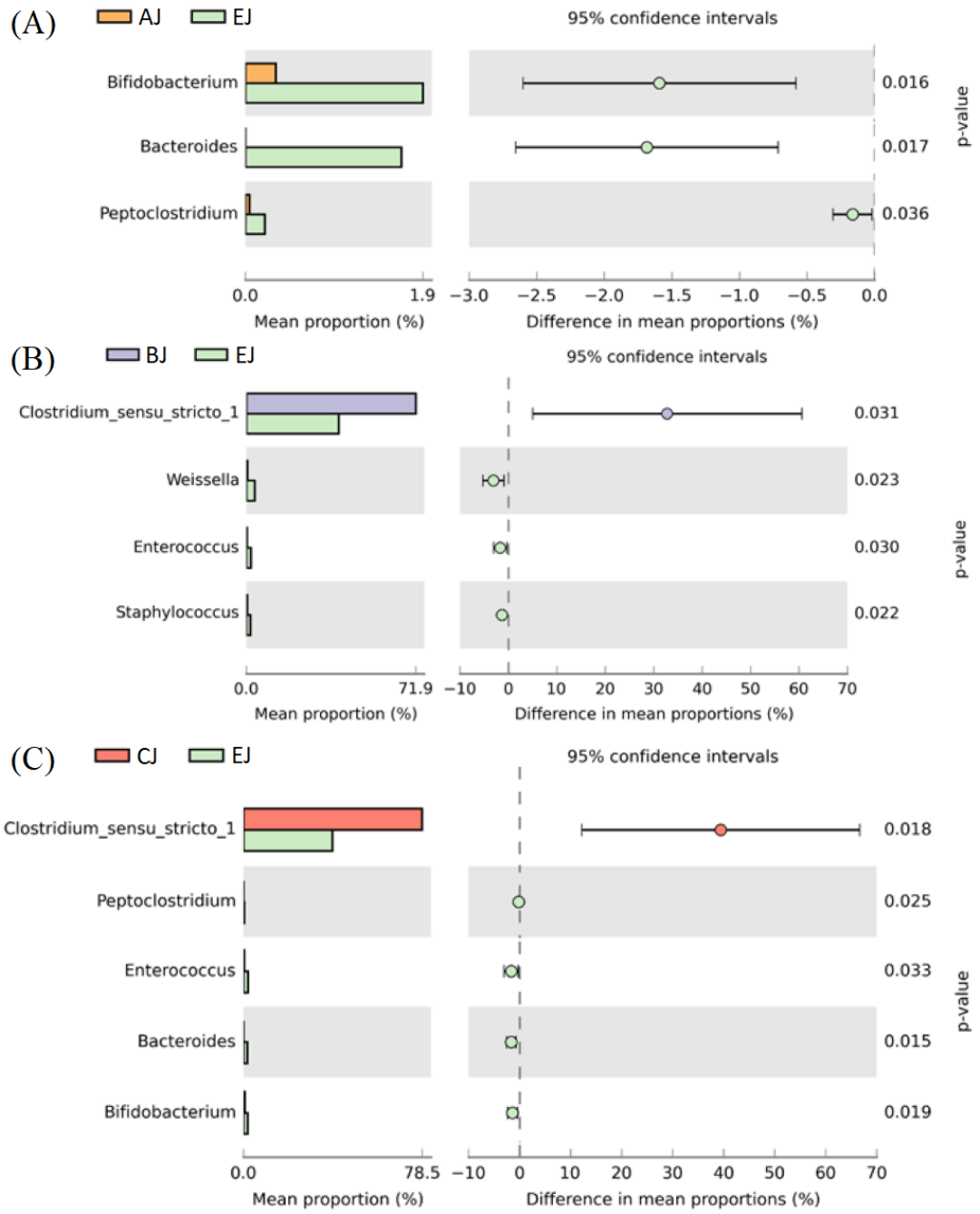


Figure 4.10 Deferential taxa between treatments and control group at the genus level by STAMP with *Welch's t-test*.

(A) Comparison between AJ and EJ, (B) BJ and EJ, and (C) CJ and EJ groups. The differential genera were assigned only to those presenting a minimum variation at a significant level ($p < 0.05$).

