EVALUATING THE *IN VITRO* CHARACTERISTICS AND PROTECTIVE EFFICACY OF CHITOSAN NANOPARTICLE VACCINES AGAINST NECROTIC ENTERITIS IN BROILER CHICKENS

by

GABRIEL AKERELE

(Under the Direction of RAMESH SELVARAJ)

Abstract

There are currently no licensed vaccines against *C. perfringens*, which causes necrotic enteritis in poultry. In aim 1, chitosan nanoparticles were formulated with native (CN) or toxoids (CT) extracellular proteins (ECP) from *C. perfringens,* both surface-tagged with *Salmonella* flagellar proteins. CN and CT were stable at pH 2 to 8, and when incubated at 100 µg/ml PBS with chicken red blood cells (cRBCs) released 1% and 0% hemoglobin, respectively. At 7.2 pH, CN had an average size of 389.7 nm and a zeta potential of -66.9 mV while CT had an average size of 352.5 nm and zeta potential of -63.6 mV. In aim 2, broilers were randomized to treatments; sham-vaccinated (Control), CN-vaccinated (CN), and CT-vaccinated (CT). Each bird was orally sham-vaccinated or vaccinated with 50µg CN or CT on d 0, 3, 7 and 14 post-hatch. On d 17 post-hatch, the CN group had higher anti-flagellar IgG than control ($P < 0.05$). On d 21 post-hatch, the CN group had higher anti-ECP IgA than control ($P < 0.05$) while the CN and CT group had higher anti-ECP IgA than control ($P < 0.05$). Splenic T-cells from the CN and CT groups ex-vivo stimulated with ECP had higher proliferation than control ($P < 0.05$). In aim 3,

broiler chicks were randomized to treatments: non-vaccinated non-challenged (NVNC), vaccinated challenge (VC), and non-vaccinated challenge (NVC). On d 0, 7, and 14 post-hatch, VC birds were orally vaccinated with CN while NVC birds were sham-vaccinated. VC and NVC birds were challenged with *E. maxima* at d 14 post-hatch, and *C. perfringens* on d 19, 20, and 21 post-hatch. On d 18 post-hatch splenic T cells from VC birds had higher CD4/CD8 ratio than control and NVC ($p < 0.05$). On d 28 post-hatch, lesion scores and mortality were numerically lower in VC than NVC birds. FCR was significantly higher in VC than NVC birds ($p < 0.05$). In conclusion, the nanoparticle vaccine was safe immunogenic and partially protective in boilers. Further studies are needed to improve the efficacy of the vaccine and understand the mechanism underlying protection in vaccinated birds.

INDEX WORDS: *C. perfringens*, chitosan, nanoparticle, vaccine, necrotic enteritis.

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DEDICATION

dedicate this dissertation to my dear Uncle and Aunty Idowu and Nike Akerele,

My grandma Mrs Remi Adedeji. Thank you for your inspiration, support, and unconditional love.

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CHAPTER 1

Introduction

Necrotic enteritis (NE) is a severe *C. perfringens*-induced disease caused predominantly by C. perfringens type A strains and to a lesser extent type C strains (Si et al., 2007; Dahiya et al., 2006). In recent times, clinical and subclinical NE infections have become exacerbated by the regulatory or voluntary withdrawal of antimicrobial growth promoters and ionophore coccidiostats. Both acute and chronic forms of infection lead to economic losses. The estimated economic burden of acute and chronic forms of infection is 2 to 6 billion USD annually. Predisposing conditions of NE include coccidiosis (Forder et al., 2012), litter change between flocks, reduced ventilation (especially during the cold months), stressors that immunocompromise the birds, diets rich in non-starch polysaccharides and/or fish meal and/or wheat (Cooper and Songer, 2009), and other poor management or biosecurity practices. Necrotic enteritis infections commonly affect broilers at 10 to 28 days of age and have been associated with the decline in protection from maternal antibodies. The infection can be acute which may result in clinical manifestations like lethargy, ruffled feathers, decreased feed intake, elevated mortality. In hyperacute cases, sudden death occurs without premonitory signs. Chronic NE has subtler outward symptoms, with reduced bird performance (lower body weight gain and higher feed conversion ratio) being the most common symptom.

There are currently no available commercial vaccines against NE in broilers. This is partly because oral vaccines based on unencapsulated subunit antigens are denatured or broken down by gut enzymes without protection from the harsh environment of the gut. There is

therefore a need to develop oral vaccines based on C. perfringens extracellular proteins that are safe and effective. Nanoparticles are well characterized as delivery vehicles for a wide array of compounds. Nanoparticle stability, inherent immunogenicity, and potential adjuvant properties make them promise as suitable vehicles to deliver oral antigens. The broad objective of this research was to design a chitosan nanoparticle vaccine based on C. perfringens proteins and assess the vaccine's safety, immunogenicity, and protective efficacy against NE. We hypothesize that chitosan nanoparticles loaded with C. perfringens proteins are safe immunogenic and can induce a protective immune response in broilers. To test this hypothesis, our specific objectives were:

Aim 1: To evaluate the safety and physicochemical properties of chitosan nanoparticles loaded with a crude extract of extracellular proteins of C. perfringens

Aim 2: To assess the immunogenicity of chitosan nanoparticles loaded with a crude extract of extracellular proteins of C. perfringens in broilers.

Aim 3: To assess the protective efficacy of chitosan nanoparticles loaded with a crude extract of extracellular proteins of C. perfringens in broilers.

CHAPTER 2

Literature Review

Clostridium perfringens

C. perfringens is a Gram positive, anaerobic (but aerotolerant), and non-motile rod species. It is an encapsulated bacterium that causes a wide range of diseases in humans and animals. Under normal conditions *C. perfringens* is non-invasive, ubiquitous and produces many toxins and hydrolytic enzymes. It is a fast-growing bacterium, with an exponential phase that lasts between 2 to 6 hours, and generation times that varies between 23 and 27 minutes. Generation times as rapid as 7.4 minutes have been recorded (Willardsen *et al* 1979). In the early stationary phase, this increases to 83 minutes (Traub *et al.*, 1987). This makes it one of the fastest-growing bacteria known and used to be referred to as an 'anaerobic flesh eater' (Shimizu et al., 2002). *C. perfringens* produces extracellular toxins that damage the intestine. At least 17 toxins have been identified. *C. perfringens* associated with NE is auxotrophic for 13 amino acids (Shimizu et al., 2002). This makes it germane for them to be able to efficiently break down tissues (necrosis) and/or acquire the nutrients they require to proliferate to high numbers in the small intestine. In the process, they cause clinical and subclinical infections. In humans, *C. perfringens* causes gangrene and gastrointestinal disease such as food poisoning and NE while in other animals, gastrointestinal and enterotoxemic diseases are more common (Petit *et al*., 1997). In fact, *C. perfringens* is the second most common source of food-borne pathogenesis in the U.S., with an estimated one-million reports each year (Scallan *et al.,* 2011).

The intestine of healthy birds typically contains approximately 10^4 CFU/g digesta of C *perfringens*/, but as little as 10^5 cfu/g of ileal digesta has been described as the threshold for the development of NE in chickens (Si et al., 2007). There are more than 800 serotypes of *C. perfringens* belonging to over 17 toxinogroups (Dahiya *et al*., 2006). Most *C. perfringens* toxin genes are borne on extrachromosomal elements and this is responsible for the wide range of pathovars seen in this group. *C. perfringens* strains are classified into 5 major toxinotypes (A, B, C, D and E) based on which combination of α, β, ϵ and ι they secrete. Each toxin produces a specific syndrome (Petit *et al*., 1999).

Available research point to plasmids as the most significant contributors to genetic diversity in NE causing strains of *C. perfringens*. Generally, 3 large plasmids that share about 35kb homology and range in size from 40 to 140kb encode the β, ϵ and ι toxins that are found in NE causing *C. perfringens* strains. These plasmids also encode other toxins such as delta, netF, beta2, cpe, TpeL as well as adhesins, catabolic enzymes and other virulence traits in NELoc regions (Parreira et al., 2012). The pJIR3536 plasmid encodes NetB in a NELoc1 region. The pJIR3535 plasmid encodes tetracycline and bacitracin resistance while pJIR3537 encodes beta2 toxin and a NELoc3 region. A fourth plasmid that has been tentatively linked to increasing severity of NE has also been found, encoding the TpeL toxin (Coursodon et al., 2012). The NetB encoding plasmid is the most conserved, highlighting the importance of its role in NE. Although the plasmids are also highly conjugative under laboratory conditions (Bannam et al., 2011), chromosomal factors may influence their stability. There is a wide variation in the occurrence and frequency of these plasmids that gives rise to strain diversity. Furthermore, mutations such as a deletion of chitinase or internalin gene on NELoc1, the presence or absence of a bacitracin

resistance gene on TpeL plasmid, or the presence or absence of *beta2* on the NELoc3 region, increase strain diversity (Lepp *et al*., 2013).

Chromosome encoded variability of *C. perfringens* associated with NE is often found in capsular polysaccharide encoding regions, prophage-related region (Myers *et al*., 2006) and adhesion-related loci (Lepp *et al*., 2013). The 2 variants of the adhesion loci include VR-10A and VR-10B. VR-10A is found in non-poultry and diseased poultry. VR-10B produces collagen adhesions proteins, is strongly linked with *netB* carrying strains, and is unique to poultry isolates (Lepp *et al*., 2013).

C. perfringens type A (*CPA*) is the most common strain associated with poultry production. It produces the alpha toxin as its major toxin and a secondary toxin, θ. *C. perfringens* type A causes gangrene in humans and diarrhea in animals such as foals and pigs (Dorner, 1986). Al-Sheikhly and Truscott (1977) were one of the first researchers to argue for the *C. perfringens* type A alpha toxin gene as the main virulence factor in NE infection. They demonstrated a high correlation between the alpha toxin content in the culture supernatant of *C. perfringens* isolates and induced NE. Since then, several other researchers have identified alpha toxin as the main virulence factor in NE (Si *et al*., 2007). However, there have is conflicting evidence about the role of alpha toxin in NE (Keyburn *et al*., 2008). Both alpha and netB toxin damage cell membrane with the aid of receptors leading to the formation of pores (figure 1a). They also release enzymes (figure 1b) which help them degrade cellular components (Petit et al., 1999).

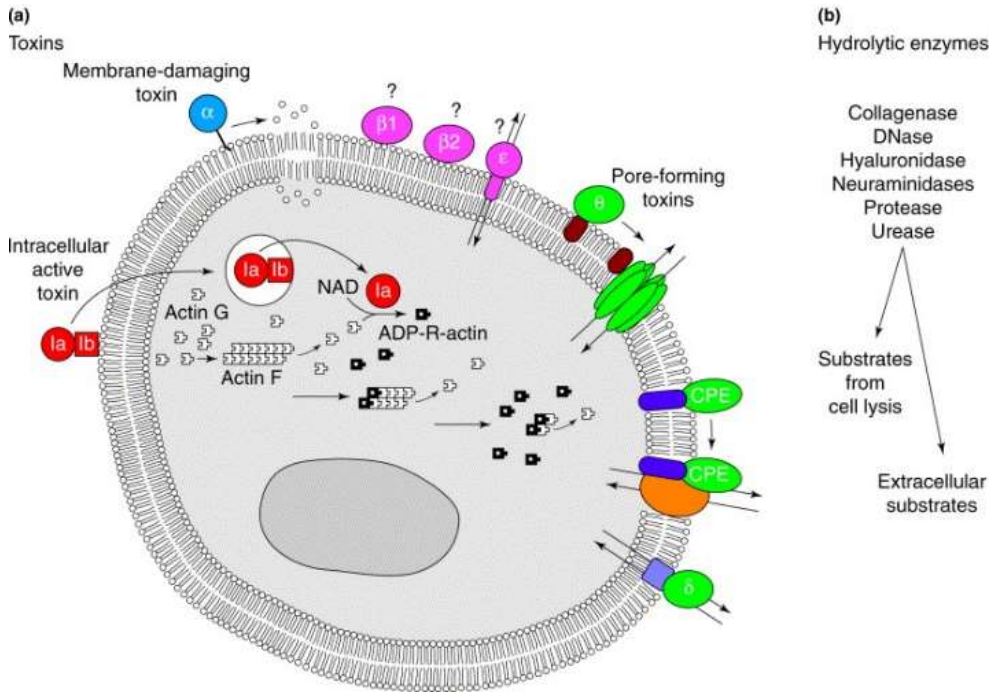


Figure 2.1. Overview of the modes of action of the *Clostridium perfringens* toxins and hydrolytic enzymes. (Petit *et al*., 1999).

There are other virulence factors (Bunting *et al*., 1997; Hunter *et al*., 1993; Petit *et al*., 1997; Perelle *et al*., 1993; Alouf *et al*., 1981; Tweten *et al*., 1988; Matsushita *et al*., 1994; Canard *et al*., 1994; Rood *et al*., 1976; Dupuy *et al*., 1997) that are also thought to play a role in *C. perfringens*-induced infections. O-glycosylated, N-glycosylated glycoproteins and chitobiose present in mucin are degraded by adhesins and glycosyl hydrolases produced by the bacteria. This leads to the colonization and degradation of the intestinal mucus layer, an essential process in the pathology of NE. The sialidases of *C. perfringens* (such as NanI) then have access to sialidase-exposed receptors on enterocyte cell surfaces (Li and MacClane, 2014). Other non-toxin genes like GAPDH, sugar transport and iron acquisition genes do not play a direct role in pathogenesis but increase the 'fitness' for the survival of the bacteria (Lepp et al., 2013). In fact, GAPDH and perfringolysin O were demonstrated to react strongly to immune serum from

protected birds in an experimental NE infection model (Kulkarni et al., 2006) while Zn metalloprotease can be a protective antigen against NE (Kulkarni et al., 2007). Virulence and metabolism are thus intimately linked in *C. perfringens.* Ohtani and Shimizu (2015) suggest a two-component (VirR/VIrS) regulon of about 150 genes including toxins that direct these functions. These genes are switched on by an accessory gene regulator (*agr*)-like quorum-sensing system. The Agr operon was first characterized in *Staphylococcus aureus*. It is comprised of 4 co-transcribed genes *agrA, agrB, agrC, agrD*. AgrD is processed from a propeptide to become an active auto inducer peptide (AIP) and transported to the extracellular environment with the help of the transporter AgrB. At critical levels, the AIP activates the two-component system of *agrC-agrA.* While there are orthologues of *agrB* and *agrD* in *C. perfringens*, there are no orthologues of *agrA* and *agrC*. Therefore, this, it is suggested, has been replaced functionally by the VirR/VirS system. This is because *Agr*-like QS system and VirR/VirS regulate many of the same toxins (Ohtani *et al.,* 2009).

Olkowski et al. (2006) suggested that initiation of NE involves proteolytic and collagenolytic factors that have not yet been fully characterized. Histopathology studies revealed that damage proceeds from the lamina propia, basement membrane and lateral sides (intercellular junctions) of the gut before luminal damage of enterocytes. A large number of bacteria then line the submucosa possibly with the help of biofilm formation

In poultry houses, *CPA* can be found in poultry litter, soil, water, chicken fluff. It is from here that it colonizes the gastrointestinal tract of 2–5-week-old chicks (Craven *et al*., 2001) and establishes itself as a normal resident of the ileum and jejunum of chicken and turkey. The overgrowth of *C. perfringens* (sometimes referred to as clostridiosis) (Engström et al., 2003) causes clinical and subclinical focal NE infections that translates to huge economic losses due to

mortality and reduced bird performance (lower body weight gain, higher feed intake and feed conversion ratio). Outbreaks of clinical infections have been reported in most countries while the epidemiology of subclinical infections are more difficult to define because the infection can only be diagnosed by post-mortem identification of lesions in the gut of randomly sampled birds. Clinical dissections of infected birds typically show dark, swollen, and firm liver with necrotic lesions (chorangiohepatitis). Depending on the severity, there may be ballooning of the intestine, tan to brownish-colored diphtheritic pseudomembranes, and copious watery brown blood-tinged fluid. The gall bladder is distended, and there is often a foul odor upon opening the intestines. Intestinal integrity is compromised leading to roughened, friable consistency (Hofacre et al., 2018). Contamination of poultry carcass with *C. perfringens* is undesirable because *C. perfringens*-associated hepatitis can lead to condemnation of both the liver and the whole carcass (Craven *et al*., 2003; Immerseel *et al*., 2004). Finally, although numerous studies have been carried out using different isolates as experimental models of infection, there is still no clear relationship between different subtypes of *CPA* and a specific pathotype and this makes the study of *C. perfringens* in relation to NE complicated. Several researchers have shown that virulent strains associated with NE within a flock tend to be clonal, inhibiting the growth of or outcompeting other strains of *C. perfringens*, irrespective of gut tissue, bird host, or origin of disease (Engström et al., 2003). The specific factors secreted by such outbreak strains that limit diversity in *C. perfringens* and other bacteria strains have not been fully elucidated (Timbermont et al., 2011) although a recent study by Timbermont et al., (2014) identified a unique 11.5 kDa bacteriocin called perfrin that was claimed not have been identified in any other bacteria. Johanssson et al., (2010) suggested that mild NE differs from severe NE with regard to *C.*

perfringens genotype diversity while *C. perfringens* species richness has been found by Gaucher et al. (2017) to correlate with clinical symptoms of NE.

C. perfringens **proteins associated with NE**

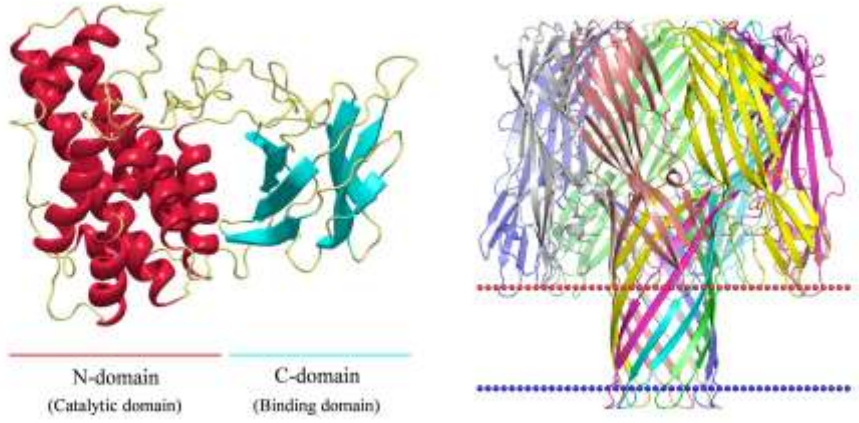
C. perfringens proteins associated with NE include toxins and metabolic enzymes that are on the surface of bacteria that are released into the environment. Metabolic enzymes moonlight on the surface of the bacteria, promoting its attachment and proliferation and thereby contributing to its virulence. From a vaccine design perspective, extracellular proteins are important therapeutic targets because they mediate the virulence of *C. perfringens* and are accessible to the host defense machinery. A number of studies characterizing the extracellular proteome of *C. perfringens* have identified a number of immunogenic antigens (Sengupta et al., 2010; Alam and Dvivedi, 2016; Matsunaga et al., 2018).

Clostridium **alpha toxin**: *C. perfringens* alpha toxin is the most studied, mostly using mice gangrene models (Mot *et al*., 2014). This toxin has molecular weight 42.528 kDa and is comprised of 370 amino acids. It shares significant homology with the phospholipase C family of genes from *Bacillus cereus* (Gilmore *et al*., 1989), *Candida. bifermentans* (Tso *et al*., 1989), and *Listeria monocytogenes* (Vazquez-Boland *et al*., 1992). The N-terminal domain consists of 9 compact alpha helical structures while the C-terminal is a beta sheet comprising 8 anti-parallel strands. It is a Zinc metalloenzyme with 3 sites for divalent cations occupied by Zinc ions in its N-terminal domain. Both the N and C-terminals interact to bring about the hemolytic activity of the toxin. At high concentrations, *C. perfringens* toxin degrades phosphorylcholine and carboxyfluorescein from membranes while in limited amounts, only causes the release of diacylglycerol and ceramide (Sakurai *et al*., 2004). This process promotes eventual cell lysis of

erythrocytes and other cell types (Titball *et al.,* 1999). Alpha toxin also helps *C. perfringens* to escape macrophage phagosomes and survive in the host tissue (O'Brien and Melville, 2004). Finally, alpha toxin can cause vasoconstriction of blood vessels facilitated by their membrane breakdown products (thromboxanes, prostaglandins, leukotrienes), thereby promoting leukostasis and necrosis of tissue.

***Clostridium* Net B (*Net*B) Toxin:** This was first discovered by Keyburn *et al*., (2008) and was shown to induce NE infections in alpha-toxin deficient mutant *CPA* strains although its receptor(s) has not yet been identified. *NetB* is potentially part of a large pathogenicity locus (Keyburn *et al*., 2010). For example, a putative fimbrial adhesin operon VR-10B identified by Wade et al (2015a) from a virulent NetB positive strain of *C. perfringens* was able to adhere to collagen IV and V *in vitro* and may contribute to the enhanced pathogenicity of NetB-containing strains of *C. perfringens*. Although the presence of NetB toxin is often associated with infections, random site-directed mutagenesis shows the possibility of non-hemolytic *netB* mutants. NetB is part of a large 42 kb pathogenicity locus (NELoc1) carried on a conjugative plasmid (*tcp*). Other distinguishing features of a NetB carrying strain includes a chromosomal 11.2 kb region (NELoc-2) and a 5.6 kb locus (NELoc-3) present on a second *tcp*-conjugative plasmid that also carries the *C. perfringens* B2 toxin gene (Parreira et al., 2012; Lepp et al., 2010). Another toxin gene, *Tpel,* found on yet a different *tcp*-conjugative plasmid may also be involved in pathogenesis (Coursodon *et al.,* 2012). Structurally, NetB is like the alpha-hemolysin, a β-barrel pore-forming toxin from Staphylococcus aureus. It is a monomer that oligomerizes when it interacts with cholesterol on cell membranes. The monomer has 16 β-strands and an α-helix, which are arranged into the β-sandwich, latch, rim, and pre-stem domains. This is characteristic of the alpha-hemolysin family. The β-sandwich domain comprises

a five-stranded and a six-stranded anti-parallel β-sheet (Yan *et al*., 2013). NetB toxin causes heptameric pores of 1.6 to 1.8 nm diameter to form in the host cell membrane leading to the formation of channels that are roughly 3-fold larger in conductance than those of alpha-hemolysin (Krasilnikov *et al*., 2000; Keyburn *et al*., 2010). The pores caused by NetB causes leakage of plasma proteins which serves as a substrate for increased *Clostridium* proliferation.



A **B**

Figure 2.2. Protein Data Bank images of Two Major Toxins That have been Associated with *C. perfringens Type A*-induced Necrotic Enteritis in Chickens. (A) alpha toxin. (B) Necrotic enteritis (NetB) toxin (https://www.wwpdb.org).

Perfringolysin O: This has a molecular weight of around 52.6kDa and theoretical isoelectric point 5.4. It is a cholesterol-dependent cytolysin, pore-forming, theta toxin. It binds as a monomer and oligomerizes as it diffuses across the membrane surface (Anderluh and Gregor, 2014). It is also under regulation of the Vir/ViS regulon. It is present in many strains of *C. perfringens*. It is the primary mediator of the escape of *C. perfringens* from macrophages and persistence in tissues (O'Brien and Melville, 2004).