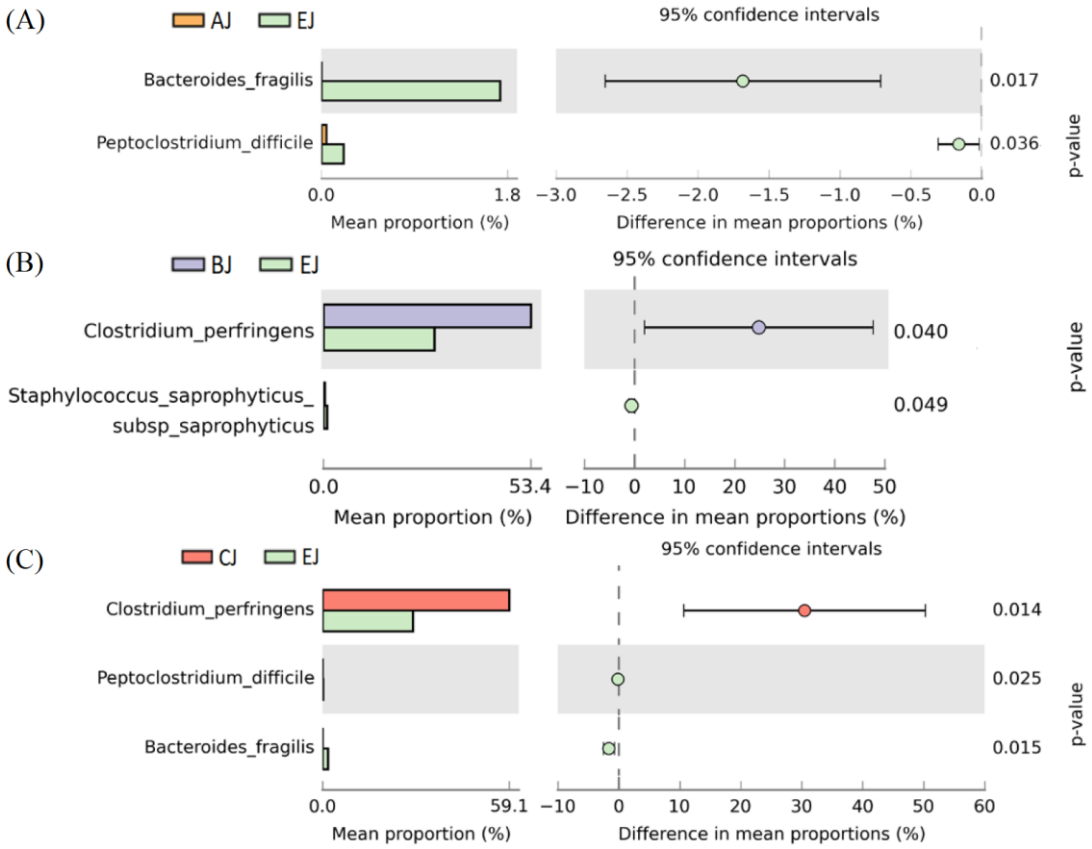


Figure 4.11 Deferential taxa between treatments and control group at the species level by STAMP with *Welch's t-test*.

(A) Comparison between AJ and EJ, (B) BJ and EJ, and (C) CJ and EJ groups. The differential species were assigned only to those presenting a minimum variation at a significant level ($p < 0.05$).

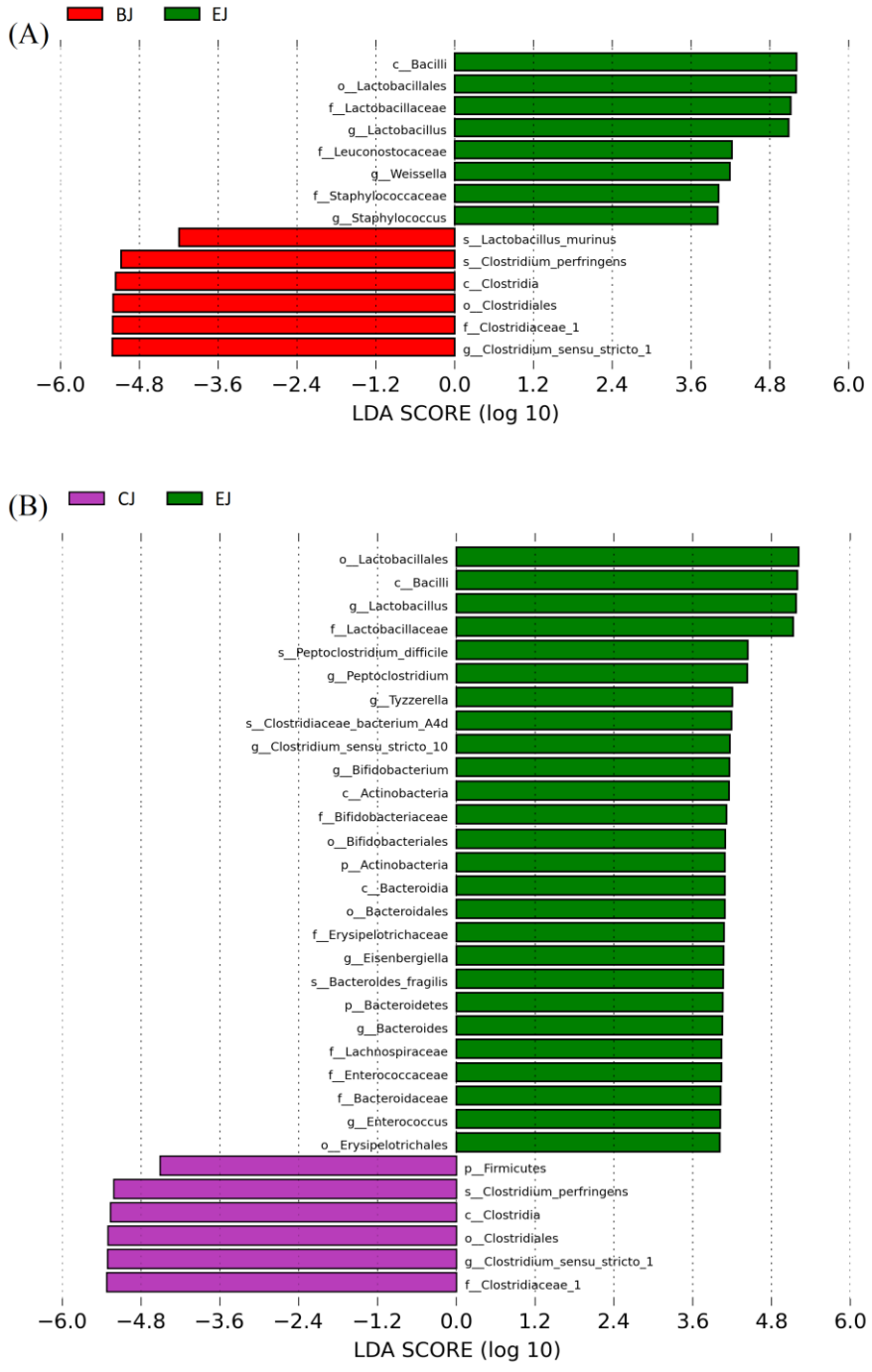


Figure 4.12 LEfSe analysis of differentially abundant clades at all taxonomic levels between CP1-challenged and control groups in jejunal microbiota.

LEfSe identified the most differentially abundant clades between groups using the LDA score of 4. Differentially abundant taxa in group BJ versus EJ (A), and CJ versus EJ (B).

4.3.7 Comparison of gut metagenomes in co-infected chickens with and without lauric acid

The addition of lauric acid increased the relative abundance of *Clostridium sensu stricto 1* and *Weissella* but decreased the relative amount of *Lactobacillus* and *Escherichia Shigella* in the jejunum compared to the co-infection group without supplementing lauric acid. Nonetheless, no significance was detected in this comparison. In addition, supplementation of lauric acid did not apparently affect the cecal microbiota between these two groups.