Elongation factor-Tu: This is a 43kDa cytoplasmic protein that helps to transfer aminoacyl tRNA during translation in prokaryotes. It is expressed intracellularly in the cytoplasm and on the cell surface where it mediates bacterial adhesion to cells and mucin. It is regulated by the VirR-VirS virulence regulon. Its presence on cells makes it a target for potential neutralizing antibodies (Lee *et al*., 2011).

Elongation factor G: This is a 76kDa cytoplasmic protein that is also involved in protein synthesis. It catalyzes the GTP-dependent ribosomal translocation step during translation elongation. It is regulated by the VirR-VirS virulence regulon of *C. perfringens*. It has been identified in both the cell surface and extracellular fractions of *Bacillus* and is thus immunogenic (Delvecchio *et al*., 2006). Its homolog in *C. perfringens* was identified by Kulkarni *et al*. (2006) as an antigenic protein in NE.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH): GAPDH is one of several metabolic enzymes that moonlight on the surface of *C. perfringens* during the pathogenesis of NE. GAPDH has a theoretical molecular weight of 35kDa and a theoretical isoelectric point of 5.5. GAPDH is primarily involved in energy metabolism such as glycolysis. GAPDH is also under regulation of the Vir/ViS regulon. On the cell surface, it is found as a tetramer in the extracellular milieu of intacT-cells where it mediates virulence, for example by binding plasmin (Modun and Williams, 1999; Matsunaga *et al*., 2018).

Fructose 1,6 biphosphate aldolase (FBA): FBA is a 34kDa glycolytic protein and therefore involved in energy metabolism. It is also under the regulation of the Vir/ViS regulon. Similar to GAPDH, it moonlights on the surface of cells where it is hypothesized to play a role in adhesion (Kulkarni *et al.,* 2006).

Pyruvate: ferredoxin oxidoreductase: PFOR is a 127.86kDa metabolic enzyme that catalyzes the conversion of pyruvate to acetyl-CoA with the release of CO_2. It is also required for carbon monoxide production and sporulation of *C. perfringens*. The 67kDa truncated protein is immunoprotective (Kulkarni *et al*., 2007, 2008).

Cell surface-associated proteins: Comparatively little is known about the contribution of *C. perfringens* cell wall proteins to virulence. Like most Gram-positive bacteria, *C. perfringens* displays a diverse array of proteins covalently anchored to its peptidoglycan. The attachment, adaptation to harsh host environments, and pathogenicity of *C. perfringens* may be linked to these proteins. They are likely to play a role in binding *C. perfringens* to damaged intestinal mucosa. There is a special family of proteins known as sortase enzymes. There are 6 members of this family (SrtA to Srt F) each playing specific roles in the survival of the bacteria. Srt A is referred to as a housekeeping enzyme that is found in bacteria such as *Staphylococcus*, and *Bacillus* helps to anchor proteins to the cell wall peptidoglycan of Gram-positive bacteria. For example, sortase anchored adhesins have been found in *Clostridium difficile.* SrtB is important for Iron acquisition under deprivation conditions, SrtC mediates the assembly of pili (from pilin sub-units), which is important for attachment and biofilm formation. SrtC may play a role in sporulation under harsh condition while the roles of SrtD, SrtE, and SrtF is still largely unknown. Sortases have been demonstrated to act through a highly evolved recognition of conserved signal peptides. For example, SrtA and SrtB from *S. aureus* recognize the LPXT–G and NPQT–N cleavage motifs respectively, with little cross-activity or redundancy (Suryadinata *et al*., 2015). Predicted cell surface anchor proteins have been found on plasmids such as NELoc-1 (Parreira et al., 2012).

Pathogenicity of *Eimeria* and proteins associated with necrotic enteritis

Eimeria is an obligate, intracellular, apicomplexan parasite that affects cattle, sheep, goats, horses, swine, and poultry. It also affects pet animals such as dogs and cats. There are eight known species that affect poultry, including *E. praecox, maxima, tenella, mivati, necatrix, acervulina, brunetti* and *imitis.* The pathogenicity of *Eimeria* in poultry is linked to its life cycle and is characterized by an exogenous and endogenous stage. In the exogenous stage, oocysts are shed in the feces of parasitized chicken. The oocysts become infective or sporocysts when exposed to optimal temperature and moisture in the litter. In the endogenous stage, sporocysts that are ingested by the bird excyst and invade the epithelial cells of the villi with the help of the grinding action of the gizzard and gastric enzymes. Infective sporocysts also invade crypt T-cells and the lamina propia. Infective sporocysts undergo several species-specific rounds of schizogony to produce merozoites. Merozoites invade cells with the help of gliding motion. Gametogony occurs following 2-3 rounds of merogony. The male and female gametocytes that result from gametogony fuse to form oocysts that are excreted in feces to complete the cycle. The gut of an apparently healthy bird contains cycling *Eimeria*. Infection with *Eimeria* is called coccidiosis. Coccidiosis in poultry results from the presence of large numbers of cycling *Eimeria* and can lead to clinical and subclinical disease (Lillehoj and Lillehoj, 2000). Coccidiosis promotes the onset of NE by compromising intestinal barrier function, causing plasma leakage into the gut lumen and inducing increased mucogenesis (Collier et al., 2008). Different species of *Eimeria* have tropisms for different anatomical sites of the gut and generate an immune response in the process. *E. maxima*, which mostly invades the jejunum, is the most associated with NE in chickens (Gilbert et al., 2011; Huang et al., 2018). *E. maxima* are highly immunogenic, have comparatively high levels of genotypic (Schwartz et al., 2009) and phenotypic (Martin et al.,

1997) variation and are globally ubiquitous (Schwartz et al., 2010). This high level of variation may explain the lack of protective immunity to E *maxima* using xenotypic antigens (Song et al., 2009). Molecular research into the biology and pathogenicity of *Eimeria* that infect chickens has focused mostly on *E. tenella* and *E. maxima*.

Surface antigen (SAG) proteins are glycosylphosphatidylinositol (GPI)-anchored SAG proteins that are constitutively expressed on the surface of *Eimeria* parasites and are therefore important for *Eimeria* attachment and invasion (Schwartz et al., 2010). *In E. tenella*, there are 62 variant SAG genes classified into two multigene families, A and B based on the positions of six conserved cysteines. (Chow et al., 2011). Other species of *Eimeria* possess similar but distinct SAG genes. Klotz et al. (2007) found 25 unique multistage secretory proteins in *E. tenella.* Six of these peptides were found to have significant homology to *E. tenella* SAG proteins: EtSAG9, EtSAG1, EtSAG10, EtSAG2, EtSAG2, EtSAG19, respectively. There are at least 19 SAG proteins expressed specifically by second-generation merozoites of *E. tenella.* In vitro, these recombinant *E. tenella* SAG proteins were able to stimulate inducible nitric oxide synthetase (iNOS) production and up-regulated Il-1β mRNA expression in chicken macrophages (Chow et al., 2011).

According to Liu et al. (2018), *E. maxima* SAG proteins are encoded in a 645bp open reading frame that codes for 214 amino acids. *E. maxima* SAG proteins are expressed in both sporozoite and merozoite stages. Vaccination of broiler birds with recombinant SAG proteins or plasmid-borne *E. maxima* SAG gene showed improved performance and shed fewer oocysts (Liu et al., 2018).

Microneme proteins are secreted in the microneme, located in the apical tip of *Eimeria* sporozoites and merozoites (Witcombe et al., 2003). Many of the molecules that comprise the

glideosome are secreted by micronemes and rhoptries. Microneme proteins are released early during invasion where they mediate attachment to the host cell and formation of the connection with the parasite's actinomyosin system (Morahan et al., 2009). Studies have shown that the major microneme adhesive repeat regions protein from *E. tenella*, microneme protein 3 (EtMIC3), is deployed at the parasite–host interface during the early stages of invasion (Lai et al., 2011).

Sporozoite antigen (SO7) protein has a theoretical molecular weight of 24kDa. This has been demonstrated to elicit humoral and cell-mediated immunity that are offer cross-species protection, possibly because of conserved epitopes (Crane et al., 1991). It is expressed in unsporulated oocysts, up to 72 hours after sporulation. SO7 is highly concentrated in both refractile bodies of sporozoites, with some limited distribution in the apical complex. SO7 is expressed *in vitro* in first stage merozoites when the residuals of the refractile body is still present, but not *in vivo*. The expression of SO7 may indicate its importance for the development of the sporozoite and the initial stages of invasion (Fetterer et al., 2007).

Innate defense mechanisms against necrotic enteritis in chickens

The innate defense mechanisms of poultry gut include the mucus lining of some epithelial cells (Juul-Madsen et al., 2008), vascular endothelium, mucosal surfaces with cilia and antimicrobial secretions (Basset et al., 2003; Berndt et. al., 2007; Butcher and Miles 2015). Other defense mechanisms include increased secretion of constitutive components and defensins. The cascade of molecules released in the complement cascade (Juul-Madsen et al., 2009), phagocytosis, autophagy, and immune activation by different families of pathogen recognition receptors (PRRs) are other mechanisms of innate defense (Mogensen, 2009). Innate immunity is

the first line of defense from pathogens. Innate immune responses lack specificity which results from its inability to distinguish between molecular subtypes, quickly dissipates, and therefore lacks the ability to 'remember' antigens in the case of repeated attacks (Kogut, 2009). The cellular components of innate immunity include antigen-presenting dendritic cells, phagocytic macrophages, and granulocytes, cytotoxic natural killer (NK) cells, and T-lymphocytes (Mogensen, 2009; Basset et al., 2003). Elevated body temperature, unique microbiome, and the absence of complementary receptors that make some avian species genetically unsusceptible to certain infections also constitute part of avian innate defense (Butcher and Miles, 2015).

Low pH: The crop contains fermenting bacteria such as lactobacillus which colonize the stratified squamous epithelium of a bird in a single layer (Fuller and Brooker, 1974; Watkins and Kratzer, 1983) shortly after hatch. The fermentation products include a host of organic acids like lactic, butyric, valeric, caproic, formic, oxalic, phenyl acetic, succinic and fumaric; acids which are responsible for the low pH that exclude the growth of many pathogenic bacteria. The relatively slow rate of crop digesta emptying at night contributes to acid build-up and thus the lowering of the pH. The proventriculus produces mucus gastric juices that contain acids such as HCl and digestive enzymes like pepsinogen (precursor to pepsin). Short and medium fatty acids contribute to the low pH of the gut.

Antimicrobial compounds: As reviewed by Bahar and Ren, (2013), antimicrobial peptides (AMPs) are small (<10KDa), ribosomal synthesized cationic, or amphipathic molecules. The chicken mouth contains salivary glands that secrete mucus and large quantities of antimicrobial peptides, in addition to digestive enzymes. Antimicrobial compounds are also secreted by gut-lining epithelial cells in mucus and other fluids. These compounds have a broad spectrum of activity against microbes. They are only composed of the canonical complement of amino acids,

unlike peptide-based antibiotics. Their proposed mode of action involves disrupting membranes and intracellular targets. Depending on their structure, they are classified as alpha, beta, extended or loop peptides. The AMPs includes cecropins, magainins, and LL-3. Beta AMPs include α-, β-, θ- defensins, plectasin, and protegrins. They are very stable, possibly because of the presence of disulfde bridges between sheets. The loop AMPs such as bactenin contain fewer disulfide links. Extended AMPs, due to the presence of a large number of amino acids such as proline, histidine, arginine, tryptophan does not form regular secondary structures (Zasloff, 2002). An example is indolicidin.

AMPs can be of host or microbial origin. The action of many probiotic organisms like lactic acid bacteria, *Bacillus* and *Enterococcus* is exerted through the secretion of bacteriocins. For example, several strains of bacillus have been shown to have antagonistic activity against *C. perfringens* through the production of bacteriocins and antimicrobial peptides *in vitro* (Teo and Tan, 2005) and *in vivo* (Jayaraman et al., 2013). Wang et al. (2015) demonstrated that the AMP sublancin ameliorates NE induced by *C. perfringens* in broilers by improving villus height and villus height: crypt depth ratio in a NE challenge study. AMPs originate from heterophils and non-heterophil cells. Avian β-defensins are the most characterized of chicken AMPs. Beta-defensins; (BD) 1, BD-6, BD7 and BD9 have been found in the crop (Hong et al., 2012). There is moderate expression of the beta defensin (BD-6) in the proventriculus (van Dijk *et al*., 2006), these factors combine to protect against pathogenic organisms present in feed during digestion.

In addition, antigen exposure can lead to rapid production of iNOS by macrophages (Thaxton *et al.,* 2006). iNOS is one of 3 enzymes that use L-arginine to produce NO. Nitric Oxide has been shown to play a major role in inflammation during infection. Dysregulated NO production can negatively impact weight, cause tissue damage and organ dysfunction (Lirk et al.,

2002) and this may be responsible for its rapid clearance by 48 hours (Thaxton *et al.,* 2006). The iNOS gene was upregulated in chicken intestinal cells in response to *Eimeria* infection (Allen, 1997) but unchanged or down-regulated in chickens when *Eimeria* was co-infected with *C. perfringens*. Feed additives that downregulate iNOS gene expression with a positive impact of the average weight gain of experimentally challenged birds have been reported (Lillehoj et al., 2016;).

Different goblet T-cells produce acidic or neutral mucin. An example is the proline-rich glycoprotein mucin 2 which has anti-microbial activity. Mucin provides target recognition sites for glycosidases, or lectin-like adhesins of both commensal and pathogenic organisms (Van Klinken et al., 1995). In addition, muc2 is a target site for secretory IgA (Zhang et al., 2015). *C. perfringens* possesses hydrolases families predicted to be involved in mucin glycoprotein breakdown (Ficko-Blean and Boraston, 2006).

Gut Microbiome: Bacteria belonging to the phyla Firmicutes, Proteobacteria, Bacteroidetes, and Actinobacteria are the most abundant taxa in the ileum of a healthy bird (Latorre et al., 2018). NE is often associated with a shift in commensal microbiota (Wu et al., 2014) and a decrease in microbial diversity (Timbermont et al., 2011). This dysbiosis may be a contributing factor and/or the result of NE (Antonissen et al., 2016). Beneficial commensals that colonize the gut villi help to exclude pathogens through a variety of mechanisms (Khalique et al., 2020). Probiotics can be used as direct-fed microbial (Latorre et al., 2015), competitive exclusion cultures (Hofacre et al., 2019), or synbiotics, to mitigate NE (Khalique et al., 2020)

Physical barriers: As reviewed by Zhang et al. (2019), the chicken intestinal epithelium consists of enterocytes, entero-endocrine cells, Paneth cells, and goblet T-cells. Epithelial cells are bound tightly together by tight and adherens junctional protein complexes that regulate paracellular

permeability. There are more than 50 tight junction proteins that make up integral transmembrane proteins such as occludin, claudins, tricellulin, and junctional adhesion molecules. Transmembrane proteins interact with cytosolic scaffold proteins such as zonula occludens (ZO). Different claudins subtypes either increase or decrease intestinal permeability. In poultry, claudin-1, claudin-2, claudin-3, claudin-5 and claudin-16 have been reported. ZO can cause the reorganization of the cytoskeleton. ZO-1 and ZO-2 have been reported in poultry. The destruction of the intestinal barrier is a major feature of NE and is characterized by increased bacteria translocation across the epithelium to internal organs, increased plasma LPS concentrations, leakage of plasma proteins into the intestinal lumen. Probiotics and prebiotics, oligosaccharides (Vieira et al., 2013), and enzymes are supplemented in feed or water to increase gut integrity (Wang et al., 2005). In addition, the crop's epithelial layer can be occasionally sloughed off to help eliminate pathogens and recolonized by bacteria present in the feed (Fuller and Brooker, 1974). Finally, in addition to having microbicidal activity, the viscous, gel-like nature of the mucus layer also serves as a physical barrier against intestinal bacteria colonization.

Toll-like Receptors: Toll-like receptors (TLRs) are a group of evolutionarily conserved type I transmembrane receptors that are involved in both the innate and adaptive arms of immunity. They are present in or on a variety of cells, including monocytes, macrophages, neutrophils, lymphocytes, NK cells, dendritic cells (Hajam *et al*., 2017). Toll-like receptors lack the trained specificity of T-cell or B cell receptors, each TLR recognizing a unique but limited range of pathogen associated molecular patterns. A Leucine-rich receptor (LRR) ectodomain recognizes the antigen, causing a signaling cascade in the TIR (Toll-interleukin-1 receptor) domain that is mediated by a variety of adaptor molecules such as Myeloid differentiation primary response protein (MyD88), TIR-domain-containing adapter-inducing

interferon-β (TRIF), TNF receptor- associated factors (TRAF) and downstream molecules such as Interleukin-1 receptor-associated kinase (IRAK). This may be accompanied by their homo or heterodimerization (Akira et al., 2006). They are important for activating dendritic cells, promoting differentiation of naïve T-cells into Th1, Th2, Th17, and Tregs (Fashina and Lillehoj, 2019).

There are unique differences in pathogen recognition and signal transduction by TLRs between humans and chickens, although such mechanisms are still relatively poorly understood in chickens. To date, at least 10 TLRs have been discovered in chickens. They include TLR-1.1, 1.2, 2.1, 2.2, 3, 4, 5, 7, 15, 21. Toll receptor 21 is a functional orthologue of human TLR 9 (Brownlie *et al.,* 2009) while no orthologue of TLR15 has been found in humans (Higgs et al., 2006). TLRs 1 and 2 have become duplicated into TLRs 1.1, 1.2, 2.1, and 2.2. Unlike the 7 other TLRs, TLR 3, 7, and 21 are located in intracellular endosomes (Chen *et al*., 2013). Human TLR-1 and 6 forms heterodimers with human TLR-2 to recognize bacteria lipoproteins and peptidoglycans while in chickens, TLR 1.1 and TLR 1.2 show the same function, recognizing bacteria lipoproteins and peptidoglycan respectively (Boyd *et al*., 2007). Toll receptors 3, 4, 5, and 7 recognize double-stranded RNA, LPS, flagellin, and ss RNA respectively (Akira *et al.,* 2006).

C. perfringens affects TLR expression in a complex manner. The up regulation of TLRs can contribute to protection against infection. However, overstimulation or prolonged exposure during NE infection can decrease performance and death (Rossignol et al., 2005). Lu *et al*., (2009) demonstrated up or down-regulation in the expression of TLR 1, 2, 4, 7, 15 and 21 in a time-dependent manner in the spleen and ileum of broilers challenged with a strain associated with NE. Although TLR2.2 is the principal receptor for peptidoglycan from Gram-negative and

Gram-positive bacteria (Higuchi et al., 2008; Du et al., 2016), some studies have found no difference in its expression in *C. perfringens*-challenged broilers (Lu et al. 2009; Alizadeh et al., 2016). The study of Lu et al. (2009), however, was based only on qPCR expression, and the gut-based immunological tissue cecal tonsils was not checked. The most significant aspect of the findings of Lu et al. (2009) was the modification of TLR15 and 21 which hitherto had been associated only with *Salmonella* Typhimurium (Higgs *et al*., 2006) and Campylobacter infections (Meade *et al*., 2009). Toll receptor 5 promotes antigen-specific innate and adaptive response as demonstrated by Vijay-Kumar *et al.,* (2010) in mice. They serve as endocytosis receptors for flagellin, and their signaling activity may be independent of MyD88 when mounting a humoral response.

E. tenella infection in chickens upregulates mRNA expression of TLR1.1, 4, 5, 7, 21 and TLR15 in a MyD88-dependent manner while *E praecox* infection in chickens up-regulates mRNA expression of TLR3, TLR4, and TLR5. In addition, *in vitro* stimulation of macrophages with heat-killed *E tenella* sporozoites leads to higher expression of TLRs and MyD88 than *in vitro* stimulation of macrophages with live *E tenella* sporozoites (Zhou et al., 2013).

Heterophils: These are orthologous to the human neutrophils and form the first line defense against pathogens. They utilize receptor-mediated phagocytosis, degranulation (Borregaard et al., 2007) and oxidative burst (Winterbourn and Kettle 2012) as mechanism (sometimes in that sequence) to fight off infections. Chicken breeds with more functionally active heterophils are generally better at resisting infections with *Salmonella* (Ferro et al., 2004), Campylobacter (Li et al., 2008) and *E. tenella* (Swaggerty et al., 2011)

Necrotic enteritis is typically associated with heterophils (in addition to lymphocyte and plasma cell) infiltration into the lamina propia of infected areas in the gut (Al-Sheikhly & Truscott, 1977).

Macrophages: Macrophages phagocytose the foreign body using toll-like or scavenger receptors by invagination into phagosomes. Within the phagosomes are acidic lysozymes that kills the bacteria. They can act as antigen presenting cells to T-cells in the context of MHC-II molecules on their cell surface. During inflammation, with the help of interferon gamma (IF-γ), they also produce cytokines such as TNF-α, IL-1, 6, 8, 12 (Arango Duque and Descoteaux, 2014). Avian macrophages play important roles in both the innate and adaptive arms of immunity. They are functional as early as embryonic day 12, therefore they are fully able to perform phagocytic functions as early as day of hatch. This property, in addition to antigen processing and presentation bodes well for in-ovo or day-of-hatch vaccinations (Gunawardana et al., 2018). The two macrophage cell lines that have been developed include MQ-NCSU (a marek's disease virus or MDV transformed) and HD11(myelocytomatosis or MC29 virus-transformed). Both are semi-adherent in cultures. The naïve macrophage becomes functionally (fc receptor expression and phagocytosis) mature in response to inflammatory signals such as LPS. In the presence of additional factors, they acquire the ability to recognize tumor targets. Macrophages can bind to non-opsonized targets, but their phagocytic functions in enhanced for opsonized antigens.

There are no resident macrophages in the abdominal cavity of birds (Qureshi and Hussain, 2000). *E tenella* infection is often accompanied by massive infiltration of the Payer's patches with leucocytes including macrophages (Lillehoj and Lillehoj, 1999). Compared to naïve chickens, infection-immunized chickens were demonstrated to have more sporozoites in their

CD3+, CD8+ αβ T-cells and fewer sporozoites in their macrophages populations 24 hours post-infection (Lillehoj and Lillehoj, 1999).

Another mechanism by which macrophages kill bacteria or parasitic pathogens is by inducing nitric oxide production (Thaxton et al., 2006) a reactive nitrogen species that disrupts the membrane of microbes. During infection with parasites such as *E. tenella*, proinflammatory cytokines are released in a bi-phasic fashion-around 5 days post-infection as part of the pathogenesis of the disease and 15-20 days post-infection for protective immunity (Byrnes et al., 1993). Vitamins D (Aslam et al., 1998), β-hydroxy-β-methylbutyrate (Peterson et al., 1999), vanadium (Qureshi et al., 1999), arginine (Khajali et al., 2020), are some nutritional factors that have been shown to modulate macrophage function. Macrophages and lymphocytes infiltrate the lamina propia during NE (Van Immerseel et al., 2002). There are currently no *in vivo* studies that elucidate the specific role of macrophages during infection with NE.

Beyond a generalized increase in the gene expression of certain pathogen recognition receptors (Lu et al., 2009), the specific role of macrophages in eliminating *C. perfringens* associated with NE is not well understood. *C. perfringens* infection is able to escape macrophage phagosomes with the help of phospholipase C and perfringolysin O (O'Brien and Melville, 2004).