4.4 Discussion

By exploring microbial composition in normal chickens, the major microbial genera in the jejunum were *Lactobacillus* and *Clostridium sensu stricto 1*, followed by other unclassified bacteria, *Weissella*, *Enterococcus*, *Escherichia Shigella*, and *Staphylococcus*. *Bacteroides* was the most abundant group in the cecum, and the remaining taxa were sequentially other unclassified bacteria, *Escherichia Shigella*, *Eisenbergiella*, and *Anaerotruncus*. Side by side treatments of *C. perfringens* and *Eimeria* altered microbial community compositions, significantly in jejunal microbiota. In this study, the challenge of CP1 increased the abundance of *Clostridium sensu stricto 1*, *Escherichia Shigella,* and *Weissella* in the jejunum, but significantly decreased the population of *Lactobacillus*. Infection of *Eimeria* significantly increased the abundance of *Weissella* and *Staphylococcus* but decreased the amount of *Lactobacillus* and *Clostridium sensu stricto 1*. Co-infection with *C. perfringens* and *Eimeria* led to the significant increment of *Clostridium sensu stricto 1*, increased the abundance of *Escherichia Shigella*, but decrements of *Lactobacillus, Weissella*, and *Staphylococcus.* Specifically, it decreases the α-diversity index of the small intestinal microbial community, promoting the single dominance of *Clostridium sensu stricto 1* reaching the relative abundance to 71.89%. On the other hand, six NE cases shared similar microbial community profile observed in PCA, indicating there exists a certain microbiota contributory to the disease. With more NE severity, the higher relative abundance of *Clostridium sensu stricto 1* but the lower relative amount of *Lactobacillus* in jejunal microbiota was noted.

Several studies have been shown *C. perfringens* challenge decreased the population of *Lactobacillus* in the ileum (Antonissen et al., 2016; Li et al., 2017b). Lactobacilli are known as lactic acid-producing bacteria and shown to have protection at the intestinal barrier by competition with pathogens. They are also able to induce immunomodulation and ferment carbohydrates into lactic acids that lower the pH of the intestinal environment to inhibit the growth of acid-sensitive pathogenic bacteria (Belenguer et al., 2007; Sengupta et al., 2013). Therefore, suppression of lactobacilli is regularly considered beneficial to the growth and colonization of enteric pathogen. This study first demonstrated the decrement of lactobacilli in jejunum following challenge of *C. perfringens* alone and in conjunction with *Eimeria.* The change of this taxon following the NE severity indicates that the decrement of *Lactobacillus* may play a role in the development of NE. In addition, the increased abundance of *Escherichia Shigella* was also observed after the challenge of *C. perfringens* and co-infection with *C. perfringens* and *Eimeria*. This genus includes enteric pathogens, which can colonize in the intestines of both humans and chickens, consequently triggering specific diseases (Mora et al., 2010). Some studies indicated that the increment of *Escherichia Shigella* in ileum was correlated with NE (Du et al., 2015; Du et al., 2016). Nevertheless, our study found that *C. perfringens* challenge could increase the abundance of *Escherichia Shigella*, but the increment was not in accordance with NE occurrence. Furthermore, the reduction of this taxa abundance was noticed in lauric acid supplementing group which has the highest number of NE cases. Those finding reflected a contradiction for this genus participating in NE development. Last but not least, a reduced abundance of *Weissella* in the jejunum of NE afflicted chickens was also noted. Another study reported similar results in cecal

microbiota after *C. perfringens* challenge (Stanley et al., 2012b). *Weissella* is a lactic acid bacterium and belongs to the family of the *Leuconostocaceae*. They harbor probiotic properties and can generate several products with probiotics potential (Fusco et al., 2015). It may interact with *C. perfringens* as other lactic acid bacteria, but its role in NE development is unclear. More studies will be needed to elucidate the relationship between *Weissella* and NE.

In the current study, significant overgrowth of *Clostridium sensu stricto 1* was associated with the NE and the infection of *Eimeria* precedent to *C. perfringens* challenge exerted synergistic effects on the overrepresentation. This correlation was consistently demonstrated by analyses of metagenomeSeq, STAMP, and LEfSe. The STAMP and LEfSe further showed *C. perfringens* was significantly overrepresented in NE groups. However, such significance was not identified by metagenomeSeq when *C. perfringens* was targeted. This result indicates that, in addition to *C. perfringens,* another member under the same genus of *Clostridium sensu stricto 1* also played a role in contributing to the development of the disease. The *Clostridium* genus is well-classified into 19 clusters by phylogenetic analysis (Collins et al., 1994). *Clostridium sensu stricto* are grouped around the type species *Clostridium butyricum* and belong to the *Clostridium* cluster 1within the *Clostridiaceae* family (Stackebrandt et al., 1999). *Clostridium sensu stricto 1* contains *C. perfringens* and other real *Clostridium* species. Their members are generally perceived as pathogenic (Rajilic-Stojanovic and de Vos, 2014) as well as interpreted as an indicator of a less healthy microbiota (Lakshminarayanan et al., 2013). This suggestion coincides with our finding that *C. perfringens* challenge on its own is not capable of causing a significant abundance of *Clostridium sensu stricto 1* and unable to

produce more NE case observed. Future research is recommended to clarify the role of other members of *Clostridium sensu stricto 1* in the pathogenesis of NE.

Single infection of *Eimeria* could not produce NE in the present study. The treatment reduced the relative abundance of *Clostridium sensu stricto 1* and *Lactobacillus* but significantly increased *Weissella* and *Staphylococcus* in jejunal microbiota. *Eimeria* infection has been shown to provide nutrients for *C. perfringens* to grow and cause physical damage to gut epithelium, thus facilitating the colonization and proliferation of *C. perfringens* (Van Immerseel et al., 2009; Williams, 2005; Williams et al., 2003). However, the inoculation of *Eimeria* into normal chickens did not elicit overgrowth of *Clostridium sensu stricto 1* and *C. perfringens* except challenging with exogenous *C. perfringens.* In contrast, the challenge of *C. perfringens* alone and in conjunction with *Eimeria* both promote proliferation of *Clostridium sensu stricto 1* and NE case. This indicates that the amount of commensal *C. perfringens* in the jejunum under *Eimeria* infection is not sufficient to reach the significant abundance of *Clostridium sensu stricto 1* or *C. perfringens*, subsequently promoting the occurrence of NE. Therefore, it is reasonable to suggest that the quantity of *C. perfringens* reaching a certain level in the jejunum is critical for the onset of proliferation. A recent study used commensal *C. perfringens,* the isolate from normal chicken, to challenge broiler and reproduce NE in conjunction with infection of *E. maxima* (Li et al., 2017a). This result also highlighted that not the specific *C. perfringens* strain, but the exogenous addition of *C. perfringens* played the key in achieving the consequence. Accordingly, the methodology to inhibit the overgrowth of *Clostridium sensu stricto 1* or *C. perfringens* in small intestines will be the straightforward strategy to prevent NE.

Recent studies have been shown that cecal microbiota had a prominent role in feed efficiency (Yan et al., 2017) and received increasing attention in terms of diseases (Wohlgemuth et al., 2011) and metabolism (Stanley et al., 2012a). In this study, the result of PCoA and PCA demonstrated that microbial communities in the jejunum were different from those in the cecum. Side by side treatments of *C. perfringens* and *Eimeria* promoted microbial shifts with biological significance in the jejunum but minimal fluctuations in taxa abundance in the cecum. Comparatively, jejunal microbiota was more significant than cecal microbiota to address the characteristic gut microbiota contributory to NE by means of metagenomeSeq and LEfSe analysis. The reason might be that cecal microbiota is demonstrated more diverse than other intestinal sections (Xiao et al., 2017) and inhibits higher amounts of microbes (10^{10}-10^{11} CFU/g) than those in the jejunum (10^{8}-10^{9} CFU/g) (Yeoman et al., 2012). Those may provide the buffer effect on microbial changes in cecal microbiota. Besides, preferential colonization of *C. perfringens* on the mucosa of the small intestine (Prescott et al., 2016a) may also contribute to less amount of *C. perfringens* into the cecum, hence adverse to elicit significant changes in cecal microbiota.