Overview of adaptive defense mechanisms against necrotic enteritis in chickens

Necrotic enteritis is an enteric infection. Therefore, the gut-associated lymphoid tissue (GALT) is the site of induction of local immune responses against NE. Organized lymphoid tissues of the GALT include the bursa of Fabricius, cecal tonsils, Payer's patch, Meckel's diverticulum, lymphocyte aggregates scattered along the intra- epithelium and lamina propia

(Lillehoj and Trout, 1996). The lamina propia, spleen, and cecal tonsils constitute important effector sites for the immune response against NE (Lillehoj et al., 2016). The cecal tonsils and peyer's patch are anatomically similar, containing T-cells, B cells, macrophages and other antigen presenting cells. The chicken's payer's patch also contains microfold (M) cells and a significant proportion of naïve pre-committed IgA-producing B cells. There are special dendritic cells called Langerhans found in the squamous epithelium of the mucosa and in the skin. These dendritic cells extend transepithelial dendrites into the intestinal lumen that together with M cells, help sample luminal contents and transport antigens from the gut to the lamina propia. At effector sites, antigen recognition and presentation lead to the maturation of B cells and T-cells into antigen-specific effector and memory cells (Lillehoj and Trout, 1996). The specific role of M cells and langerhan cells of the broiler intestine in transporting *Eimeria* or *C. perfringens* antigens across the lumen of the gut has not been investigated. The potential for adaptive immunity to protect against NE is the basis of vaccination studies.

Overview of cell-mediated immunity and its role against necrotic enteritis in chickens

The thymus becomes colonized in 3 waves with precursors of T-cells in the consecutive order of $TCR1^+$ $TCR2^+$ and $TCR3^+$ at embryonic day 15, 19- and 2-days post-hatch (DPH) respectively (Coltey et al., 1987). From here they home to the periphery in the same order. The $TCR1^+$ subpopulation is composed of TCR γδT-cells, $TCR2^+$ subpopulation is composed of TCRαβ Vβ1 cells and $TCR3^+$ subpopulation is composed of TCRαβ Vβ2 cells. Gamma-delta T-cells are more commonly associated with epithelia-rich surfaces such as the gut mucosa and constitute a larger proportion of chicken peripheral T-cells (20 -60%) compared to mammals (5-10%) (Thome et al., 2015). In contrast, TCRαβ Vβ2 T-cells are extremely rare in the intestine

(Imhof et al., 2000). Similar to mammals, the peptide-recognizing diversity of TCR is the product of similar somatic recombination processes that occur to generate the diversity of T cell receptors.

The avian CD4 is a monomer with 4 N-terminal extracellular Ig-like domains. Positively charged amino acids present in this region are involved in their interaction with MHC-II molecules. The avian CD8 cell is either a homodimer ($\alpha\alpha$) or more predominantly, a heterodimer ($\alpha\beta$) (Luhtala, 1997). Immunity against *Eimeria* infections strongly depends on the cellular arm of the immune response including CD4+ and CD8+ T-cells (Rose et al., 1996). Immune responses are generated by invasive zoites and metabolically active schizonts (Jenkins et al., 1991). A study of the dynamics of the invasion of immunized birds by *Eimeria* sporozoites by Augustine and Danforth (1986) shows that immunity prevents sporozoite development but not sporozoite invasion. Gut-associated lymphoid T-cells are primarily responsible for the immune response to *Eimeria* infection. As evidenced by the varying populations of T-cells, proinflammatory cytokines and increased/decreased oocyst output during primary or challenge infections, the relative contributions of $CD4^+$ and $CD8^+$ T-cells to resistance and immunity appears to vary with infecting *Eimeria* strain (Trout and Lillehoj, 1996). For example, $CD4^+$ cells appear to be more important effectors of resistance to primary *E. tenella* infections than to primary *E. acervulina* infections. Meanwhile, $CD8^+$ cells may be more important for immunity to *Eimeria* challenge infections (Trout and Lillehoj, 1996). In addition, it is believed that the invasion of $CD8^+$ TCR$\gamma\delta$ T-cells (Lawn and Rose, 1982) and $CD8^+$ $\alpha\beta$ T-cells (Lillehoj and Lillehoj, 2000) by sporozoites contributes to primary infection of epithelial crypT-cells. In the most likely scenario, CD4 and CD8 cells are stimulated to different degrees at different times because chickens are often simultaneously infected by different *Eimeria* strains.

γδ-T-cells which are abundant in the intestinal mucosa and ceca of chickens are involved in chicken response to *C. perfringens* infection (Pieper et al., 2008). *C. perfringens* peptidoglycan cell wall is recognized by TLR 2 on the surface of macrophages, dendritic cells, and other innate cells (Du et al., 2016). The activation of innate cells helps to trigger a cytokine cascade that results in the differentiation of naïve T-helper cells to Th1, Th2 and Th17 cells. The activation of T helper cells culminates in the secretion of IL-1, interferon gamma by Th1 cells, IL-13 by Th2 cells, IL-17 by Th17 cells, and transforming growth factor (TGF)-β and IL-10 by regulatory T-cells (Cosmi et al., 2014). Fasina and Lillehoj (2019) suggest that inflammatory response to *C. perfringens* challenge is mediated by Th2 and Th17 cytokines at 7 days post-infection. The upregulation of systemic antibodies against *C. perfringens* further supports the involvement of CD4 helper T-cells (Fasina and Lillehoh, 2019). Till date, there is no information about how spatial and temporal T cell activation affects clinical symptoms of clostridiosis or NE in chickens**.**

Overview of humoral immunity and its role against necrotic enteritis in chickens

Lymphoid follicles in the crop mucosa and isolated lymphoid follicles in the lamina propia (Vaughn et al., 2008a) contribute to a humoral response by producing antigen-specific antibodies from B-lymphocytes present. In chickens, IgM-expressing B cells from spleen at embryonic day 8 migrate to the bursa where they develop. Mature B cells start seeding the periphery from E d18 (i.e., just before hatch), undergoing further development typically until 5-7 weeks. The bursa then involutes several months later. Broadly, the 2 populations of B cells that emigrate from the bursa are the short-lived ones that originate from the cortex and follicular B cells that have a longer half-life (Paramithiotis and Ratcliffe, 1993). Within the bursa somatic

rearrangement and gene conversion events leads to the generation of antibody-producing B cells capable of recognizing different antigens.

Unlike in *C. perfringens* infections, humoral responses to *Eimeria* infections play a minor role in defense against coccidiosis in chickens (Rose et al., 1996). The secretory IgA antibody is produced by plasma cells in the GALT and selectively transported through epithelial cells into external secretions. Mucosal IgA response against alpha toxin, NetB and other extracellular proteins of *C. perfringens* was reported in chickens that were partially protected against NE (Kulkarni et al., 2007, 2010; Jang et al., 2012). The administration of recombinant immunogenic proteins elicits systemic IgG and IgA responses that reach the mucosa (Kulkarni et al., 2007, 2010).

Overview of current intervention strategies against necrotic enteritis

Necrotic enteritis being a multifactorial disease requires a combination of different approaches in mitigation efforts. Coccidiosis is one of the most common predisposing conditions to NE (Baba et al., 1992). Therefore, interventions strategies that target coccidiosis will have an indirect effect on the NE. According to Dahiya et al., (2006), the control of NE falls under 3 broad categories: dietary modification and/or use of feed additives, pathogen reduction and amplification of immune response.

Dietary modification and/or use of feed additives: This includes the use of enzymes, organic acids, pro/pre-biotics, and various phytochemicals in naked or encapsulated forms to reduce the risk of coccidiosis and NE. Birds on diets that are high in energy, rich in protein, wheat- or barley are ten times more susceptible to NE infections than birds on maize-based diets (Cooper and Songer, 2009). Therefore, the addition of enzymes xylanase to wheat base diets decrease

digesta viscosity and fermentation, increased nutrient digestion and digesta passage, and reduces the amount of nutrients available to the pathogenic microflora, (Choct et al., 1999). Organic acids such as formic, acetic, propionic, butyric acids, or lactic, malic, and tartaric acids have antimicrobial activity. Organic acids can help to enhance performance, therefore ameliorating the effects of subclinical NE. The mode of action of organic acids may include decreasing the pH of the gut and the cytoplasm of pathogenic bacteria, making it harder for the pathogens to proliferate (M'Sadeq et al., 2015). However, the efficacy of organic acids to protect against clinical NE is debatable.

Probiotics are live microbials fed to chickens to improve their immunity intestinal morphology or stimulate the metabolism, in the process reducing the risk of infection by opportunistic pathogens. Probiotics can be used as direct-fed microbials (Latorre et al., 2015), competitive exclusion cultures (Hofacre et al., 2019), synbiotics (Mortada et al., 2020). Some common probiotic strains that have been widely investigated include member of the genus *Enterococcus*, *Bacillus*, lactic acid bacteria and yeast (Caly et al., 2015). There are numerous studies that have shown *in vitro* and *in vivo* activity antimicrobial activity of these probiotics against *C. perfringens* or *Eimeria*. Generally, probiotics exert their effect using competitive exclusion, secretion of bacteriocins (Pan and Yu, 2014) secretion of enzymes (Hatoum et al., 2012), stimulation of immune development, improvement of gut morphology and enhancement of adaptive immune response against infections (Vieira et al., 2013). However, the range of protection in NE experimental studies appears to vary with a probiotic cocktail, time/duration of administration, mode of administration, model and severity of infection. Chicken probiotics are marketed as prophylactics, with a potential beneficial effect of reducing the risk or severity of NE.

Prebiotics are mostly indigestible oligosaccharides that stimulate the commensal gut flora and enhance the beneficial effects of probiotics to the exclusion of pathogenic microorganisms (Patel and Goyal, 2012). Common prebiotics include mannanooligosaccharides (MOS), fructooligosaccharides (FOS), xylooligosaccharides (XOS). Prebiotics act using mechanisms similar to those of probiotics. Prebiotic are broken down by probiotics and commensal bacteria, releasing short chain fatty acids (SCFC). Short chain fatty acids have been shown to improve gut morphology (Xu et al., 2003; M'Sadeq et al., 2015) although the exact mechanism is not fully known. Broilers that were fed short and medium chain fatty acids have had less necrotic lesions than broilers that were not (Timbermont et al., 2017).

Pathogen reduction: *Clostridium* and *Eimeria* are ubiquitous in poultry houses and therefore practically impossible to get rid of. *C. perfringens* is also a commensal in the gut of flocks, with majority being harmless. Studies have shown that NE outbreaks tend to be clonal, with pathogenic strains inhibiting the growth of or outcompeting other strains of *C. perfringens*. Therefore, the institution of sound management and biosecurity measures help minimize the conditions that lead to the infection or predominance of pathogenic *C. perfringens* strains. Birds should be provided with proper feed and drinking water intake, adequate bedding, temperature, humidity, lighting and ventilation. Heat stress, for example, was demonstrated to increase the severity of NE lesions in experimentally infected broiler chicks (Tsiouris et al., 2009). Although *Eimeria* generally thrives in atmospheric conditions that are beneficial to birds, factors such as stocking density and litter conditions can be optimized to maintain trickle infection while discouraging oocyst and bacteria accumulation (Peek and Landman, 2011). Good animal husbandry also minimizes the risk of introducing pathogens such as Marek's disease virus, Infectious bursal disease virus that can also predispose birds to NE.

Amplification of immune response: Currently, antibiotics and anticoccidials are among the most effective means of controlling NE. Antibiotics had been most commonly used as growth promoting agents and for prophylaxis for subclinical NE (Prescott et al. 2016). However, the potential for antibiotic residues contaminating carcass and eventually contributing to multidrug resistant food-borne pathogenesis in human beings has led to limitations of their use or outright ban. Example of this ban is in Europe in 2006, under feed additives regulation 1831/2003/EC (Kemper 2008). European farmers rely mostly on therapeutic use of antibiotics such as amoxicillin and tylosin to control outbreaks of clinical NE (Lanckriet et al., 2010). In the U.S., international and public health agencies have increasingly targeted antibiotic resistance as an emerging public health concern (Kilonzo-Nthenge et al., 2008). Example of this ban in the U.S. is under the veterinary feed directive, coded in the 21 CFR (https://www.federalregister.gov/documents/2015/06/03/2015-13393/veterinary-feed-directive).The mode of action of antibiotics includes selective modification of the gut microflora, decreasing bacterial fermentation, reducing thickness of the intestinal wall and suppressing bacterial catabolism (Carlson and Fangman, 2000). Other common poultry antibiotics are bacitracin, chlortetracycline, erythromycin, and penicillin.

Anticoccidials can be classified as synthetics (chemicals), ionophores, or mixed products of ionophores and/or chemicals. Ionophores can be monovalent, monovalent glycosidic or divalent (Peek and Landman, 2011). Lasalocid, Monensin, and Salinomycin are examples of ionophores. Amprolium, Diclazuril, and Nicarbazin are examples of chemical anticoccidials. Ionophore mode of action is similar to antibiotics. Hence, ionophores can inhibit Gram-positive organisms (Dutta and Devriese 1984) and some Gram- negative bacteria (Ford et al. 1981). Chemicals have a range in their mechanism of action against *Eimeria*, but generally involves the

disruption of certain biochemical processes during the intracellular stages of the parasite. For example, amprolium competes with the parasite for the absorption of thiamine (vitamin B1) (Peek and Landman, 2011). The increasing reduction in susceptibility of *Eimeria* to anticoccidials permits trickle infections and may be the reason why reduced sensitivity is yet to be linked to significant outbreaks (McDougald and Shirley 2009). Furthermore, rotation and bioshuttle programs, making use of different anticoccidials and/or anticoccidials with different modes of action within a defined rearing period have been suggested as effective in limiting loss of sensitivity and antibiotic resistance in *Eimeria* (Peek and Landman, 2011).

Vaccine development against necrotic enteritis

The 2 prominent targets of current NE vaccination efforts are *C. perfringens* and *Eimeria*. *Eimeria* vaccination can be alternated with anticoccidial drugs in feed within rotation programs and in combination with biosecurity. Broadly speaking, vaccination can be classified based on route of administration and type of antigen into parenteral/non-parenteral and live/non-live vaccines respectively.

Live (attenuated) vaccines: Live vaccines against coccidiosis consist of attenuated or non-attenuated sporulated oocysts. Attenuated coccidiosis vaccines consist of precocious mutants of various species administered in feed or orally at day of hatch. The application of non-attenuated vaccines has a relatively higher risk of coccidiosis outbreaks (Chapman et al., 2002). Vaccination is generally advantageous for commercial breeding flocks when used in addition to anticoccidials such as ionophores (Matthis et al., 2014). Anticoccidial vaccines can be administered to laying hens for maternal antibodies (Michael, 2007) (Keyburn et al., 2013). Coccidiosis vaccines can be applied to laying hens, as well as some Antibiotic-Free (ABF) or Raised Without Antibiotics

(RWA) broiler flocks. Coccidiosis vaccines can also be used in bio shuttle or rotation programs to prevent resistance or restore sensitivity of infective parasites to anticoccidial control programs (Peek and Landman, 2011).

Studies have shown that infection-immunization with *Clostridium* can induce protection against homologous challenge by reducing lesions associated with NE (Thompson et al., 2006). Thompson et al (2006) found out while immunizing with a virulent strain could protect against avirulent challenge, avirulent infection-immunizing conferred little protection against virulent strains. There is relatively little research that have explored live attenuated vaccines using clostridium against NE. This may have to do with the difficulty in finding the right balance between attenuation and protection (Mot et al., 2014).

Subunit vaccines: Subunit vaccines composed of a purified antigenic determinants of *C. perfringens* and *Eimeria* have been explored with limited success. Subunit vaccines may consist of native antigens or recombinant proteins expressed from DNA of various developmental stages (sporozoites, merozoites and gametes) of the *Eimeria* parasite (Lillehoj et al., 2017) or extracellular proteins of Clostridium (Saleh et al., 2011; Fernandes da Costa et al., 2013). Some studies have shown that chickens could be protected against *C. perfringens*-induced NE by injection with inactive and active toxins (Kulkarni et al., 2007) or other antigenic proteins (Jiang et al., 2009). Lillehoj et al., (2017) found some protective efficacy in vaccination with a combination of a subunit of *C. perfringens* and *Eimeria*. One of the central goals of current vaccine design approaches is oral delivery of antigens. This vaccination strategy is potentially efficient, economical and reliable for vaccinating large flocks (Hoelzer, et al., 2018). The mucosa, however, is one of the first points of contact between the host and external environment, making it one of the most important hubs for innate defense mechanisms. This ‘harsh’

environment is largely acidic, contains a relatively large number of proteolytic enzymes and mucus that is continuously shed off. These mechanisms prevent efficient delivery of oral unencapsulated antigens to gut-associated lymphoid tissues, requiring the use of active adjuvants, high and/or multiple vaccine doses to elicit sufficient immune response comparable to parenteral administration. Another significant challenge is the tendency of the mucosal immune system to generate tolerance to protein antigens.

Nanoparticle Vaccines: Nanoparticles are being increasingly used as vaccine adjuvants to stimulate specific and robust immunity (Carrillo-Conde *et al*., 2011). Targeting M-cells by intranasal particulate vaccine carriers has also been explored in mice (Nochi et al., 2007; Hase et al., 2009) but not in chickens. There are several advantages in using nanoparticles in vaccine design.

The nanoparticle is an antigen delivery system that facilitates closer interaction between antigen and antigen-presenting cells (APCs). Also, some act as TLR 2, -4, and -5 agonists. Thus, when delivered with immunizing antigens such as proteins (figure 2.2), they help trigger inflammatory costimulatory signals that are potentially immunoprotective against the antigen (Tamayo *et al.,* 2010). Their antimicrobial activities activity can also be modified through the addition of inorganic elements or compounds like Gold (Wei *et al*., 2007), Silver (Wei *et al*., 2009; Salem *et al*., 2015), zinc oxide (Salem *et al*., 2015), Copper (Ahmed *et al*., 2015) as well as organic compounds. ZnO-based nanoparticles has been demonstrated to be effective against attachment of Vibrio cholerae and enterotoxic *Escherichia coli* in mice (Salem *et al*., 2015). They induce death when they disrupt prokaryotic membranes by releasing toxic oxygen radicals or interacting with their DNA (Apperlot *et al*., 2009; Krishnamoorthy *et al*., 2012). Antigens

loaded onto nanoparticulates are effectively protected from enzymatic degradation within the gastrointestinal tract (Salman *et al.,* 2009).

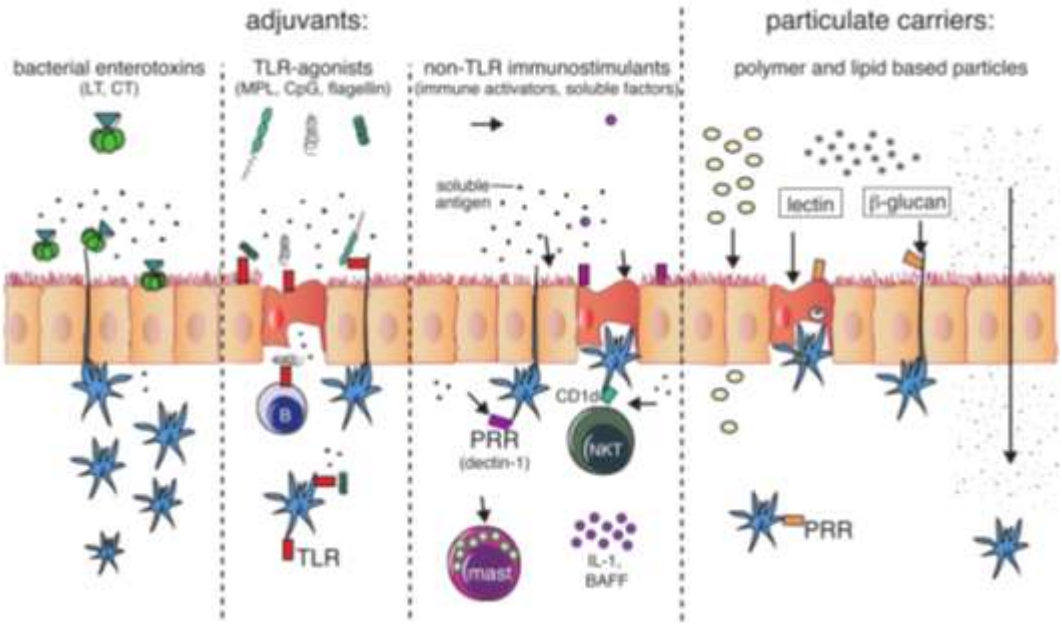


Figure 2.3. **The role of adjuvants in stimulating immune response in mucosal surfaces (Lawson et al., 2011)**

Chitosan nanoparticle is a polycationic, N-deacetylated derivative of chitin composed of more than 5000 glucosamine (N-acetylglucosamine) polymer units also known as 2-amino-2-deoxy-(1 → 4)-β-D-glucopyranose (Prabaharan and Mano, 2005; Anusha and Fleming, 2016) with unique physical and chemical properties. They have high nitrogen content and are useful as chelating agents. They are present in present in plant cell walls, fungal cell walls, crustaceans such as shrimps and crabs. It can also be fermented from Aspergillus niger (Pochanavanich and Suntornsuk, 2002).

Chitosan-based nanoparticles have been extensively studied. This is because they are biodegradable, biocompatible, non-toxic (Zhao *et al*., 2014) and can be chemically modified to withstand varying pH, temperature, solubility (Yata and Gosh, 2011), microbial and component adsorption (Shang *et al*., 2011), with the purpose of delivering biomolecules and drugs (Prabaharan and Mano 2005) and removing contaminants from waste water treatment (Shang *et al*., 2011). Like a lot of other nanoparticles, they can be easily resized through ionic gelation (Anusha and Fleming, 2016) or reshaped. Chitosan has numerous stimulating and inhibitory activities in different animal cell types. They have applications in the food, medical, pharmaceutical, and biomedical industries (Shang *et al*., 2011). Chitosan DNA administered intranasally has been shown to successfully induce serum anti-*Campylobacter jejuni* IgG and intestinal mucosal antibody (IgA) and help reduce its colonization in the intestine and cecum (Jiao *et al*., 2010).

Chitosan has shown potential as an adjuvant to drive cell-mediated immunity. Unlike alum, they do not inhibit interleukin 12 while they promote the production of antigen-specific Th1 and Th17 (Mori *et al*., 2012) and type-1 interferons which contribute to the maturation of dendritic cells. In dendritic cells, their cationic polymers engage STING-cGAS in order to activate innate and adaptive immune pathways (Carroll *et al*., 2016).

Chitosan plays a role in prodrug mediated suicide gene therapy against a wide range of tumors. Sustained release of up to one week was achieved by (Yata and Ghosh, 2011) using chitosan-based cytosine deaminase nanocomposite.

In conclusion, NE is a disease that can lead to huge economic losses in the poultry industry. Current mitigation efforts include the use of live vaccines, and this has been partially successful in controlling NE. However, subunit antigens offer some advantages over live

antigens. There are potentially protective subunit antigens secreted by *C. perfringens* but the route of administration of the antigen affects its immunogenicity and efficacy. Therefore, there is a need to explore rational vaccine design strategies that will complement current invention efforts. The well-documented advantages of nanoparticle technology suggest that it can be used as an encapsulating agent to boost the safety and immunogenicity of subunit antigens. The emergence of chitosan nanoparticles as potential adjuvants for antigen delivery in livestock suggests that in should be explored as vaccine designs against NE using *C. perfringens* subunits.

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CHAPTER 3

Design, and *in vitro* characterization of chitosan nanoparticles loaded with native and inactivated extracellular proteins from a field strain of *Clostridium perfringens* associated with necrotic enteritis.

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Abstract

There are currently no licensed vaccines against *C. perfringens* which causes NE in poultry. Chitosan nanoparticles were formulated with native (CN) or toxoids (CT) of extracellular proteins of *C. perfringens,* both surface-tagged with *Salmonella* flagellar proteins. In a pH stability assay, CN and CT nanoparticles released 6% and 0% of their cargo proteins at 8.0 pH. In a protein release assay, CN and CT nanoparticles also released 16% and 10% of their protein respectively at 7.4 pH after 24 hours. CN and CT nanoparticles incubated at 100 µg/ml PBS with Chicken RBCs released 1% and 0% proteins respectively. CN and CT were nanoparticles incubated at 0.1mg/ml, 0.25mg/ml and 0.125mg/ml loaded protein in 1X PBS of pH 2, 3, 4, 5, 6, 7, and 8. The lowest average diameter of the synthesized CN nanoparticle was 339.2 nm, at 0.1mg/ml and pH 2. At pH7.2, the synthesized CN nanoparticles had an average size of 389.7 nm and a zeta potential of -66.9 mV while the synthesized CT nanoparticles had an average size of 352.5 nm and a zeta potential of -63.6 mV. This vaccine was therefore safe, and stable invitro. The range of the nanoparticle's physicochemical properties suggest its potential for use in *in vivo* studies for immunogenicity and protection studies.

Introduction

Necrotic enteritis is a severe *C. perfringens* induced disease and is caused by type A and type C strains (Dahiya et al., 2006; Thompson et al., 2006). Previous research has shown that there are immunogenic and partially protective proteins secreted by *C. perfringens* in broth cultures (Kulkarni et al., 2006; Saleh et al., 2011). These cultures contain both toxins such as alpha toxin (Saleh et al., 2011) and moonlighting proteins such as GAPDH (Kulkarni et al., 2006). Subunit protein antigens are safer than live antigens but not immunogenic when administered orally without encapsulation or adjuvants. This is because orally administered subunit antigens induce tolerance, or they are digested by enzymes in the chicken's gut (Kim et al., 2014). There is a need to develop means of 'protecting' antigens by encapsulating them in order to enhance their potential as oral vaccines.

Chitosan-based nanoparticles have been extensively studied and have applications in the food, medical, pharmaceutical, and biomedical industries (Shang *et al*., 2011). They are biodegradable, biocompatible, non-toxic in some animals studied (Zhao *et al*., 2014). Chitosan can be easily resized as nanoparticles or microparticles through ionic gelation (Anusha and Fleming, 2016) or reshaped. Their physicochemical properties can be chemically modified to withstand varying pH, temperature, solubility (Yata and Gosh, 2011), microbial and component adsorption (Shang *et al*., 2011). Therefore, chitosan can be used to deliver biomolecules and drugs in animals (Prabaharan and Mano 2005). Chitosan nanoparticles protect their cargo until delivery to target sites (Prabaharan and Mano, 2005). Depending on how they are designed, chitosan has a range of stability, cargo release time points (Hou et al., 2012). They stimulate antigen presenting cells and T cells (Xiang et al., 2006). The physicochemical properties and safety of chitosan as an oral delivery vehicle of influenza proteins has been characterized in

swine (Dhakal et al., 2018). To the best of our knowledge, this is the first study that will evaluate the encapsulation of *C. perfringens proteins* extracellular proteins with chitosan. The objective of this study was to characterize the *in vitro* safety and physicochemical properties of nanoparticles loaded with *C. perfringens* proteins extracellular proteins and *Salmonella* Typhimurium flagella proteins.

3.2. Materials and methods

3.2.1. Collection of *C. perfringens* extracellular proteins in native form

A field strain of *C. perfringens*, *C. perfringens* (a gift from Dr. C. Hofacre, Southern Poultry Research Group) was cultured on blood agar for 24 hours at 37°C. Isolated colonies of *C. perfringens* were inoculated into 200 ml of thioglycollate broth (Sigma Aldrich, St Louis, MO) for 24 hours at 37°C under anaerobic conditions. The 200 ml culture was inoculated into 2 L of thioglycolate broth for 24 hours at 37°C under anaerobic conditions. At 24 h of incubation, the optical density of the culture, measured at 600 nm with a spectrophotometer (Biochek, Scarborough, ME) was 0.6. The supernatant was collected after centrifugation at 11,420 X g for 30 min and filtered using a 0.22 µm filter (Sarstedt, Newton, NC). Extracellular proteins (ECP) in the native form were precipitated from the supernatant using 106g $(NH_4)_2SO_4$ per 200 ml of the supernatant at 4°C overnight. The native ECP was concentrated and purified by a process modified from the method by Keyburn et al. (2008). The native *C. perfringens* was reconstituted in 40 ml of PBS, concentrated and desalted 5X using a 10 kDa Amicon® 8400 Ultra filter membrane (Sigma Aldrich, St. Louis, MO). The protein concentration in the ECP suspension was determined using a Bradford reagent kit (Bio-Rad, Hercules, CA) following the manufacturer's protocol. Optical density was measured at 595 nm as previously described. The concentration of the native ECP was 42 mg/ml. A single batch of proteins was prepared and used

for the entire study. Aliquots of the ECP were lyophilized, with sucrose as cryoprotectant, and stored at −80°C until further use.

3.2.2. Preparation of *C. perfringens* toxoid

Preparation of ECP toxoid was carried out as described previously (Fernandes Da Costa et al., 2013) with modifications. Purified native ECP (100 mg) was reconstituted in 100 ml 1X PBS, treated with 1ml of formaldehyde solution (ThermoFisher Scientific, Waltham, MA), and incubated at 37°C for 5 d to synthesize the ECP toxoid. The reaction was stopped by adding 54.6 mg L-lysine. The toxoid was dialyzed against 1X PBS to remove excess formaldehyde, lyophilized as described previously, and stored at −80°C until further use.

3.2.3. Preparation of chitosan native and toxoid ECP nanoparticles

Chitosan nanoparticles were prepared by ionic gelation as described earlier (Dhakal et al., 2018). Purified native ECP (5mg) or toxoid ECP (5mg) was reconstituted in 1 ml of 1 X MOPS buffer. The reconstituted native ECP or toxoid ECP was added dropwise to 5 ml of a solution of 10 mg/ml of chitosan polymer (Sigma Aldrich, St. Louis, MO) in nano-pure water under magnetic stirring. 12.5 mg of sodium tripolyphosphate (TPP), dissolved in a 25 ml 1X PBS, was added to the above mixture dropwise for a chitosan to TPP ratio of 4:1. Flagella proteins were extracted from *Salmonella* as described previously (Komoriya et al., 1999), reconstituted to a final concentration of 0.5 mg/ml, and added to the above mixture dropwise to synthesize the native ECP chitosan (CN) or toxoid ECP chitosan (CT) nanoparticle. The CN and CT nanoparticles were collected by centrifugation at 10,000 X g for 30 minutes, lyophilized and stored at -80°C until further use.

3.2.4. The entrapment efficiency of ECP and flagellar proteins in the synthesized CN and CT nanoparticles

The entrapment efficiency was measured by quantifying the amount of proteins leftover in the supernatant after centrifuging the CN and CT nanoparticles at 10,000 X g for 30 minutes. This experiment was carried out twice to obtain mean entrapment efficiency. The protein content in 200 µl of the supernatant was measured using Bradford reagent as described above. The entrapment efficiency was determined as follows:

Entrapment efficiency (%) = (Total *protein for nanoparticle synthesis – Total protein in supernatant)* / Total *protein for nanoparticle synthesis* × 100.

3.2.5. Particle size distribution and Zeta potential of synthesized CN nanoparticles

Only the synthesized CN nanoparticles were suspended at 0.1mg/ml, 0.25mg/ml and 0.125mg/ml loaded protein in 1X PBS adjusted with HCl or NaOH to pH 2, 3, 4, 5, 5, 7, and 8. The particle size distribution was measured with a zeta sizer (Zetasizer Nano ZS, Malvern, U.K).

Both the synthesized CN and CT nanoparticles were suspended only at 0.1mg/ml loaded protein in 1X PBS of pH 7.2. The particle size distribution and zeta potential of the synthesized CN and CT nanoparticles were measured with a zeta sizer as previously stated. The diameter of the dispersed particles was determined using the autocorrelation function and reported as percentage intensity. Particle size distribution and zeta potential assay were conducted once. For particle size distribution, the reading that had the least deviation from the mean of four readings was reported.

3.2.5. Cumulative protein release assay and pH stability of CN and CT nanoparticles

The stability of the synthesized CN and CT nanoparticles was measured using *in vitro* protein release assay as described previously (Dhakal *et al.* 2018). Suspensions of 0.5 mg/ml of CN or CT nanoparticles in 3 ml of 1X PBS at 7.4 pH were incubated at 41°C for 0, 2, 3, 10, 17 and 24 hours. 350µl of supernatants were collected and centrifuged at 10,000 X g at 4°C in triplicates. The protein content in 200 µl of the supernatant was analyzed using Bradford reagent as described above. The cumulative protein released at each time point was determined as follows:

cumulative protein released (%) = *(cumulative protein released in supernatant*/0.5) x 100

The pH stability of the synthesized CN and CT nanoparticles were measured by reconstituting 0.1 mg/ml of CN or CT nanoparticles in 1X PBS at 3.0, 3.5, 4.5, 5.5, 6.5, and 8 pH. The suspension of CN or CT nanoparticles was incubated at 41°C for 3 hours and centrifuged at 10,000 X g for 5 minutes at 4°C. The protein content in 200 µl of the supernatant was analyzed using Bradford reagent as described above. The experiment was carried out twice. The protein released at each pH was determined as follows:

protein released (%) = *(protein released in supernatant*/0.1) x 100

3.2.6. Effect of CN and CT nanoparticles on cRBCs

The effect of CN and CT nanoparticles on cRBCs was measured by analyzing the amount of hemoglobin released from chicken RBCs using the hemolysis assay as described previously (Pan *et al.* 2016). Blood from 3-week-old broiler chickens (1ml) was collected in EDTA treated tubes. The blood was centrifuged at 750 X g to collect cRBC. The cRBCs were washed four times with 1X PBS and reconstituted in 2 ml of 1 X PBS. 10 µl of cRBC suspension was

incubated with 0.5 ml of PBS (negative control) or 25 μg/ml or 50 μg/ml or 100 μg/ml CN and CT nanoparticles or pure deionized water (positive control). The suspensions were incubated for 3 hours at 37°C on a shaker (ThermoFisher Scientific, Waltham, MA) with rotating agitation at 100 rpm, and centrifuged at 750 x g for 5 minutes. The absorbance values of 200 μl of the supernatant was determined at 595 nm using a microplate reader. The experiment was carried out twice. The hemolysis of cRBC was determined as follows:

hemolysis (%) = *([OD_{595nm} Absorbance (treatment - negative control)] / [OD_{595nm} Absorbance (positive control - negative control])* ×100.

3.3. Results

3.3.1. The entrapment efficiency, Particle size distribution and zeta potential of the synthesized nanoparticles.

The entrapment efficiency for both CN and CT nanoparticles was 70 % each.

The average diameter of the synthesized CN nanoparticle at pH 4 and pH 5 for all dispersion concentrations was greater than 10μm. Data for pH 4 and pH 5 were therefore omitted (Figure 3.1A to D). Only CN was used to assess particle size distribution. At 0.1mg/ml concentration, the synthesized CN nanoparticles had an average diameter of that ranged from 342 nm at pH 2 to 10μm or more at pH 5, at its largest peak. At 0.125mg/ml concentration, the synthesized CN nanoparticles had an average diameter of that ranged from 396.1 nm at pH 2 to 10μm or more at pH 5, at its largest peak. At 0.25mg/ml concentration, the synthesized CN nanoparticles had an average diameter of that ranged from 531.2 nm at pH 2 and pH 7, to 10μm or more at pH, 5 at its largest peak.

At pH 7.2, the synthesized CN nanoparticles had an average size of 389.7 nm at its largest peak and a zeta potential of -66.9 mV (Figure 3.2A) while the CT nanoparticles had an average size of 352.5 nm at its largest peak and a zeta potential of -63.6 mV (Figure 3.2B).

3.3.3. pH stability and cumulative protein release from CN and CT nanoparticles.

CN nanoparticles released 4 % and 6 % of proteins at 2.5 pH and 8.0 pH respectively while CT nanoparticles did not release measurable antigens at any of the pH assayed (Figure 3.3A).

At pH 7 in PBS, CN nanoparticles released 2 % of its protein while CT nanoparticles released 4 % of its protein during the first two hours of incubation. At 24 h of incubation, CN nanoparticles had released 16 % of protein while CT had released 10 % of its protein cargo (Figure 3.3B).

3.3.4. Effect of CN and CT nanoparticles on chicken red blood cells

Chicken RBCs (cRBC) incubated with 25 µg/ml, 50 µg/ml and 100 µg/ml of CN nanoparticles had 2.5 %, 1.4 % and 1.0 % hemolysis respectively (Table 3.1). Chicken RBC incubated with 50 µg/ml and 100 µg/ml of CT nanoparticles had 0 % hemolysis.

3.4. Discussion

This trial identified the physicochemical properties of chitosan nanoparticles loaded with *C. perfringens* extracellular proteins and *S.* Typhimurium flagella proteins. The entrapment efficiencies of CN and CT nanoparticles were 70 %. The size of a nanoparticle affects which cells take it up and how it triggers the immune system (Xiang et al., 2006). The presence of particles above 10 µm in CN particle size distribution at pH 4 and pH 5 may be due to aggregation. This suggests that pH 4 and pH 5 may not be optimal for resuspension of the nanoparticles CN nanoparticles at 0.1mg/ml at pH 2 and pH 7-7.2 had the smallest particle size

distribution and may be optimal for resuspension. The tendency of the nanopartilces to aggregate more at higher concentrations may be due to increased hydrophobic interactions (Vaisman et al., 2006). Nanoparticles with zeta potentials of greater than +30 mV or less than −30 mV are considered strongly cationic or anionic respectively (Clogston et. al., 2011) and this is related to their stability and efficacy (Kiaie et al., 2016). Therefore, the zeta potentials of -66.9 mV and -63.6 of the CN and CT nanoparticles respectively may indicate stability in PBS at 7.2 pH. Both the CN and CT nanoparticles had an average size of less than 500 nm. A previous study demonstrated that particle sizes around 500 nm or less are optimum for uptake by dendritic cells and macrophages (Motram et al., 2007). The presence of particles above 1µm in CT particle size distribution may be due to aggregating particles.

The CN and CT nanoparticles released 16 % and 10 % of their cargo respectively, after 24 hours incubation at 7.4 pH. Earlier studies reported that Chitosan nanoparticles loaded with Newcastle disease virus F protein released 30 % protein (Zhao et al., 2012) and chitosan nanoparticles loaded with killed swine influenza antigen released 10 % protein (Dhakal et al., 2018) after 24 hours. Chitosan: TPP ratio of 4:1 was used for this study. Hou et al., (2012) demonstrated that as the chitosan: TPP ratio increases, protein loading efficiency decreases and protein release from the nanoparticle increases. CN and CT nanoparticle were stable and released less than 8 % of their antigen cargo at pH between 2.5 and 8. The pH of the avian gastrointestinal tract typically ranges from 3 in the gizzard to 7 in the ceca (Sadeghi *et* al., 2016).

The *C. perfringens* isolate used for this study was shown by PCR to be alpha toxin positive and NetB negative and TpeL negative but has been used to induce mild NE (data not shown). CN and CT nanoparticles released less than 3 % and less than 0.1 % hemoglobin from cRBCs indicating that the CN and CT nanoparticles do not have an adverse effect on cells.

Chitosan nanoparticles have been demonstrated to be safe in chicken embryo kidney (CEK) cells (Zhao et al., 2012).

In conclusion, this study was carried out to assess the cytotoxicity and physicochemical properties of chitosan nanoparticles loaded with native or inactivated extracellular proteins of *C. perfringens* and *Salmonella* flagellar proteins. Both nanoparticle formulations were safe in red blood cells, releasing their antigen slowly over time. Both formulations were also stable over a wide range of pH. The zeta potential for both nanoparticles indicate that they are likely to remain poly dispersed in neutral solution. The results from this preliminary assessment suggest that either of the CN or CT formulations can be safely assessed in broilers for safety, immunogenicity and protective efficacy.

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Table 3.1. Effect of CN and CT nanoparticles on chicken red blood cells.

Treatment	RBC Hemolysis (%)		
	25µg/ml	50 µg/ml	100 µg/ml
CN	2.500 ± 0.002	1.364 ± 0.001	1.023 ± 0.001
CT	0.021 ± 0.030	0 ± 0.000	0 ± 0.000

Chitosan-native (CN) and chitosan-toxoid (CT) nanoparticles were synthesized by entrapping *C. perfringens* extracellular proteins and flagellar proteins. Mean ± standard deviation. (n=2).

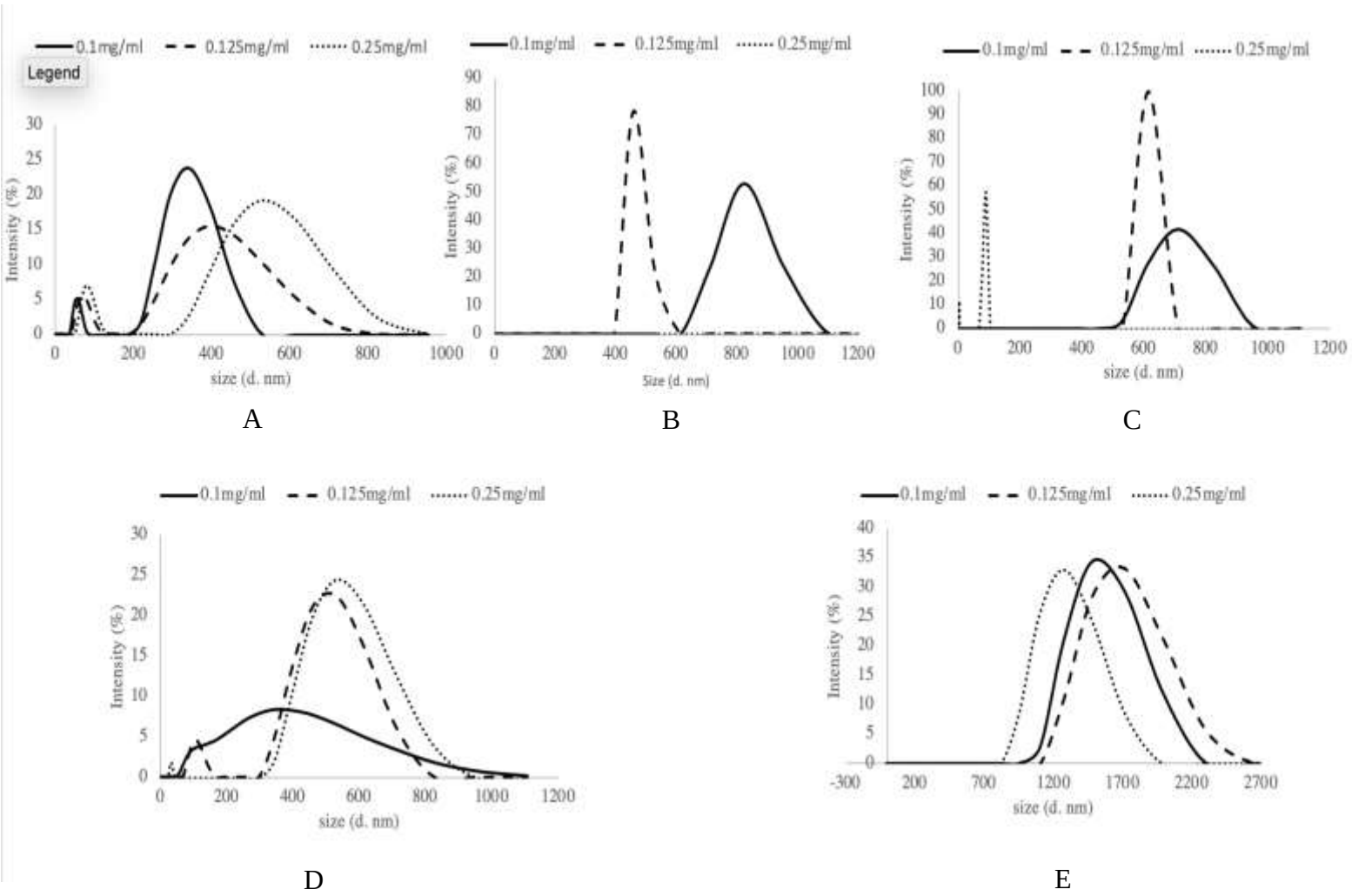
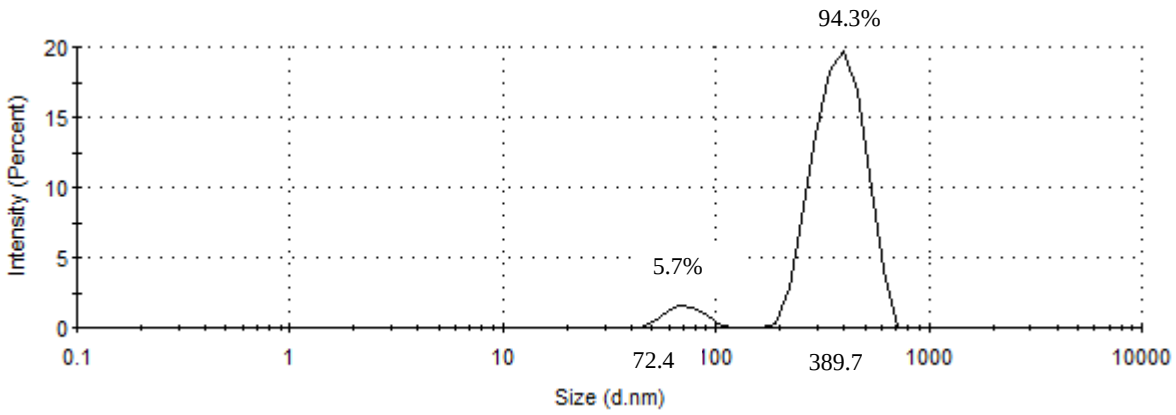
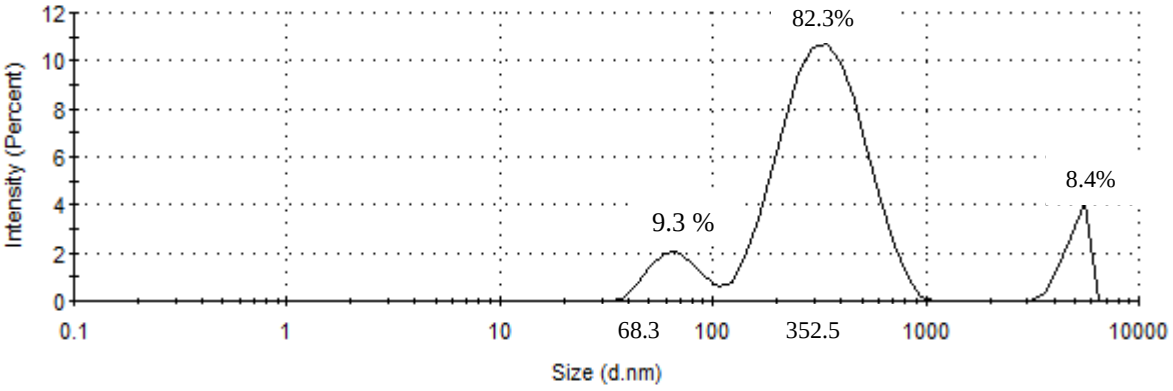


Fig. 3.1. Particle size distribution and zeta potential of CN nanoparticles at different concentrations and pH. The particle size distribution of 0.1mg/ml, 0.125mg/ml and 0.25mg/ml Chitosan-native (CN) nanoparticles dispersed in 1x PBS at pH 2 (A), pH 3 (B), pH 6 (C), pH 7 (D) and pH 8 (E) was measured at 25°C and 173° scattering angle with a zeta sizer. The diameter of the dispersed particles was determined using the autocorrelation function and reported as percentage intensity.