Medium-chain fatty acids (MCFAs) such as lauric acid are a family of saturated 6- to 12-carbon fatty acids from plants and documented beneficial effects on intestinal health and microbial growth inhibition (Bertevello et al., 2012; Dierick et al., 2004; Zentek et al., 2012). The mechanism of their bactericidal activity is not fully understood. Relative studies showed that they could act as nonionic surfactants to become incorporated into the bacterial cell membrane, as well as diffuse through cell membranes and create pores, changing membrane permeability and leading to cell death (Altieri et

al., 2009; Bergsson et al., 2001; Desbois and Smith, 2010). In this work, lauric acid attracted interest due to its inexpensiveness and natural properties, including strong antibacterial effects against *C. perfringens* and no inhibitory effect on *Eimeria* infection (Mathis et al., 2018). Based on Timbermont's study, lauric acid was most effective in inhibiting the growth of *C. perfringens* strain *in vitro*. Given a supplementary dose of 0.4 kg/ton in feed caused a significant decrease in NE incidence (from 50% down to 25%) compared with the infected, untreated control group (Timbermont et al., 2010). This study followed the dose and used experimental grade product of lauric acid to evaluate the effects on NE incidence and intestinal microbiota. However, the addition of lauric acid did not reduce the incidence of NE. For intestinal microbiota, lauric acid neither exerted the inhibitory effect against the proliferation of *C. perfringens* nor elevated the level of beneficial bacteria, such as *Lactobacillus* and *Bifidobacterium*. But, the relative abundance of *Escherichia Shigella* was decreased without affecting the incidence. Since lauric acid has different grade of products, such as experimental or food grade, the contradictory result may attribute to the influence of different formula on the absorptive efficiency of this compound. MCFAs are hydrophobic and partly absorbed through the stomach mucosa. Hence, their triacylglycerols are considered as a desirable formula for feed additive because they can be absorbed intact into intestinal epithelial enterocytes via this form (Zentek et al., 2011).

In summary, significant overgrowth of *Clostridium sensu stricto 1* in jejunum was recognized as the major microbiota contributory to the NE. In addition to *C. perfringens*, the other member within *Clostridium sensu stricto 1* was also found to participate in disease development. The decrement of *Lactobacillus* following the NE severity

indicated that lactobacilli also participate in the progress of the disease. These taxa showed counteractive effects in their functions as well as in the bacterial abundance, attempting to maintain the homeostasis of jejunal microbiota in chickens. Therefore, manipulations to inhibit multiplication of *Clostridium sensu stricto 1* and *C. perfringens* and to rehabilitate the dominant *Lactobacillus* population in the jejunum should be the niche for developing effective strategies to prevent NE.

4.5 Reference

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CHAPTER V

CONCLUSION

NE reaches economic significance to the poultry industry because of its acute mortality and subclinical infections in broiler flocks. After phasing out of antibiotics in poultry production, it becomes a continuous burden to the industry worldwide without a prompt and effective strategy to prevent and control this disease. The review of the literature demonstrated that NE is a multifactorial disease, involving in the participation of *C. perfringens*, contributory factors, and host responses. It is evident that contributory factors create environmental conditions favorable for colonization and multiplication of *C. perfringens* that predispose chickens to the disease. In addition, causative *C. perfringens* produces virulent proteins such as attachment factors, collagenolytic enzymes, bacteriocins, and toxins to promote bacterial colonization, single dominance, invasion, and then the transmission. The host immune status, physiological integrity of the intestines, and gut microbiota also participate in the development and severity of NE. Although advances in NE research have greatly contributed to identifying predisposing factors and preventative resources, including vaccines, feed additives, and others, desirable protection or prevention strategies are still lacking. To provide practical insights in preventing and controlling NE, this dissertation addressed several knowledge gaps in the pathogenesis and disease induction, then generated the following conclusion.

A novel qPCR primer set targeting *netB*, mainly recognized in NE-producing *C. perfringens* and demonstrated as a key virulence factor for NE, was developed and optimized to qualify and quantify this gene in *C. perfringens* isolated from NE and non-NE chickens. Sixty percent of NE and non-NE isolates both carried *netB and* no significant difference was detected between these two types of isolates regarding the carriage and the quantity of this gene. These results suggest that the presence and the copy number of *netB* in *C. perfringens* are not the determinants for clinical NE. Moreover, no significant difference was identified between NE and non-NE isolates in the carriage of other toxin genes, *cpb2*, and *tpeL.* We conclude that *netB* exists in the majority of *C. perfringens* type A isolates. The presence as well as the quantity of *netB* are insufficient to predict an association with the outbreaks of NE or the pathogenicity of *C. perfringens*. Although *netB* has been demonstrated to fulfill molecular Koch's postulates in experimental models, the virulence of *netB* may be in effect under certain conditions that are met, further promoting the development of NE.