A



B

Fig. 3.2. Particle size distribution and zeta potential of CN and CT nanoparticles. Chitosan-native (CN; A) and chitosan-toxoid (CT; B) nanoparticles were dispersed in 1x PBS at pH 7.2 and their particle size distribution and zeta potential were measured at 25°C and 173° scattering angle with a zeta sizer. The diameter of the dispersed particles was determined using the autocorrelation function and reported as percentage intensity.

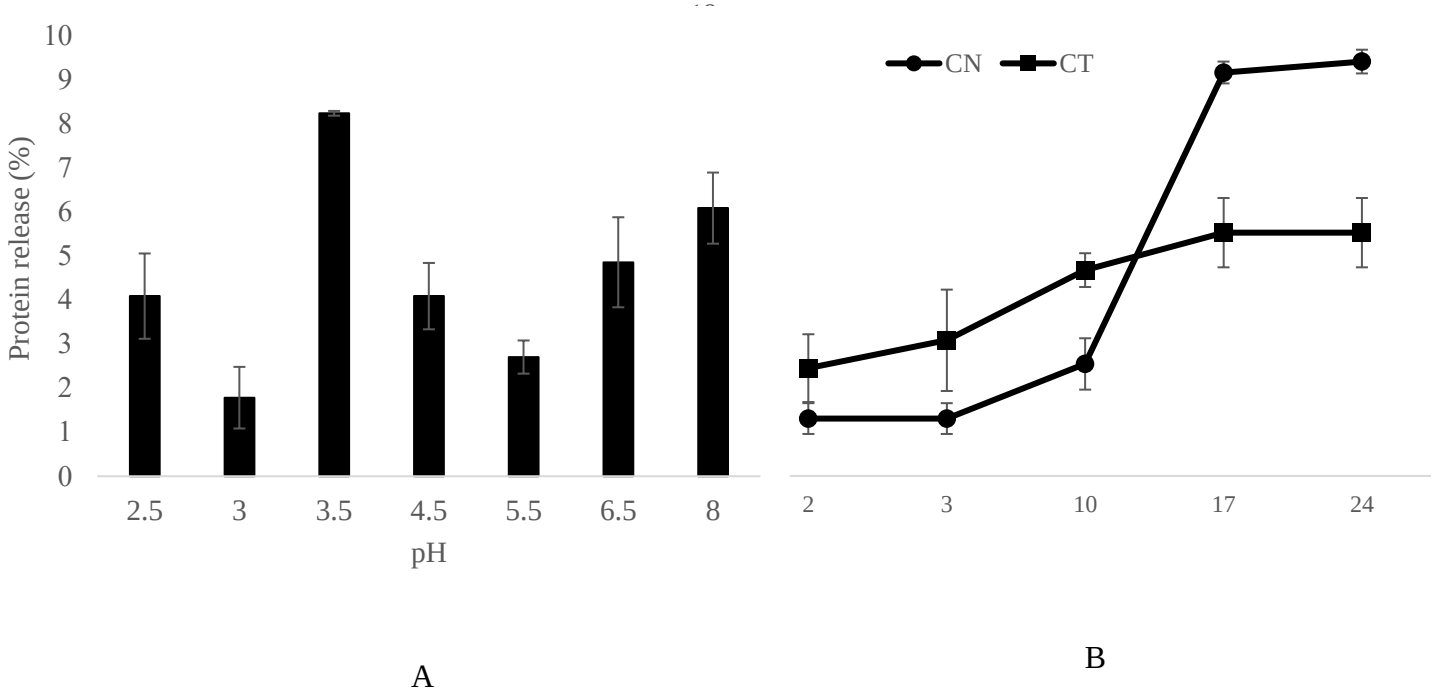


Fig. 3.3. *In vitro* cumulative protein release assay and pH stability of CN and CT nanoparticles. Chitosan-native (CN) and chitosan-toxoid (CT) nanoparticles were incubated in solutions of different pH for 3h and the percentage of total protein released was measured using the Bradford assay (Panel A). Protein release from CT was below the detection limit of the assay. Chitosan-native (CN) and chitosan-toxoid (CT) nanoparticles were incubated in PBS and the cumulative antigen release was measured at indicated time points using the Bradford assay (Panel B). Means ± SD. n = 2.

CHAPTER 4

Immunogenicity of chitosan nanoparticles loaded with native and inactivated extracellular proteins from a field strain of *Clostridium perfringens* associated with necrotic enteritis.

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Abstract

There are currently no licensed vaccines against *Clostridium perfringens* which causes NE in broilers. Chitosan nanoparticles were formulated with native (CN) or toxoids (CT) of extracellular proteins (ECP) of *C. perfringens,* both surface-tagged with *Salmonella* flagellar proteins. Ninety broilers were randomly assigned to treatments: sham-vaccinated (Control), CN-vaccinated (CN), and CT-vaccinated (CT). Each bird was orally gavaged with 0.5ml PBS or 50µg vaccine in 0.5ml PBS on d 0, 3, 7 and 14 of age. At 10 d of age, the CN group had higher anti-flagellar IgA than control ($P < 0.05$). At 17 d of age, the CN group had higher anti-flagellar IgG than control ($P < 0.05$). At 21 d of age, the CN group had higher anti-ECP IgA than control ($P < 0.05$) while both the CN and CT group had higher anti-ECP IgA than control ($P < 0.05$). Splenic T-cells from chickens in the CN and CT group ex-vivo stimulated with 0.05 mg/ml ECP, had higher proliferation control ($P < 0.05$, $P < 0.01$ respectively). Splenic T-cells from chickens in the CN and CT groups ex-vivo stimulated with 0.1 mg/ml ECP had proliferation than control ($P < 0.05$). Pooled serum from 17 d of age CN and CT-vaccinated birds partially neutralized toxins in 50µg of ECP ($P < 0.05$). Pooled serum from 28 d of age CN-vaccinated birds also partially neutralized toxins in 50µg of ECP. The result from this study indicates the potential for chitosan loaded with *C. perfringens* extracellular proteins to be applied to NE challenge studies.

Key words

Chitosan; Nanoparticle; Poultry; *Clostridium perfringens*; Vaccination; Necrotic enteritis

4.1.Introduction

Necrotic enteritis is a severe *C. perfringens* induced disease and is caused by type A and type C strains (Dahiya et al., 2006). Clinical NE infections occur when *C. perfringens* (*C. perfringens*) proliferates in the small intestine and produces exotoxins that can damage the intestinal epithelium. In recent times, clinical and subclinical NE infections have become exacerbated by the withdrawal of antibiotic growth promoters and ionophore coccidiostats. Currently, there is only one commercially available *C. perfringens* toxoid vaccine for layer birds. This "killed" vaccine needs to be injected which can decrease production performances and the value of breast meat. Though some research to deliver the *C. perfringens* antigens orally have been attempted, all such research involves either attenuating a live strain (Thompson et al., 2006) or using a live vector such as *Salmonella* (Wilde et al., 2019). The disadvantages of applying live vaccines in poultry production for NE control has been documented (Mot et al., 2014) Therefore, developing a potent killed or subunit *C. perfringens* vaccine that can be applied orally would have potential application for the poultry industry.

The major *C. perfringens* virulence factors include genes for the production of 17 different toxins (Bokori-Brown et al., 2011). Most of the current vaccines against NE targets coccidia parasites because NE has been frequently associated with coccidiosis. Earlier reports identified that vaccines designed against *C. perfringens* alpha-toxin, glyceraldehyde-3-phosphate dehydrogenase, pyruvate: ferredoxin oxidoreductase, fructose 1,6-biphosphate aldolase, and a hypothetical extracellular protein successfully induced immunity (Kulkarni et al., 2007). ECP of *C. perfringens* (ECP) are immunogenic (Sengupta et al., 2010) and have been explored as antigens for subunit vaccines (Kulkarni et al., 2007) either in inactivated (Saleh et al., 2011) or in native form (Lanckriet et al., 2010).

Nanoparticle delivery systems can act as vaccine adjuvants and stimulate specific and robust immunity (Carrillo-Conde et al., 2011). Nanoscale materials (<500 nm) can be used for vaccine delivery and can be modified to meet the desired application (Nel et al., 2006). Several biodegradable and biocompatible natural and synthetic polymers have been approved by the FDA for drug and vaccine delivery (Thomas et al., 2011)

Chitosan is a polycationic, N-deacetylated derivative of chitin and composed of more than 5000 glucosamine (N-acetylglucosamine) polymer units (Li et al., 2015). Chitosan is biodegradable, biocompatible, non-toxic (Zhao et al., 2014) and can be chemically modified to resist degradation by gut digestive enzyme, pH changes, temperature changes (Yata and Ghosh, 2011), and can be loaded with several microbial antigens (Shang et al., 2011). Chitosan nanoparticles protect their antigen cargo until delivery to target sites (Prabaharan and Mano 2005). Anti-*Campylobacter jejuni* DNA vaccine, encapsulated in chitosan and administered intranasally, induce serum anti-*C. jejuni* IgG and IgA and decreased *C.jejuni* colonization in the intestine and cecum (Huang et al., 2010). A Newcastle disease F protein encapsulated in chitosan and administered intranasally reduced viral load in several tissues and decreased viral shedding (Zhao et al., 2012).

The objective of this study is to develop an oral vaccine based on nanocarriers that will mitigate the effect of NE in the poultry industry. It is hypothesized that chitosan loaded with extracellular proteins and delivered orally to broilers will stimulate antigen-specific humoral and cell-mediated immune responses.

4.2. Materials and methods

4.2.1. Experimental animals: All animal protocols were approved by the Institutional Animal Care and Use Committee at the University of Georgia.

A total of 90 one-day-old chicks (Cobb 500; Cobb hatchery, Cleveland, GA) were randomly distributed to one of the three treatments. A cage was treated as a replicate. Each treatment was replicated in six cages (n = 6) of five chicks per cage. Birds were vaccinated by oral gavage on day 0 (day of hatch), day 3, day 7, and day 14 with either 0.5 ml PBS (CTRL) or 50 µg nanoparticles in 0.5 ml PBS (CN) or 50 µg CT nanoparticles in 0.5 ml PBS (CT). Birds were raised under standard management practices. Feed intake and body weight were measured on d 14 and d 28 of age.

4.2.2. Anti-ECP- and anti-flagellar-specific IgG and IgA antibodies in serum and bile of chickens orally gavaged with CN and CT nanoparticle vaccine: Serum and bile were collected from one bird per cage (total 6 birds/treatment) at d 3, 10, 17, 21, 28 of age. The amounts of anti-ECP- and anti-flagellar- specific IgG and IgA antibodies in serum and bile were determined by ELISA as previously described (Sengupta et al., 2010) with modifications. Native ECP was coated at 10 µg/ml (IgA) or 20 µg/ml (IgG) on ELISA plates (Nunc Maxisorp^{TM}, ThermoFisher Scientific, Waltham, MA). Bile was diluted to 1:200 and serum was diluted to 1:20 in PBS containing 2.5 %, non-fat dry milk and 0.1 % Tween 20 (VWR, Radnor, PA). Horseradish peroxidase (HRP) conjugated polyclonal goat anti-chicken IgG (Bethyl, Montgomery, TX) at 1:20,000 dilution or HRP-conjugated polyclonal goat anti-chicken IgA (SouthernBiotech, Birmingham, AL) at 1:10,000 was used as a secondary antibody. Optical density was measured as absorbance at 450 nm using a spectrophotometer (Biochek, Scarborough, ME) and values are reported as OD_{450}.

4.2.3. Ex vivo recall response of splenic mononuclear cells of chickens orally gavaged with CN and CT nanoparticle vaccine: Spleen samples were collected from one bird/cage on d 17 and d 21 of age. Single cell suspensions of mononuclear cells were collected as described earlier (Shanmugasundaram et al., 2015). Approximately $5x10^5$ mononuclear cells were plated in triplicates per sample in 100 µl of RPMI-1640 (Sigma Aldrich, St. Louis, MO) supplemented with 10 % fetal bovine serum and 1 % Penicillin and Streptomycin. Zero (PBS) or 0.05 mg/ml or 0.1 mg/ml or 0.25 mg/ml or 0.5 mg/ml native ECP was added to each well and incubated for 5 d at 37 ºC in the presence of 5 % CO_2. Lymphocyte proliferation was measured using [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) assay as described earlier (Wang *et al.,* 2012). Optical density was measured at 570 nm wavelength using a spectrophotometer as described above and values are reported as OD_{570}.

4.2.4. Effect of serum antibodies from chickens orally gavaged with CN and CT nanoparticle vaccine on ECP neutralization: The effect of serum antibodies on ECP neutralization was analyzed as described previously (Eby et al., 2017) with modifications. Serum was collected at d 17, d 21 and d 28 of age from four birds/treatment and diluted to 1:20 in RPMI-1640 supplemented with 10 % fetal bovine serum and 1 % Penicillin and Streptomycin. Splenocytes were collected from naïve birds as described earlier (Dhakal *et al*., 2018) and seeded in 96-well plates for 6 hours. Non-adhered cells were discarded. Adhered cells were seeded in 96 well plates at approximately 10^6 splenocytes/well in 100 µl RPMI containing 10 % fetal bovine serum and 1 % penicillin and streptomycin, in triplicates per sample. The cells were incubated at 41°C and 5 % CO_2 for 6 hours. A neutralization plate was set up in which 75 µl of 1 mg/ml ECP was incubated with either 75 µl of 1:20 dilution of serum, or RPMI only as previously described (toxin positive control). A set of wells with 150 µl of RPMI only was maintained as toxin

negative control. Incubation was for 1hr at 37°C, with rotating agitation to neutralize toxins. 100µl of the neutralizing solution was added to splenocytes and further incubated for 4 d at 41°C and 5 % CO_2. Splenocyte proliferation was measured using the MTT assay as described above. Optical density was measured at OD_{570} using a spectrophotometer as described above. The toxin neutralization was determined as follows:

toxin neutralization (%) = [OD_{570nm} *Absorbance (treatment – toxin positive control)* / OD_{570nm} *Absorbance (negative control – toxin positive control*)] × 100.

4.2.5. SDS-PAGE and western immunoblotting

Approximately 67 µg native ECP and 2 µg flagellar proteins were separated on a 10 % SDS-PAGE gel at 100 V for 1 hour 30 minutes and stained with Coomassie R250 stain as described earlier (Zhao *et al*., 2012). The separated proteins were transferred to a PVDF membrane which was incubated with 1:50 dilution of pooled sera from all 6 birds per treatment, at d 21 of age. HRP-conjugated polyclonal goat anti-chicken IgA (SouthernBiotech, Birmingham, AL) at 1:10,000 was used as a secondary antibody. The bands were visualized using an imager (Biorad, Hercules, CA) and analyzed using Image Lab ™ software (v. 6.0.1).

4.2.6. Statistical analysis: All statistical analyses were carried out with statistical software (SAS, v. 9.4, SAS Institute Inc., Cary, NC, USA). Analysis of BWG, FCR, toxin neutralization, antibody and T lymphocyte responses was carried out using a one-way analysis of variance (ANOVA) and pre-planned orthogonal test to contrast CTRL vs CN, CTRL vs CT and CN vs CT. Analysis of mortality was carried using Kruskal-Wallis chi-square test, while chisquare rest was used to analyze lesion scores. Significance was determined at $P < 0.05$ and/or at $P < 0.01$.

4.3. Results

4.3.1. Production performance of chickens orally gavaged with CN and CT nanoparticles

CN and CT nanoparticle administration did not significantly affect the body weight gain and feed conversion ratio at d 0 to 7, d 0 to 14, d 0 to 21 and d 0 to 28 (Table 4.1). CN and CT nanoparticle administration did not significantly affect mortality.

4.3.2. Anti-ECP- and anti-flagellar- specific IgG and IgA antibodies in serum and bile of chickens orally gavaged with CN and CT nanoparticle vaccine: At 17 d of age, chickens in the CT group had statistically significant higher anti-ECP IgA than that in the control group ($p < 0.05$, Figure 4.1A). At 21 d of age, chickens in the CN group had statistically significant higher anti-ECP IgA than that in the control group ($p < 0.05$), while chickens in the CT group had comparable anti-ECP IgA to that in the control (Figure 4.1A). At 10 and 28 d of age, chickens in the CN and CT group had comparable anti-ECP IgA to that in the control group (Figure 4.1A).

At 21 d of age, chickens in the CN group had statistically significant higher anti-ECP IgG than that in the control group ($p < 0.05$, Figure 4.1B) and statistically significant higher anti-ECP IgG than that in the CT group ($p < 0.05$, Figure 4.1B). At 10, 17, and 28 d of age, chickens in CN and CT group had comparable anti-ECP IgG to that in the control group (Figure 4.1B).

At 10 d of age, chickens in the CN group had statistically significant higher anti-flagellar IgA than that in the control group ($p < 0.05$, Figure 4.1C). At 17, 21, and 28 d of age, chickens in CN, CT and control groups had comparable anti-flagellar IgA (Figure 4.1C).

At 17 d of age, chickens in the CN group had statistically significant higher anti-flagellar IgG than that in the control group ($p < 0.05$, Figure 4.1D). At 10, 21, and 28 d of age, chickens in CN, CT and control groups had comparable anti-flagellar IgG (Figure 4.1D).

4.3.3. Ex vivo recall response of splenic mononuclear cells of chickens orally gavaged with CN and CT nanoparticle vaccine: At 0 mg/ml ECP stimulation concentration, splenic mononuclear cells obtained from chickens in the control group had statistically significantly higher proliferation than mononuclear cells from the CN and CT group ($p < 0.05$, Figure 4.2). At 0.05 mg/ml and 0.1 mg/ml ECP stimulation concentrations, splenic mononuclear cells from the CN and CT groups had statistically significantly higher proliferation than that in the control group ($p < 0.05$). At 0.25 mg/ml and 0.5 mg/ml ECP stimulation concentrations, mononuclear cells from the CN group had statistically significant higher proliferation than that in the control group ($p < 0.05$) while mononuclear cells from the CT group had comparable proliferation to the control group.

4.3.4. Effect of serum antibodies from chickens orally gavaged with CN and CT nanoparticle vaccine on ECP neutralization: At d 17 of age, splenocytes incubated with ECP and serum from CN-vaccinated and CT-vaccinated birds had statistically significant higher proliferation than splenocytes incubated with only ECP ($p < 0.05$, Figure 4.3). Ad d 17 of age, splenocytes incubated with ECP and serum from control birds had comparable proliferation with splenocytes incubated with only ECP ($p < 0.05$).

At d 28 of age, splenocytes incubated with serum from CN-vaccinated birds had statistically significant higher proliferation than splenocytes incubated with only ECP ($p < 0.05$). At d 28 of age, splenocytes incubated with ECP and serum from CT-vaccinated birds or ECP and serum from control birds had comparable proliferation.

4.3.5. SDS-PAGE and Western Immunoblot:mAn SDS-PAGE identified several proteins in the ECP of *C. perfringens* and two proteins in flagella (Figure 4.4A).

Serum from the control and CT group at d 21 of age identified only a 50 kDa immunoreactive protein in the ECP of *C. perfringens* (Figure 4.4B and 4.4D).

Serum from the CN group at d 21 of age identified 125 kDa, 71 kDa and 50 kDa immunoreactive proteins in the ECP fraction of *C. perfringens* (Figure 4.4C).

4.4. Discussion

This trial identified the immunogenicity of chitosan nanoparticles loaded with *C. perfringens* extracellular proteins and *S.* Typhimurium flagella proteins. The ECP of this strain had a prominent band at 56 kDa for flagella and several bands including 121 kDa, 71 kDa and 50 kDa for ECP. Earlier reports identified a 53 kDa flagellar protein (Sophie, et al., 2006) and hence the antigen used in the study is very likely to be flagellar protein

In vivo, CN and CT nanoparticle vaccination did not significantly affect BWG, FCR, and mortality. Earlier studies reported that chickens gavaged with chitosan nanoparticles did not have any effect on mortality (Zhao et al., 2012; Dhakal et al., 2018).

A 50 µg dose of chitosan loaded with *Salmonella* flagella and OMP was immunogenic when administered orally in laying hens (unpublished data). The oral vaccination of chickens with the CN and CT nanoparticles induced anti-ECP or anti-flagella IgG or IgA. Flagella antigens were included for their adjuvant properties in this study (Gupta, et al., 2014). There was a significant induction of IgG and IgA in the CN vaccinated group at day 21 of age, with birds in the CN group having higher serum IgG than that in the CT group. The 21-day time point is critical because maternal antibodies against *C. perfringens* wanes at 2 to 3 weeks post-hatch leading to Clostridial enteritis in chickens (Shojadoost, et al., 2012). Also, while the CN

nanoparticle vaccine induced humoral response in bile and serum against ECP and flagella, the CT nanoparticle induced a humoral response only in bile against ECP. The difference in humoral response between the CN and CT group may be related to the inactivation of the antigen in CT nanoparticles. Although, there is currently no research that has explored the effect of attenuation on the immunogenicity of *C. perfringens* proteins, Thompson et al. (2006) showed that immunizing ability is associated with virulence. Jang et al., (2012) demonstrated that subcutaneous vaccination of chickens with *Clostridium* proteins induced antigen specific IgG in serum at 20 days post-hatch and decreased intestinal lesions (Jang et al., 2012). Oral vaccination of chickens with live *C. perfringens* induced serum and mucosal antibody responses at day 22 post-hatch and decreased intestinal lesions (Mishra and Smyth, 2017). This study also identified that antibodies in the serum of chickens vaccinated with CN and CT nanoparticles partially neutralized the cytotoxicity of ECP on splenocytes. Kulkarni et al. (2006) identified that antibodies in serum and intestinal washes of birds vaccinated with purified recombinant alpha-toxin of *C. perfringens* reduces the lecithinase activity of alpha-toxin on 5 % egg yolk agar.

Serum from vaccinated birds were analyzed to identify if the antibodies in the serum of vaccinated birds immunoreacted with the antigen that was used to synthesize the nanoparticle vaccine. Assays carried out twice indicated that serum from birds vaccinated with the CN nanoparticle vaccine immunoreacted weakly with two unique proteins in ECP compared to serum from control birds and birds vaccinated with the CT nanoparticle. The difference in the number and intensity of bands between the CN and CT group may be related to differences in their immunogenicity. Similar to this study, Kulkarni et al. (2006) demonstrated that sera from chickens immunized with *C perfringens* recognize antigens in the vaccine.

The ECP used for this study was cytotoxic to adherent splenocytes but not Leghorn Male

Hepatoma cells (data not shown). Toxin neutralization by serum antibodies in macrophage-like cells cultures has been demonstrated previously in mice (Eby et al., 2017). Serum collected from CN vaccinated birds on d 17 and d 21 of age neutralized toxins in ECP, while serum collected from CT vaccinated birds only on d 17 of age neutralized toxins in ECP.

Splenic mononuclear cells from chickens that were vaccinated with CN and CT nanoparticles had a higher recall response than controls. In a similar study, Zhao et al. (2012) demonstrated that splenic lymphocytes of chickens vaccinated with chitosan nanoparticles loaded with antigen leads to a higher recall response. Fasina and Lillehoj (2019) showed that T-cells are involved in the immune responses of chickens to enteritis caused by *Clostridium*. A higher recall response may be important for mounting a faster immune response by T-cells of vaccinated birds when exposed to toxins during an infection. It is not immediately clear why splenic mononuclear cells from the CT-vaccinated birds had comparable proliferation to control birds, unlike CN-vaccinated birds.

In conclusion, immunogenicity does not always correlate with protection. However, the induction of measurable cellular and humoral responses in broilers by the synthesized nanoparticles when administered by oral gavage is promising. The ability of CN nanoparticles to induce a better humoral response than CT nanoprticles, the ability of CN nanoparticles to induce a recall response in splenic mononuclear cells when stimulated with higher concentrations of ECP, and the absence of a formaldeyde inactivation step in the preparation of CN nanoparticles makes CN nanoparticles a promising candidate for further studies. Research is therefore ongoing to assess the protective efficacy in broilers and futher characterize the immunogenicity of the CN nanoparticle vaccines during an experimental challenge of Clostridial enteritis.

Further research will refine the antigen selection, and also explore the nature of the immune response and/or protection by the different antigens in the crude mixture.

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Table 4.1. The performance of broilers birds vaccinated with CN and CT vaccine

Treatment	0 to 7		0 to 14		0 to 21		0 to 28		% mortality
	BWG (g)	FCR	BWG (g)	FCR	BWG (g)	FCR	BWG (g)	FCR	
Control	8.8	0.6	340	1.80	0.6	1.3	1410	1.9	2/30
CN	7.9	0.5	350	1.55	0.7	1.2	1440	1.7	0/30
CT	7.8	0.5	340	1.71	0.6	1.3	1485	1.6	0/30
SEM	0.01	0.04	13	0.14	0.06	0.05	76	0.2	0.02
P-value	0.4	0.4	0.80	0.45	0.2	0.9	0.82	0.44	0.13

Chitosan-native (CN) and chitosan-toxoid (CT) nanoparticles were synthesized by entrapping *C. perfringens* extracellular proteins and flagellar proteins. Day-old chicks were orally gavaged with either 0.5 ml PBS (Control) or 50 µg CN or 50 µg CT nanoparticles in 0.5ml PBS on 0 (day of hatch), 3, 7, and 14 d of age. Bodyweight gain (BWG) and feed conversion ratio (FCR) were measured on d 14 and 28. Mean of 6 replicates (n = 6). SEM = standard error of mean.

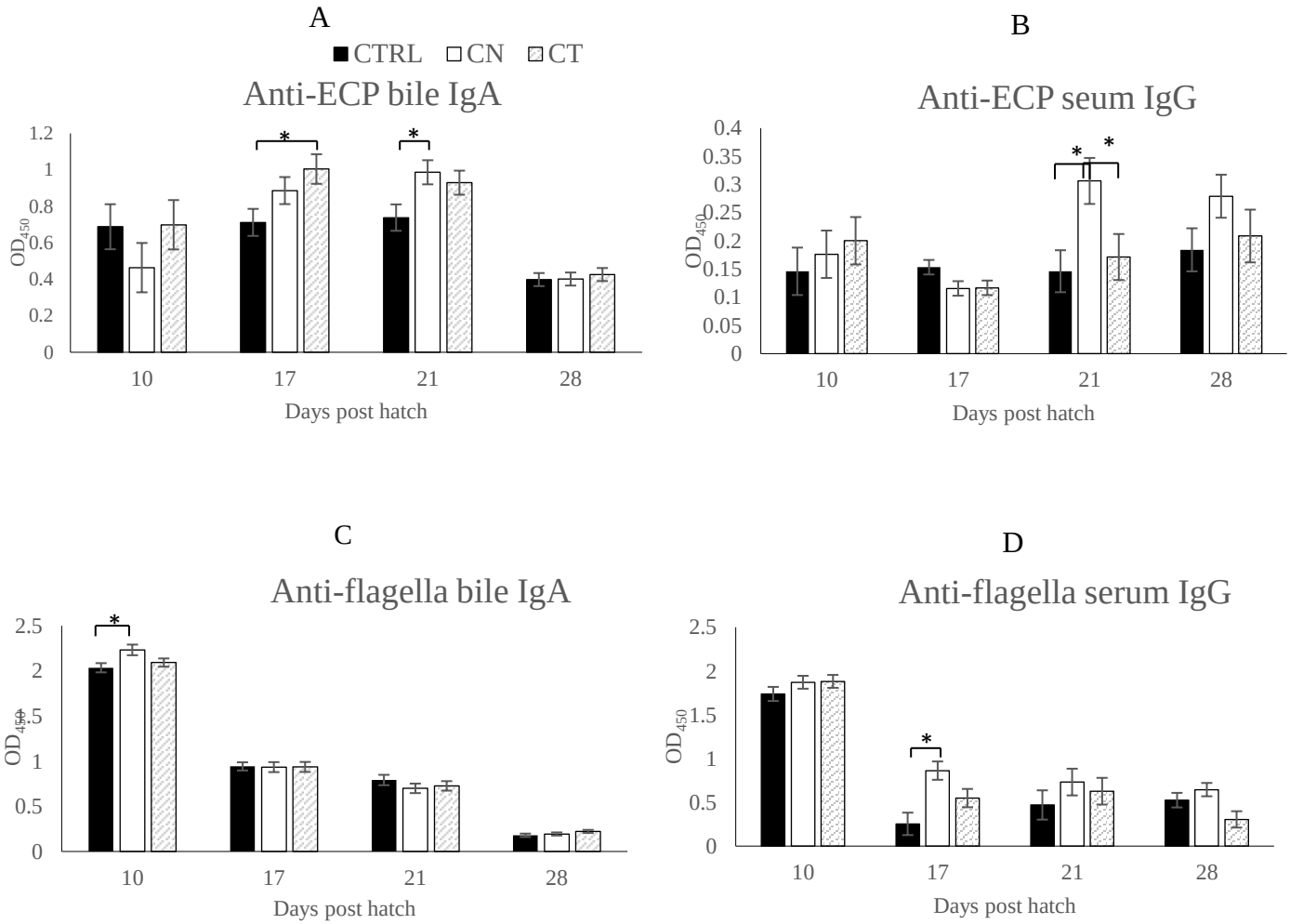

Fig. 4.1. Anti-ECP- and anti-flagellar- specific IgG and IgA antibodies in serum and bile. Chitosan-native (CN) and chitosan-toxoid (CT) nanoparticles were synthesized by entrapping *C. perfringens* extracellular proteins (ECP) and flagellar proteins. Day-old chicks were orally gavaged with either 0.5 ml PBS (Control) or 50 µg CN or 50 µg CT nanoparticles in 0.5 ml PBS on 0 (day of hatch), 3, 7, and 14 d of age. On d 10, 17, 21 and 28 of age, anti-ECP IgA (A), anti-ECP IgG (B), anti-flagellar IgA (C) and anti-flagellar IgG (D) amounts were analyzed by ELISA and values reported as Optical density (OD) values. Mean + SEM. n=6 replicates. $P < 0.05$.

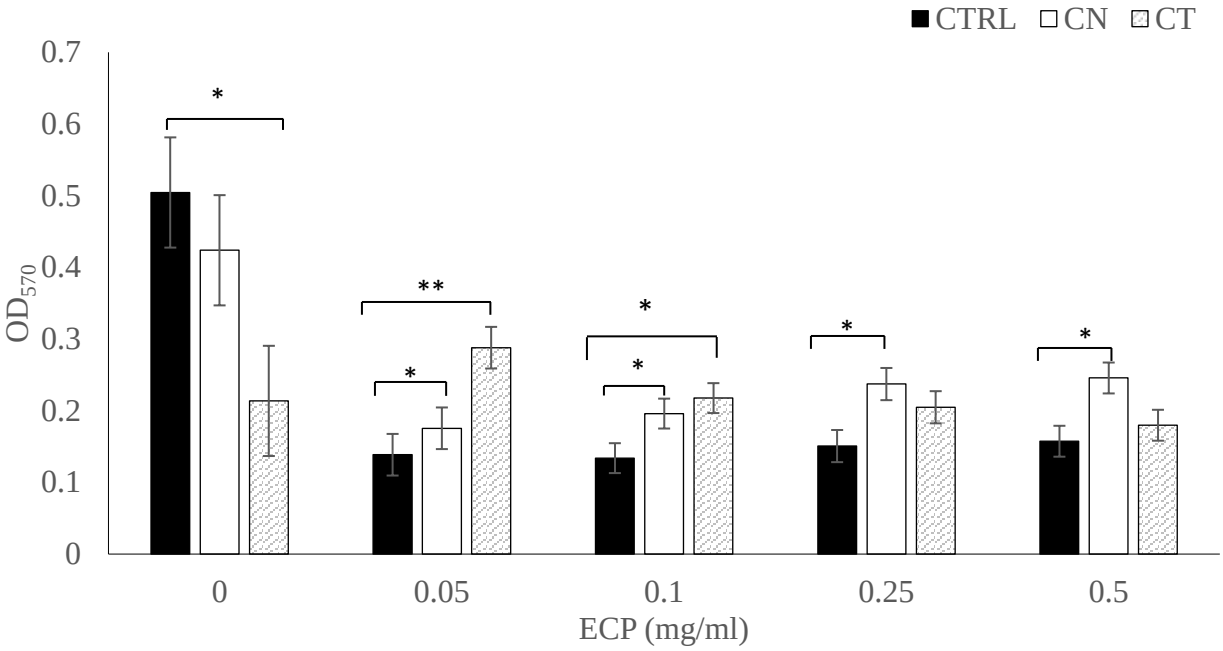

Fig. 4.2. Ex vivo recall response of splenic T-cells of chickens orally gavaged with CN and CT nanoparticle vaccine. Chitosan-native (CN) and chitosan-toxoid (CT) nanoparticles were synthesized by entrapping *C. perfringens* extracellular proteins (ECP) and flagellar proteins. Day-old chicks were orally gavaged with either 0.5 ml PBS (Control) or 50 µg CN or 50 µg CT nanoparticles in 0.5 ml PBS on 0 (day of hatch), 3, 7, and 14 d of age. Splenic mononuclear cells were collected on d17. Splenic mononuclear cells were stimulated with 0, 0.05, 0.1, 0.25 or 0.5 mg/ml ECP for 5 d. Lymphocyte proliferation was measured using MTT assay and values reported as Optical Density (OD) values. Mean + SEM. n = 6 replicates. '*' = $P < 0.05$; '**' = $P < 0.01$

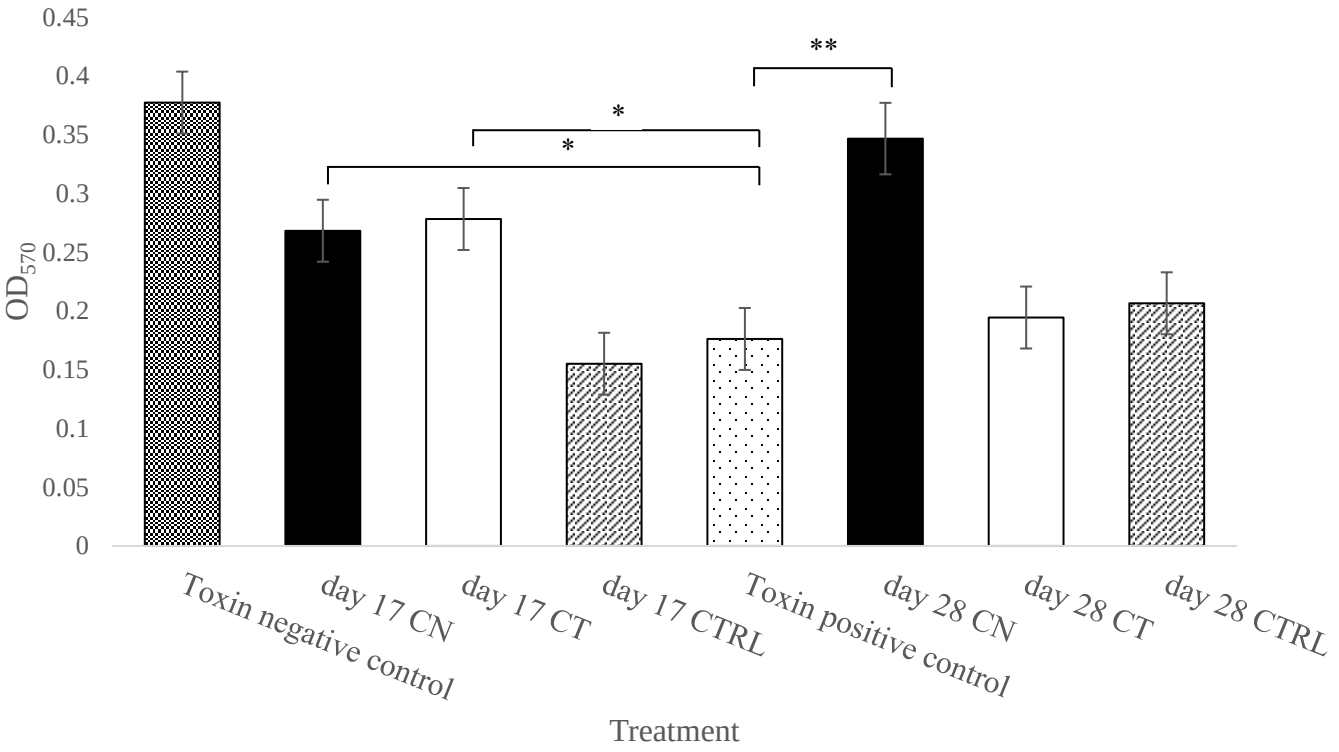

Fig. 4.3. Effect of serum antibodies from chickens orally gavaged with CN and CT nanoparticle vaccine on ECP neutralization. Chitosan-native (CN) and chitosan-toxoid (CT) nanoparticles were synthesized by entrapping *C. perfringens* extracellular proteins (ECP) and flagellar proteins. Day old chicks were orally gavaged with either 0.5 ml PBS (Control) or 50 µg CN or 50 µg CT nanoparticles in 0.5 ml PBS on d 0 (day of hatch), 3, 7, and 14 of age. Serum was collected at d 17, d21 and d 28 of age and incubated with 1 mg/ml native ECP to neutralize ECP. Splenocytes from normal birds were incubated with the above neutralizing solution for 4 days. Splenocyte proliferation was measured using MTT assay and values reported as Optical Density (OD) values. Mean + SEM. n = 6 replicates. '*' = $P < 0.05$; '**' = $P < 0.01$

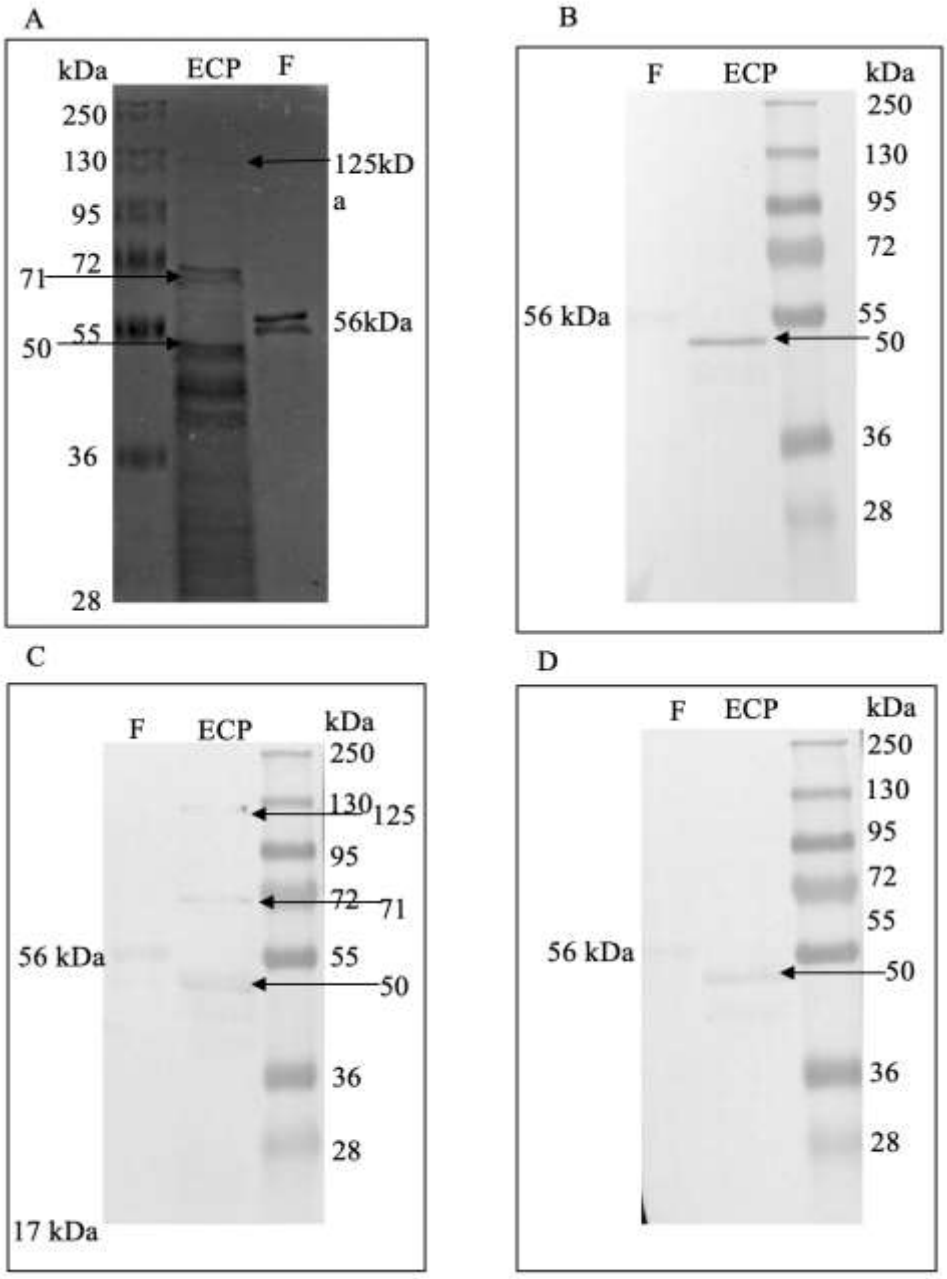

Fig. 4.4. SDS-PAGE and Western immunoblot. Extracellular proteins (ECP) were extracted from *C. perfringens* and flagellar proteins were extracted from *Salmonella enterica* serotype Enteritidis and separated on a gel and stained with Coomassie Blue (Panel A). Chitosan-native

(CN) and chitosan-toxoid (CT) nanoparticles were synthesized by entrapping ECP and flagellar proteins. Day-old chicks were orally gavaged with either 0.5 ml PBS (Control) or 50 µg CN or 50 µg CT nanoparticles in 0.5 ml PBS on d 0 (day of hatch), 3, 7, and 14 of age. At d 21 of age, serum was collected from birds in control (B), CN (C) and CT (D) and used as a primary antibody in the western immunoblot of ECP and flagellar proteins.

CHAPTER 5

Immunogenicity and protective efficacy of a chitosan nanoparticle vaccine loaded with native extracellular proteins of *C. perfringens* and *Salmonella* flagella in a necrotic enteritis challenge model

[1]G. Akerele, N. Ramadan, S. Renu, G. J. Renukaradhyab, R. Shanmugasundarama, R.K Selvaraj. To be submitted to *PLOS ONE*.

Abstract

There are currently no commercial vaccines against necrotic enteritis (NE) in broilers. Chitosan nanoparticles were formulated with extracellular proteins (ECP) of *C. perfringens* surface-tagged with *Salmonella* flagellar proteins. One-day-old male broiler chicks were completely randomized to treatments: Non-vaccinated non-challenged (NVNC), Vaccinated challenge (VC), and non-vaccinated challenge (NVC). At day of hatch (doh), d 7, and d14 post-hatch, VC birds were orally gavage with 50µg vaccine in 0.5ml PBS while NVC birds were gavage with 0.5ml PBS only. On d 14 post-hatch, birds in the VC and NVC groups were infected by oral gavage with 5,000 oocysts/bird of *E. maxima* followed by *C. perfringens* on d 19, d 20, and d 21 post-hatch. The average lesion score was higher in the VC group compared to the NVC group though not statistically significant. At d 28 post-hatch, mortality was higher in the VC group compared to the NVNC group though not statistically significant, but lower than that in the NVC group, also not statistically significant. At d 28 post-hatch, the VC group had comparable FCR to the NVNC group but had statistically significantly higher FCR than that in the NVC group ($p < 0.05$). At d 28 post-hatch, the VC group also had comparable FCR to the NVNC group but had statistically significantly higher feed intake than that in the NVC group ($p < 0.05$). All three treatment groups had comparable body weight gain at d 14, d 21 and d 28 post-hatch. At day 21 post-hatch the VC group had higher antigen specific recall response than NVNC and NVC birds. Vaccination also resulted in an increase in the CD4 / CD8 ratio of T-cells in the spleen on day 18 post-hatch. In conclusion, there were numerical but not statistically significant decrease in NE lesions and mortality in vaccinated and challenged broilers. Further studies are needed to improve the efficacy of the vaccine and understand the mechanism underlying protection in vaccinated birds.

5.1. Introduction

NE is a debilitating disease of *C. perfringens* that affects poultry gut health. Estimated losses is estimated to be at least 2 billion USD annually from clinical and subclinical infections. The resurgence in infection is due to the voluntary or regulatory withdrawal in the use of antibiotics as growth promoters (M'Sadeq et al., 2015). NE is a multifactorial disease with a complex etiology. However, the overgrowth of commensal or virulent *C. perfringens* strains is the primary cause of infection (Cooper and Songer, 2009). Current intervention strategies include the use of feed additives, improving biosecurity/management practices, anticoccidial drugs and vaccinations (Peek et al., 2011; Hofacre et al., 2019). There are currently no available commercial vaccines against NE in broilers. Most commercial vaccine products are anti coccidia vaccines because coccidiosis is one of the most important predisposing conditions for NE (Collier et al., 2008).

Two important considerations for any vaccine design strategy are antigen selection and route of delivery. Subunit vaccines are generally safer than live vaccines. Proteins subunits in the extracellular secretion of *C. perfringens* have been explored as antigens for candidate vaccines against NE with mixed results, with the most promising results involving parenteral routes of administration (Saleh et al., 2011; Lillehoj et al., 2017; Mot et al., 2017). Parenteral antigen delivery is neither economical nor practical for large commercial operations. However, unencapsulated, proteins are denatured or broken down by the low pH conditions and gut enzymes when administered by oral gavage, leading to inefficient delivery to inductive sites (Kim et al., 2017). As a result, research is constantly seeking ways to improve the immunogenicity and protective efficacy of orally administered antigens. There is a need to

develop vaccines based on *C. perfringens* extracellular proteins that are safe, effecatious, and can be administered by orally to help mitigate production losses to NE in the poultry industry.

The safety and immunogenicity of nanoparticles as encapsulating and antigen delivery vehicles have well documented (Nel et al., 2006; Carrillo-Conde et al., 2011; Thomas et al., 2006). As a result, chitosan has emerged as potential candidate for antigen delivery. Further studies are required to characterize its immunogenicity and efficacy against NE in broiler.