NE is not similar to other clostridial diseases, regularly depending on their secreted toxins to develop the disease. It is a multifactorial disease, and we further demonstrated that the use of *netB*-positive *C. perfringens* without a predisposing factor failed to induce NE in the *C. perfringens* and *Eimeria* side by side induction model. At least one of the predisposing factors was required for the induction of disease. Incorporation of fishmeal in conjunction with the challenge of *netB*-positive strain reproduced NE but did not significantly affect the incidence of the disease. However, chickens challenged with *Eimeria* and *C. perfringens* and fed with the diet containing fishmeal were more susceptible to the NE and the severity of disease increased. As the

result, we conclude that both *netB*-positive *C. perfringens* and predisposing factor(s) were required for NE reproduction. In addition, with more predisposing factors participating in the induction, the severity and incidence of NE increased.

We investigated gut microbiota in NE-positive and NE-negative jejunal and cecal samples collected from a side by side *C. perfringens* and *Eimeria* challenge model. Our results demonstrated microbial shifts with biological significance in the jejunum but minimal fluctuations in taxa abundance in the cecum. Jejunal microbiota was shown more significant than cecal microbiota to address characteristic gut microbiota related to the NE. The majority of NE cases shared similar microbial community profile, presenting a higher abundance of *Clostridium sensu stricto 1*, the genus contains *C. perfringens*, but the relatively low amount of *Lactobacillus* in jejunal microbiota. With more NE severity, the degree of changes in their abundance increased. Although lauric acid was applied in the feed as an alternative prevention for its antibacterial effects against *C. perfringens*, given a supplementary dose of 0.4 kg/ton neither exerted the inhibitory effect on the abundance of *Clostridium sensu stricto 1* nor reduced the incidence and severity of NE. Analyses of metagenomeSeq, STAMP, and LEfSe further displayed that significant overgrowth of *Clostridium sensu stricto 1* was associated with the NE and *Eimeria* infection precedent to *C. perfringens* challenge exerted synergistic effects on the overrepresentation. In addition to *C. perfringens*, the other member under *Clostridium sensu stricto 1* was correlated to disease development. Therefore, we conclude that significant overgrowth of *Clostridium sensu stricto 1* in jejunum was the major microbiota contributory to the NE. Besides, the decrement of *Lactobacillus* following the NE severity also indicated that lactobacilli participate in the progress of the disease. The

observation that commensal *C. perfringens* under *Eimeria* infection without challenging exogenous *C. perfringens* did not reach the significant abundance of *Clostridium sensu stricto 1* and unable to promote NE. It suggests that the quantity of *C. perfringens* reaching a certain level in jejunum is critical for the onset of proliferation.

In summary, the projects included in this dissertation have demonstrated that the carriage and quantity of toxin gene are not directly correlated with the pathogenicity of NE. Under certain condition, regulatory mechanisms may trigger the expression of virulence genes into effect and further investigations are needed to illustrate the detailed pathogenesis. For disease induction, both causative *C. perfringens* and predisposing factor(s) are required for consistent reproduction of the disease, and *Eimeria* exerts significant effects on NE induction. Fishmeal and *Eimeria* can act synergistically to promote a contributory microbiota, exhibiting significant overgrowth of *Clostridium sensu stricto 1* in jejunum, under the introduction of exogenous *C. perfringens*, in turn contributing to NE development in the hosts. Therefore, the measures to inhibit over-proliferation of *Clostridium sensu stricto 1* and *C. perfringens* in small intestines will be practical to prevent NE. Based on our findings, multi-techniques including farm sanitation, coccidial control, and optimized feed formula are recommended in the field to avoid introducing causative pathogen and creating a favorable gut condition for overgrowth of *Clostridium sensu stricto 1* and *C. perfringens.*