The objective of this study was therefore to determine the immunogenicity and protective efficacy of an oral chitosan nanoparticle vaccine against experimentally induced NE. It is hypothesized that chitosan loaded with extracellular proteins and delivered orally to broilers will stimulate antigen-specific humoral and cell-mediated immune responses and will reduce the severity of NE in broilers.

5.2. Materials and methods

5.2.1. ***Experimental animals***: All animal protocols were approved by the Institutional Animal Care and Use Committee at the University of Georgia.

A total of 144 one-day-old male broiler chicks obtained from a commercial hatchery were raised in Petersime battery cages for 28 days at the Southern Poultry Research facility (Athens, GA). Chicks were weighed by pen and then randomly distributed to one of three treatments in a completely randomized design: Non-vaccinated non-challenged (NVNC) or negative challenge control group consisted of chicks that were neither vaccinated nor challenged. Vaccinated challenge (VC) consisted of chicks that were vaccinated and challenged. Non-vaccinated challenge (NVC) or positive challenge control group consisted of chicks that were not vaccinated but were challenged. A cage was treated as a replicate. Each treatment was replicated in six cages (n = 6) of eight chicks per cage. Birds were raised under standard management practices. Feed intake, body weight and mortality were measured weekly.

5.2.2. ***Preparation and administration of vaccine, necrotic enteritis challenge and lesion scoring***: A field strain of *C. perfringens* (a gift from Dr. C. Hofacre, Southern Poultry Research Group) was obtained. A chitosan nanoparticle vaccine was synthesized with native extracellular proteins from the *C. perfringens* isolate according to methods detailed in a previous publication (Akerele et al., 2020). Synthesized nanoparticles were resuspended to 0.1 mg/ml loaded protein in PBS at pH 7.2. On d 0 (day of hatch), d 4 and d 14 of age, birds in the VC and NVC groups were orally gavage with 0.5 ml nanoparticle suspension (50 µg loaded protein) or 0.5 ml PBS respectively.

At day 14 post-hatch, birds in the VC and NVC groups were infected by oral gavage with 5,000 oocysts/bird of *E. maxima*. At days 19, 20 and 21 post-hatch birds in the VC and

NVC groups were also infected by oral gavage with 1.0×10^8 colony forming units /bird of the same field isolate of *C. perfringens* used to prepare the vaccine.

At d 21 post-hatch, three birds from each pen were lesion scored for NE. The scoring system was based on a score of 0-3 as follows: 0 for a normal intestine, 1 for the presence of a slight mucus covering and loss of tone, 2 for severe NE and 3 for severe NE with the presence of blood in the lumen.

5.2.3. ***Ex vivo recall response of splenic and cecal tonsil mononuclear cells of chickens***: Spleen and cecal tonsil samples were collected from one bird/cage on d 19, and two birds/cage on d 21 and d 28 of age. Single cell suspensions of mononuclear cells were collected as described earlier (Shanmugasundaram et al., 2015). Approximately $5x10^4$ mononuclear cells/well were plated in duplicates per sample in 100µl of RPMI-1640 culture media (Sigma Aldrich, St. Louis, MO) supplemented with 10 % fetal bovine serum and 1 % Penicillin and Streptomycin. 100µl of RPMI-1640 culture media only (negative control) or 25µg, or 50 µg or 100µg of native ECP or 0.1 µg of conclanavin A (con A) in 100 µl of RPMI-1640 culture media was added to each well and incubated for 5 d at 37 ºC in the presence of 5 % CO_2. Lymphocyte proliferation was measured using [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) assay as described earlier (Zhao *et al.,* 2012). Values are reported as Optical density measured at 570 nm using a spectrophotometer.

5.2.4. ***$CD4^+$ and $CD8^+$ cell percentages in cecal tonsils of chickens vaccinated with native-ECP PNP vaccine:*** Cecal tonsil and spleen samples were teased over a 0.4µm cell strainer (Sigma–Aldrich, St. Louis, MO) with RPMI to obtain a single-cell suspension. Single-cell suspensions from all 3 treatment groups at d 7 and d 14 post challenge (n = 6) were concentrated for lymphocytes by density centrifugation over Histopaque (1.077 g/mL; Sigma–Aldrich, St. Louis,

MO). For CD4+/CD8+ analysis, single-cell suspensions of the cecal tonsils (1×10^6 cells) were incubated with FITC-conjugated mouse anti-chicken CD4, PE-conjugated mouse anti-chicken CD8 (Southern Biotech, Birmingham, AL) at 1:200 dilution, and unlabeled mouse IgG at 1:200 dilution in a 96-well plate for 20 minutes. After incubation, cells were washed twice by centrifugation at 400 x g for 5 minutes using wash buffer (1× PBS, 2 mM EDTA, 1.5% FBS) to remove unbound primary antibodies. After washing, cells were analyzed using cytosoft software (Guava Easycyte, Millipore, Billerica, MA). The $CD4^+$ and $CD8^+$ and $CD4^+/CD8^+$ cell percentage was analyzed after gating cells based on forward-scatter and side-scatter plot for lymphocytes.

5.2.5. ***Nitric oxide production from adherent splenic and cecal tonsil mononuclear cells of chickens***: This was according to the method of Acevedo et al., (2020) with some modifications. Spleen and cecal tonsil samples collected and plated as previously stated were incubated for 18 to 24 hours at 37 ºC in the presence of 5 % CO_2 to allow cells attach. Unattached cells were removed with media and replaced with freshly prepared RPMI prepared as stated above. RPMI-1640 culture media (200 µl) only (negative control) or 25 µg, 50 µg or 100 µg of ECP or 0.1 µg of *Salmonella Enteritidis* lipopolysaccharide (LPS) in 200 µl of RPMI-1640 culture media was added to each well and incubated for 5 d at 37 ºC in the presence of 5 % CO_2. Lymphocyte proliferation was measured as stated above.

5.2.6. ***Anti-ECP- and anti-flagellar-specific IgG and IgA antibodies in serum and bile of chickens orally gavaged with*** **the formulated** ***nanoparticle vaccine***: Serum and bile were collected from one bird per cage (total 6 birds/treatment) at d 18, d 21 and d 28 post-hatch. The amounts of anti-ECP- and anti-flagellar- specific IgG and IgA antibodies in serum and bile were determined by ELISA as previously described (Sengupta et al., 2010) with modifications. Native

ECP was coated at 10 µg/ml (IgA) or 20µg/ml (IgG) on ELISA plates (Nunc Maxisorp™, ThermoFisher Scientific, Waltham, MA). Bile was diluted to 1:200 and serum was diluted to 1:20 in PBS containing 2.5 %, non-fat dry milk and 0.1% Tween 20 (VWR, Radnor, PA). Horseradish peroxidase (HRP) conjugated polyclonal goat anti-chicken IgG (Bethyl, Montgomery, TX) at 1:20,000 dilution or HRP-conjugated polyclonal goat anti-chicken IgA (SouthernBiotech, Birmingham, AL) at 1:10,000 was used as a secondary antibody. Optical density was measured as absorbance at 450nm using a spectrophotometer (Biochek, Scarborough, ME).

5.2.7. ***Effect of serum antibodies from chickens on ECP neutralization***: Liver male hepatoma cells (LMH) were seeded in 96-well plates at 40,000 cells per well for 30 minutes in 100µl of Dulbecco's modified eagle medium (DMEM). The DMEM was supplemented with 10% Fetal bovine serum and 1% Penicillin and Streptomycin. Antigen-antibody reaction was prepared in duplicates by incubating 15µl of *C. perfringens* supernatant containing 825µg protein with 15µl serum from each replicate for 60 minutes. The antigen-antibody mixture was added to the growing LMH culture. Cells were incubated in 5 % CO_2 at 37°C for 8 hours. LMH cytotoxicity was measured according to the protocol outlined by OPS diagnostics (OPS diagnostics Lebanon, NJ) to measure the release of lactate dehydrogenase (LDH) from dying cells. Briefly, 50µl of cell-free supernatant of LMH culture was added to 50µl of 50mM Lithium Lactate and 50µl of a mixture of 100µl Phenazine methosulfate or PMS (9mg/ml), 100µl iodonitrotetrazolium 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium) or INT (33mg/ml) and 2.3ml Nicotinamide adenine dinucleotide or NAD (3.7mg/ml) Solution. Color development was allowed for 5 minutes. Optical density measurement was at 490nm with a spectrophotometer.

5.2.8. Statistical Analysis: All statistical analyses were carried out with statistical software (SAS, v. 9.4, SAS Institute Inc., Cary, NC, USA). Analysis of body weight gain (BWG), feed conversion ratio (FCR), feed intake (FI), toxin neutralization, nitritc oxide production, antibody and T lymphocyte responses was carried out using a one-way analysis of variance (ANOVA) and pre-planned orthogonal contrasts to compare NVNC vs VC, and VC vs NVNC. Analysis of mortality and gut lesions was carried using Kruskal-Wallis chi-square test. Significance was determined at $P < 0.05$ and/or at $P < 0.01$.

5.3 Results

5.3.1. ***Protective efficacy of vaccination of broiler birds against mortality and jejunum lesions***: One-week post *Eimeria* infection, mortality was similar for the VC and NVNC, but higher in the NVC group approaching significance (Table 5.1).

At d 28 post-hatch, mortality was higher in the VC group compared to the NVNC group though not statistically significant, but lower than that in the NVC group, also not statistically significant.

The average lesion score was higher in the VC group compared to the NVC group though not statistically significant (Table 5.1), but lower than that in the NVC group, also not statistically significant.

5.3.2. ***Effect of vaccination and challenge on feed intake, body weight gain and feed conversion ratio of broiler birds***: Analysis of performance was carried out at three time intervals: d 0 to d 14, d 14 to d 21 and d 14 to d 28 post-hatch. From d 0 to d 14 post-hatch, all three treatment groups had comparable feed intake. However, from d 14 to d 21 post-hatch, the NVNC group had numerically higher feed intake than the VC group which approached

significance (P = 0.08, Figure 5.1). From d 14 to d 28 post-hatch, the VC group had statistically significantly higher FI than that in the NVNC group but had comparable FI to the NVC group ($p < 0.05$, Figure 5.1).

All three treatment groups had comparable body weight gain at the three time points (Figure 5.2).

From d 0 to d 14 post-hatch, all three treatment groups had comparable feed conversion ratios (Figure 5.3). From d 14 to d 21 post-hatch, the VC group had comparable FCR to the NVNC group but had statistically significantly lower FCR than that in the NVC group ($p < 0.01$, Figure 5.3). From d 14 to d 28 post-hatch, the VC group had comparable FCR to the NVNC group but had statistically significantly lower FCR than that in the NVC group ($p < 0.05$, Figure 5.3).

5.3.3. ***Effect of vaccination and challenge on the proliferation of spleen PBMCs of broilers birds***: At day 18 post-hatch, at 0µg, 50µg ECP stimulation and 0.1µg con A stimulation, splenic mononuclear cells obtained from chickens in all 3 treatment groups had comparable proliferation (Figure 5.4A). At day 18 post-hatch, at 25µg ECP stimulation concentration, mononuclear cells from the VC group had comparable proliferation to that in the NVNC group but had lower proliferation which approached significance ($p = 0.08$, Figure 5.4A) than that in the NVC birds. At 100µg ECP stimulation, splenic mononuclear cells from the VC group had comparable proliferation to that in the NVNC group but statistically significantly lower proliferation than that in the NVC group ($p < 0.01$, Figure 5.4A).

At day 21 post-hatch, at 0µg, 50µg, 100µg, ECP stimulation and 0.1µg con A stimulation, splenic mononuclear cells obtained from chickens in all 3 treatment groups had comparable proliferation (Figure 5.4B). At day 21 post-hatch, at 25µg ECP stimulation, splenic

mononuclear cells obtained from chickens in the VC group had statistically significantly higher proliferation than that in the NVNC and NVC group ($p < 0.05$, Figure 5.4B).

At day 28 post-hatch, at 0µg, 25µg, and 50µg, ECP stimulation, splenic mononuclear cells obtained from chickens in all 3 treatment groups had comparable proliferation (Figure 5.4C). At day 28 post-hatch, at 100µg stimulation, splenic mononuclear cells obtained from chickens in the VC group had statistically significantly lower proliferation than that in the NVNC group ($p < 0.05$, Figure 5.4C) but had comparable proliferation to the NVC group. At day 28 post-hatch, at 0.1µg ConA stimulation, splenic mononuclear cells obtained from chickens in the VC group had comparable proliferation to that in the NVNC group but had statistically significantly lower proliferation than that in the NVC group ($p < 0.05$, Figure 5.4C).

5.3.4. ***Effect of vaccination and challenge on the CD4/CD8 ratio of broiler birds*****:** At d 18 post-hatch in the spleen, the VC group had numerically higher CD4/CD8 ratio which approached significance ($p = 0.05$, Figure 5.5) than that in the NVNC group but had comparable CD4/CD8 ratio to the NVC group.

At d 21 post-hatch in the spleen and cecal tonsils, all three treatment groups had comparable CD4/CD8 ratios (Figure 5.5).

At d 28 post-hatch in the spleen, all three treatment groups had comparable CD4/CD8 ratios (Figure 5.5). On d 28 post-hatch in the cecal tonsils, the VC group had comparable CD4/CD8 ratio to that in the NVNC group but had statistically significantly lower CD4/CD8 ratio to that in the NVC group ($p < 0.01$, Figure 5.5).

5.3.5. ***Effect of vaccination and challenge on nitric oxide production from cecal tonsil mononuclear cells of broiler birds***: At day 18 post-hatch, at 0µg, 25µg, 50µg, and 100µg ECP stimulation, cecal tonsils mononuclear cells obtained from chickens in all 3 treatment groups had

comparable proliferation (Figure 5.6A). At day 18 post-hatch, at 0.1µg LPS stimulation concentration, splenic mononuclear cells obtained from chickens in the VC group had comparable nitric oxide production to that in the NVNC group but had statistically significantly lower nitric oxide production than that in the NVC group ($p < 0.05$, Figure 5.6A).

At day 28 post-hatch, at 0µg, 100µg, ECP stimulation concentrations, as well as at 0.1µg LPS stimulation concentration, cecal tonsils mononuclear cells obtained from chickens in all 3 treatment groups had comparable proliferation (Figure 5.6B). At day 28 post-hatch, at 25µg ECP stimulation, splenic mononuclear cells obtained from chickens in the VC group had statistically significantly lower nitric oxide production than that in the NVNC group but had comparable nitric oxide production to that in the NVC group ($p < 0.05$, Figure 5.6B).

At day 28 post-hatch, at 50µg ECP stimulation, splenic mononuclear cells obtained from chickens in the VC group had comparable nitric oxide production to that in the NVNC group but had statistically significantly lower nitric oxide production than that in the NVC group ($p < 0.05$, Figure 5.6B).

5.3.6. ***Effect of vaccination and challenge on nitric oxide production from splenic mononuclear cells of broiler birds at d 18 and d 28 post-hatch***: There was no statistically significant difference in nitric oxide production between all 3 treatments at d 18 post-hatch (Figure 5.7A). At 0µg, 50µg and 100µg antigen stimulation as well as at 0.1µg LPS stimulation, splenic mononuclear cells obtained from chickens in all 3 treatment groups had comparable proliferation (Figure 5.7B). At 25µg antigen stimulation concentration, splenic mononuclear cells obtained from chickens in the VC group had comparable nitric oxide production to that in the NVNC group but had statistically significantly lower nitric oxide production than that in the NVC group ($p < 0.05$, Figure 5.7B).

5.3.7. ***Effect of vaccination anti-ECP-specific serum IgG and bile IgA***: All treatments had comparable anti-ECP antibodies in serum and bile at d 18, 21, and 28 pos hatch (Figure 5.8).

5.3.8. ***Effect of vaccination and challenge on ECP neutralization***: On d 18 post-hatch, LMH cells incubated with ECP and serum from all three treatment groups had comparable LDH release (Figure 5.9A). On d 18 post-hatch, LMH cells incubated with ECP and bile from the VC group had comparable LDH release to that in the NVNC group but had higher LDH release which approached significance ($p = 0.07$, Figure 5.9B) than that in the NVC group.

On d 21 post-hatch, LMH cells incubated with ECP and serum from the VC group had lower LDH release which approached significance ($p = 0.08$, Figure 5.9A) than that in the NVNC group but had statistically significantly higher LDH release than that in the NVC group ($p < 0.05$, Figure 5.9A). On d 21 post-hatch, LMH cells incubated with ECP and bile from all three treatment groups had comparable LDH release (Figure 5.9B).

On d 28 post-hatch, LMH cells incubated with ECP and serum from the VC group had comparable LDH release to that in the NVNC group but had statistically significantly higher LDH release than that in the NVC group ($p < 0.05$, Figure 5.9A). On d 28 post-hatch, LMH cells incubated with ECP and bile from the VC group had comparable LDH release to that in the NVNC group but had statistically significantly lower LDH release than that in the NVC group ($p < 0.01$, Figure 5.9B).

5.4. Discussion

To the best of our knowledge, this study is the first to identify the immunogenicity and protective efficacy of nanoparticles loaded with *C. perfringens* extracellular proteins and *Salmonella* flagella proteins. The study was designed on the back of promising results from a

previous study carried out by our lab that demonstrated the safety and immunogenicity of chitosan nanoparticle vaccine that was administered by oral gavage in broilers (Akerele et al., 2020). The NE challenge model employed was successful in reproducing clinical NE as shown by 33% mortality and 0.72 average NE gut lesions in the unvaccinated challenged group. Over the 2-week period post challenge, the vaccinated and challenged group had numerically higher mortality and intestinal lesions than negative controls but had numerically lower mortality and intestinal lesions than non-vaccinated challenged birds. Vaccination also improved the feed conversion ratio and feed intake of challenged birds. Increased mortality, feed intake, lower feed conversion, body weight gain and the presence of intestinal lesions have been associated with clinical NE in broilers (Latorre et al., 2018). Vaccination did not significantly improve body weight gain of challenged bids in the intervals examined. The improvement in the performance suggests that the vaccine is partially protective in broilers. Hu et al. (2013) demonstrated that nanoparticle detained toxins are immunogenic and protective in mice. In addition, Zhao et al. (2012) demonstrated that chickens that were gavaged with chitosan nanoparticles vaccines against influenza were better protected.

It has been shown that a cell-mediated immune response is more important for immunity to Eimeria infections (Rose et al., 1996) and chitosan has shown potential as an adjuvant to drive cell-mediated immunity (Mori et al., 2012). Splenic mononuclear cells from non-vaccinated, challenged birds had higher antigen specific recall response than splenocytes from vaccinated challenged birds and negative controls at day 18 post-hatch. Antigen specific proliferation was found in vaccinated challenged birds compared to controls at the peak of infection, on day 21 post-hatch. Zhao et al. (2012) also found increased lymphoproliferation of splenocytes of chickens vaccinated with chitosan nanoparticles compared to non-vaccinated controls. ConA is a

T cell mitogen from plants that can be used to measure T cell proliferation in response to vaccination (Linghua et al., 2007). However, there was no increased T cell proliferation in vaccinated challenged birds suggesting either a different dose of ConA may be appropriate for exvivo stimulation or vaccination was inducing a diminished T cell response. Increased T cell proliferation as induced by ConA was found in the non-vaccinated challenged group on day 28 post-hatch. Because conA is non-specific, the phenotype of proliferating T cells may be dominated by effector or regulatory cells (Yuan et al., 2012). *Eimeria* has previously been demonstrated to induce Il-10 which can increase regulatory T cells (Rothwell et al., 2004). Overall, there appears to be an antigen concentration-dependent effect on splenocyte proliferation. The decrease in proliferation of splenic mononuclear cells at 100µg ECP concentration at d 28 post-hatch may be due to the cytotoxicity of the ECP to a population of cells present at this time point (Akerele et al., 2020).

There was a numerical increase in the CD4/CD8 ratio of T-cells in the spleen of vaccinated and non-vaccinated, challenged birds on day 18 post-hatch. The increase in the CD4/CD8 ratio at day 18 may be indicative of a Th2 response that generally results in antibody production (Alam et al., 2016). At day 28 post-hatch, an increase in the CD4/CD8 T cell ratio was found only in the cecal tonsils, in the non-vaccinated challenged group compared to the vaccinated challenge group. The implication for this is not clear as the mechanisms underlying the temporal and spatial CD4 and CD8 response during NE is not yet fully understood. However, Hong et al. (2006) demonstrated the persistence of relatively higher numbers of CD4 T-cells during a secondary or challenge infection with Eimeria.

Ex vivo stimulation of cecal tonsil mononuclear cells for nitric oxide production was carried out to assess innate priming. Vaccination resulted in an antigen-dependent increase or

decrease in nitric oxide production from ex-vivo stimulated cecal tonsil cells. Non-vaccinated, challenged birds up-regulated NO production in response to LPS stimulation on day 18 post-hatch, but this reaction was largely down-regulated by day 28 post-hatch. Nitric oxide is usually induced early during an infection and then rapidly cleared; else it can have a negative impact on bird performance. iNOS has been demonstrated to be upregulated in chicken intestinal cells in response to *Eimeria* infection (Allen, 1997) but unchanged or down-regulated in chickens when *Eimeria* was co-infected with *C. perfringens*. The non-vaccinated, challenged birds compared to vaccinated and control birds, had increased NO production from ex vivo stimulated spleen mononuclear cells on d 28 post-hatch. Decreased iNOS gene expression has been associated with improved body weight gain (Lillehoj et al., 2016).

Antigen neutralization assay was carried out to determine whether antibodies in serum or bile can neutralize toxins in ECP, thereby protecting LMH cells from lactose dehydrogenase release. LMH cells have been demonstrated to be vulnerable to LDH release by toxins such as NetB in the supernatant of virulent *C. perfringens* (Keburn et al., 2008). The strain of *C. perfringens* used for this study is *netB* positive (data not shown). Contrary to expectations, increased cytotoxicity or decreased neutralization was observed in the serum and bile of vaccinated, challenged birds, compared to non-vaccinated, challenged birds at all time points except in bile at day 28 post-hatch. The reason for this trend is not immediately clear, although complement was not inactivated for this study. Antibody-dependent and independent complement system has been characterized in poultry such as duck (Koppenheffer, et al., 1999) and demonstrated to play a role *in vitro* in pathogen neutralization (Sahin et al., 2003). However, non-specific comeplement cytotoxicity to certain cell line is possible (Sugimoto, et al., 1979).

In conclusion, chitosan nanoparticles loaded with extracellular proteins of *C. perfringens* and *Salmonella* flagellar proteins are immungenic and protective. The vaccine produced cell mediated and humoral immune response in the birds. Further studies are required to understand the underlying mechanisms that mediate protection of vaccinated birds. The vaccine was designed with only *C. perfringens* proteins with a homologous challenge. Further studies may be needed to determine heterologous protection, and if protection can be improved by adding eimeria proteins, varying the route of adminstration, and refining the antigen selection.

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Table 5.1. Protective efficacy of vaccination of broiler birds against mortality and jejunum lesions

Experimental group	14-21	14-28	Lesion score
	mortality (%)	mortality (%)	
NVNC	2.4 ± 0.02	8.3 ± 0.05	0 ± 0
VC	2.4 ± 0.02	13.0 ± 0.09	0.45 ± 0.14
NVC	7.0 ± 0.03	33.3 ± 0.12	0.72 ± 0.16
P-value	0.07	0.07	0.03

Chitosan-nanoparticles were synthesized and entrapped with *C. perfringens* extracellular proteins and *Salmonella* Enteritidis flagellar proteins. There were 3 treatment groups. At day 0 (day of hatch), 3, and 14 post-hatches, each broiler chick in the non-vaccinated non-challenged (NVNC), vaccinated challenged (VC) and non-vaccinated challenged (NVC) groups were orally gavaged with 0.5ml PBS, 0.5µg chitosan nanoparticles, and 0.5ml PBS respectively. At day 14 post-hatch, chicks in the VC and NVC groups were orally infected with 5,000 oocysts of *E. maxima*, followed by an oral gavage with log 8 cfu/ml *C. perfringens* on d 19, 20 and 21 post-hatch. At day 21, lesions from 3 birds/cage were scored from 0 to 3. Mean ± SEM of 6 replicates (n = 6). Mortality was recorded as it occurred.

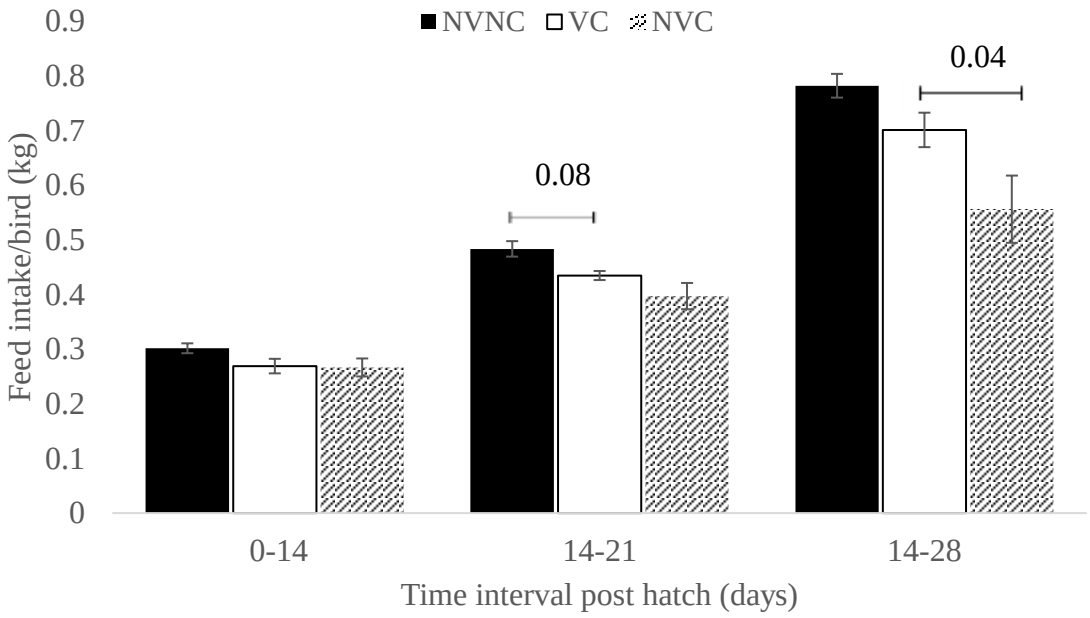

Figure 5.1. Effect of vaccination and challenge on the feed intake of broiler birds. Chitosan-nanoparticles were synthesized and entrapped with *C. perfringens* extracellular proteins and *Salmonella* Enteritidis flagellar proteins. There were 3 treatment groups. At day 0 (day of hatch), 3, and 14 post-hatches, each broiler chick in the non-vaccinated non-challenged (NVNC), vaccinated challenged (VC) and non-vaccinated challenged (NVC) groups were orally gavaged with 0.5ml PBS, 0.5µg chitosan nanoparticles, and 0.5ml PBS respectively. At day 14 post-hatch, chicks in the VC and NVC groups were orally infected with 5,000 oocysts of *E. maxima*, followed by an oral gavage with log 8 cfu/ml *C. perfringens* on d 19, 20 and 21 post-hatch. Feed intake was measured from day of hatch. Mean ± SEM of 6 replicates (n = 6).

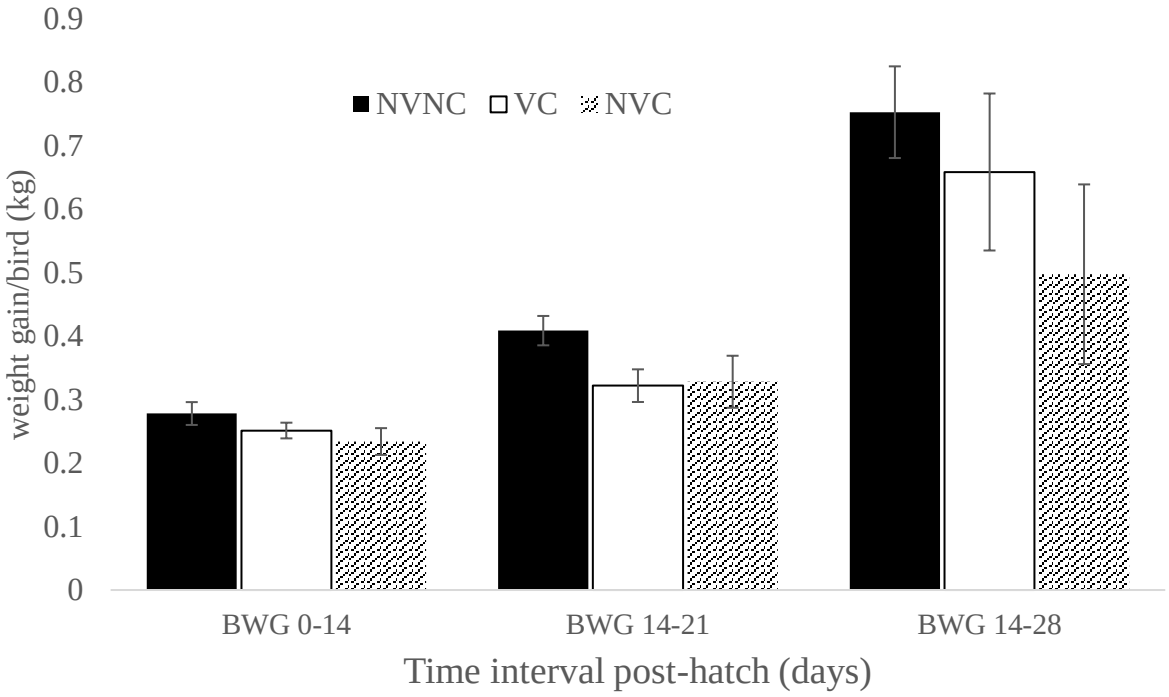

Figure 5.2. Effect of vaccination and challenge on the average body weight gain of broilers birds. Chitosan-nanoparticles were synthesized and entrapped with *C. perfringens* extracellular proteins and *Salmonella* Enteritidis flagellar proteins. At day 0 (day of hatch), 3, and 14 post-hatches, each broiler chick in the non-vaccinated non-challenged (NVNC), vaccinated challenged (VC) and non-vaccinated challenged (NVC) groups were orally gavaged with 0.5ml PBS, 0.5µg chitosan nanoparticles, and 0.5ml PBS respectively. At day 14 post-hatch, chicks in the VC and NVC groups were orally infected with 5,000 oocysts of *E. maxima*, followed by an oral gavage with log 8 cfu/ml *C. perfringens* on d 19, 20 and 21 post-hatch. Body weight was measured from day of hatch. Mean ± SEM of 6 replicates (n = 6).

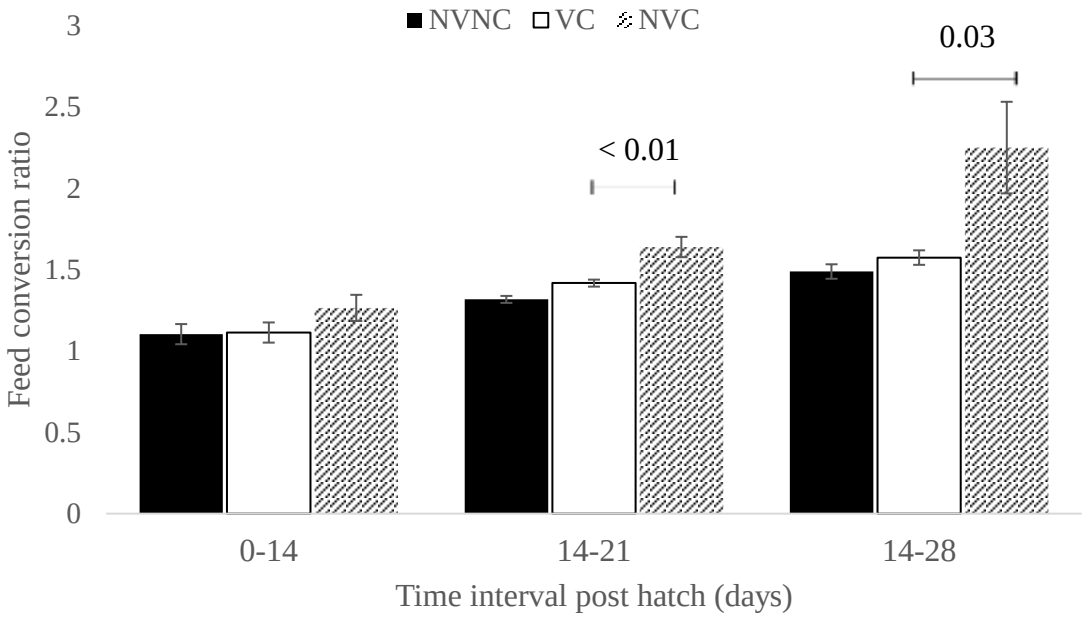

Figure 5.3. Effect of vaccination and challenge on the feed conversion ratio of broiler birds. Chitosan-nanoparticles were synthesized and entrapped with *C. perfringens* extracellular proteins and *Salmonella* Enteritidis flagellar proteins. There were 3 treatment groups. At day 0 (day of hatch), 3, and 14 post-hatches, each broiler chick in the non-vaccinated non-challenged (NVNC), vaccinated challenged (VC) and non-vaccinated challenged (NVC) groups were orally gavaged with 0.5ml PBS, 0.5µg chitosan nanoparticles, and 0.5ml PBS respectively. At day 14 post-hatch, chicks in the VC and NVC groups were orally infected with 5,000 oocysts of *E. maxima*, followed by an oral gavage with log 8 cfu/ml *C. perfringens* on d 19, 20 and 21 post-hatch. FCR was measured from day of hatch. Mean ± SEM of 6 replicates (n = 6).

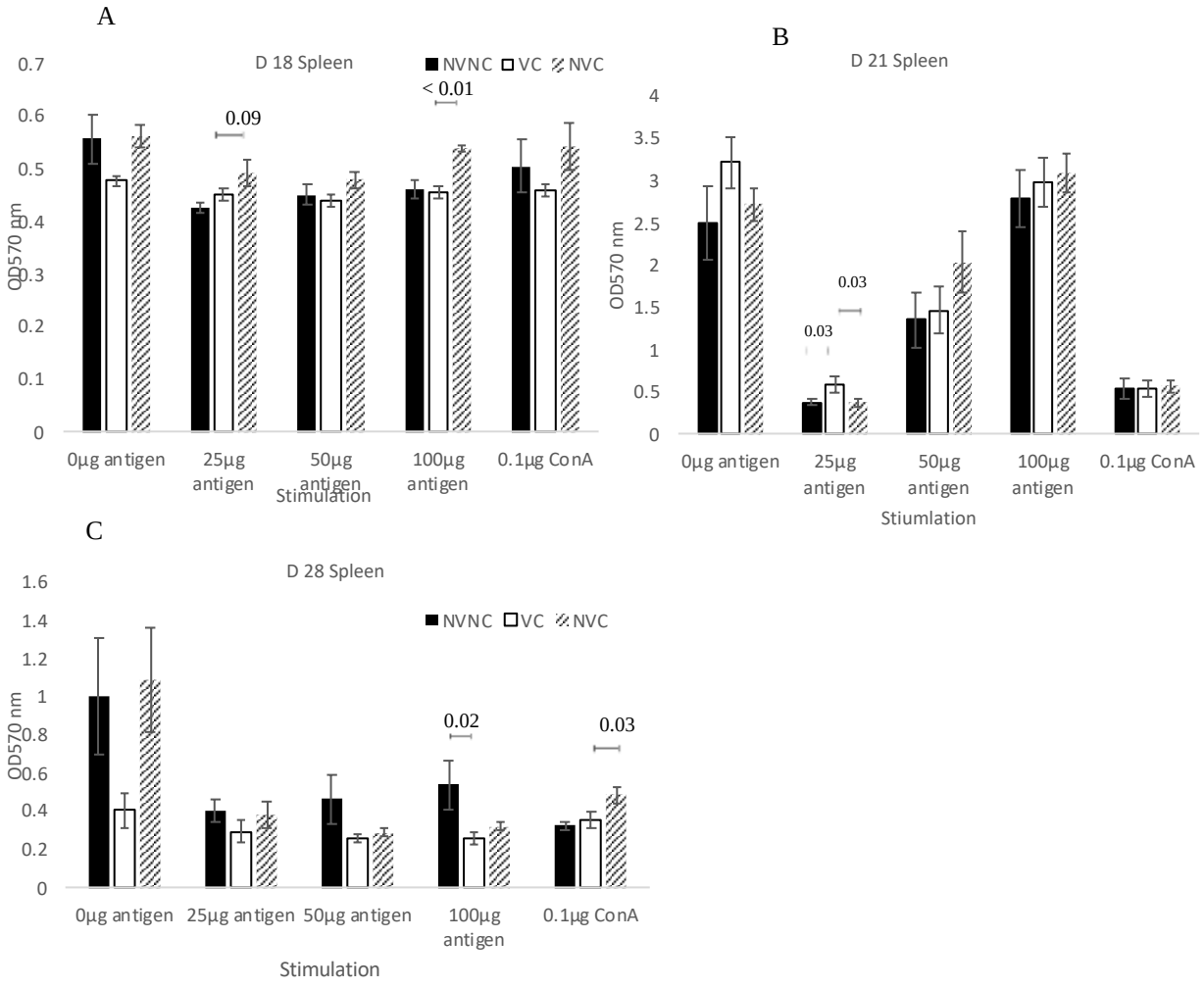

Figure 5.4. Effect of vaccination and challenge on the proliferation of spleen PBMCs of broilers birds. Chitosan-nanoparticles were synthesized and entrapped with *C. perfringens* extracellular proteins and *Salmonella* Enteritidis flagellar proteins. At day 0 (day of hatch), 3, and 14 post-hatches, each broiler chick in the non-vaccinated non-challenged (NVNC), vaccinated challenged (VC) and non-vaccinated challenged (NVC) groups were orally gavaged with 0.5ml PBS, 0.5µg chitosan nanoparticles, and 0.5ml PBS respectively. At day 14 post-hatch, chicks in the VC and NVC groups were orally infected with 5,000 oocysts of *E. maxima*, followed by an oral gavage with log 8 cfu/ml *C. perfringens* on d 19, 20 and 21 post-hatch. On d

18 (Panel A), 21 (Panel B) and 28 (Panel C) post-hatch, splenic mononuclear cells were stimulated with 0, 0.05, 0.1, 0.25 or 0.5 μg ECP for 5 d. Lymphocyte proliferation was measured using MTT assay and values reported as Optical Density (OD) values. Mean ± SEM of 6 replicates (n = 6).

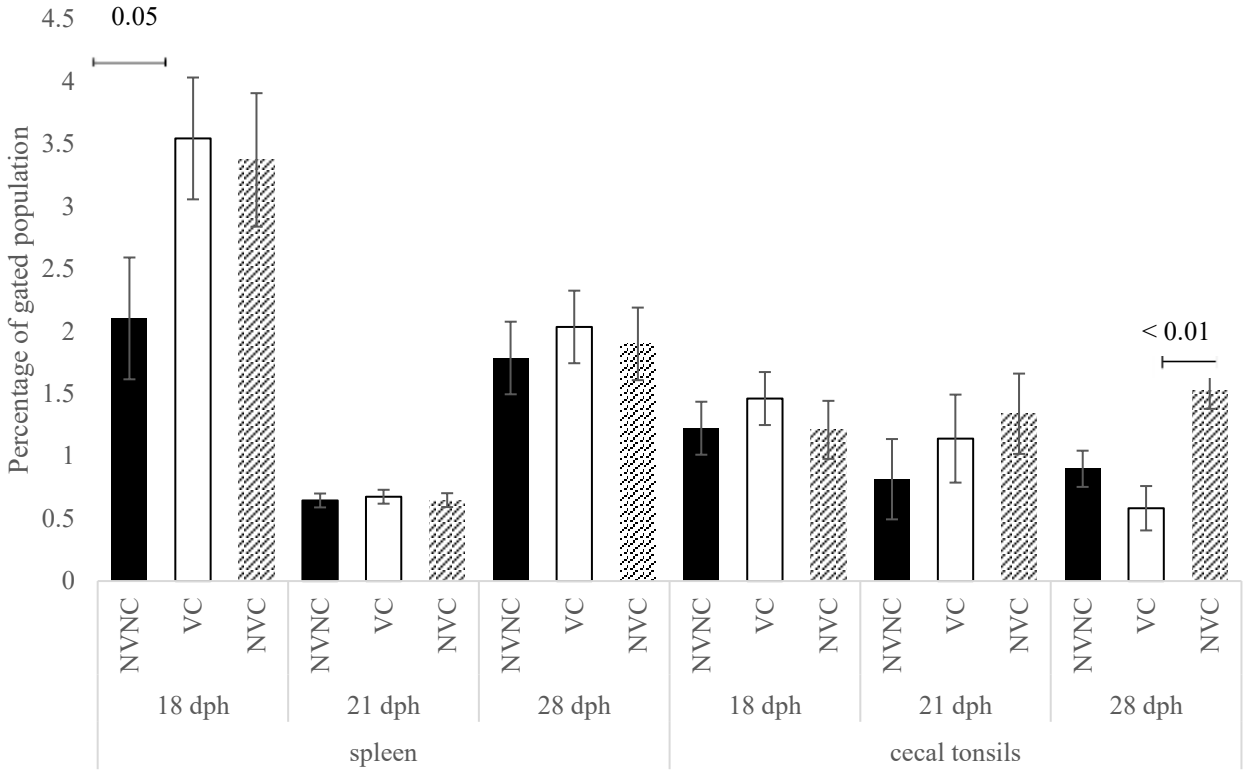

Figure 5.5. Effect of vaccination and challenge on CD4/CD8 ratio of broiler birds. Chitosan-nanoparticles were synthesized and entrapped with C. perfringens extracellular proteins and Salmonella Enteritidis flagellar proteins. At day 0 (day of hatch), 3, and 14 post-hatches, each broiler chick in the non-vaccinated non-challenged (NVNC), vaccinated challenged (VC) and non-vaccinated challenged (NVC) groups were orally gavaged with 0.5ml PBS, 0.5µg chitosan nanoparticles, and 0.5ml PBS respectively. At day 14 post-hatch, chicks in the VC and NVC groups were orally infected with 5,000 oocysts of E. maxima, followed by an oral gavage with log 8 cfu/ml *C. perfringens* on d 19, 20 and 21 post-hatch. Single cell population of mononuclear cells from the spleen and cecal tonsils cells were collected at d 18, 21, and d 28 post-hatch and stained with anti-CD4 and anti-CD8 antibodies. Mean ± SEM. n = 6 replicates. $P < 0.05$.

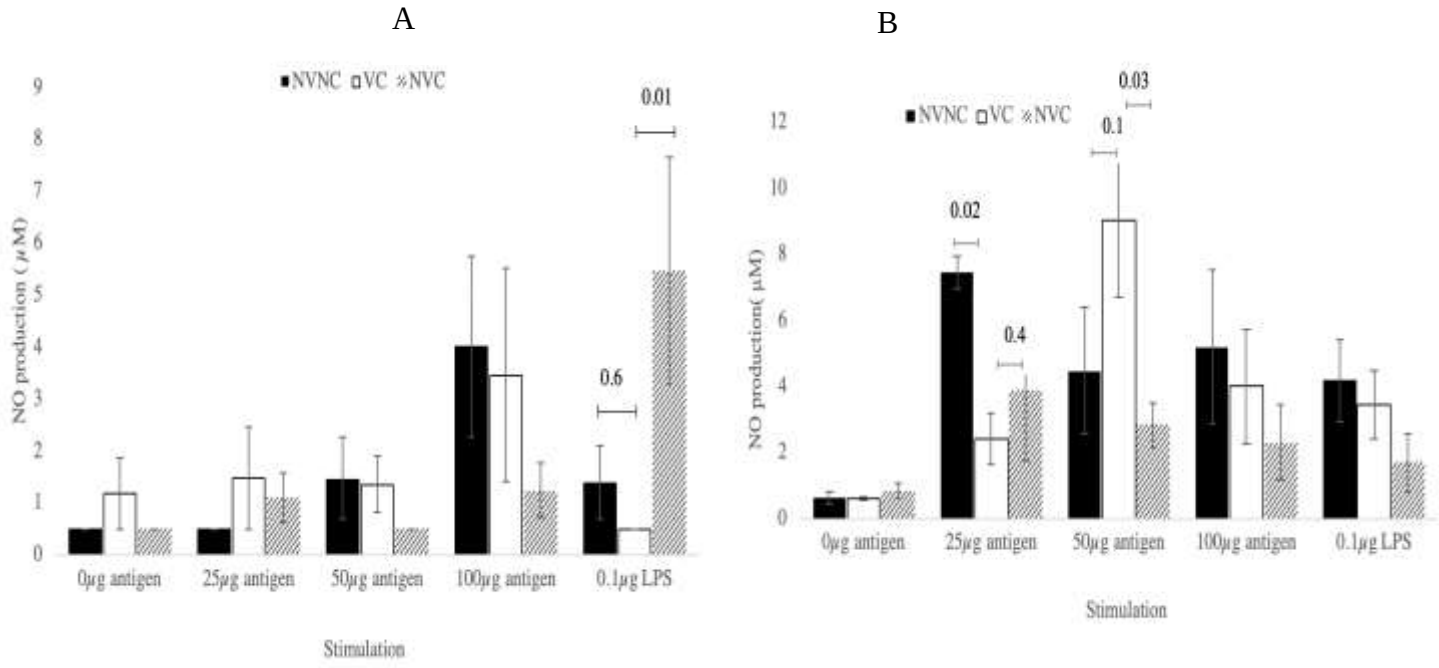

Figure 5.6. Effect of vaccination and challenge on nitric oxide production from cecal tonsil mononuclear cells of broiler birds. Chitosan-nanoparticles were synthesized and entrapped with *C. perfringens* extracellular proteins and *Salmonella* Enteritidis flagellar proteins. At day 0 (day of hatch), 3, and 14 post-hatches, each broiler chick in the non-vaccinated non-challenged (NVNC), vaccinated challenged (VC) and non-vaccinated challenged (NVC) groups were orally gavaged with 0.5ml PBS, 0.5μg chitosan nanoparticles, and 0.5ml PBS respectively. At day 14 post-hatch, chicks in the VC and NVC groups were orally infected with 5,000 oocysts of *E. maxima*, followed by an oral gavage with log 8 cfu/ml *C. perfringens* on d 19, 20 and 21 post-hatch. Cecal tonsil mononuclear cells were stimulated with 0, 0.05, 0.1, 0.25 or 0.5 μg ECP for 48 hrs. On d 18 (Panel A), and d 28 (Panel B) Nitric oxide production was measured using Greiss reagent and standard curve analysis of Na nitrate reduction. Mean ± SEM of 6 replicates (n = 6).

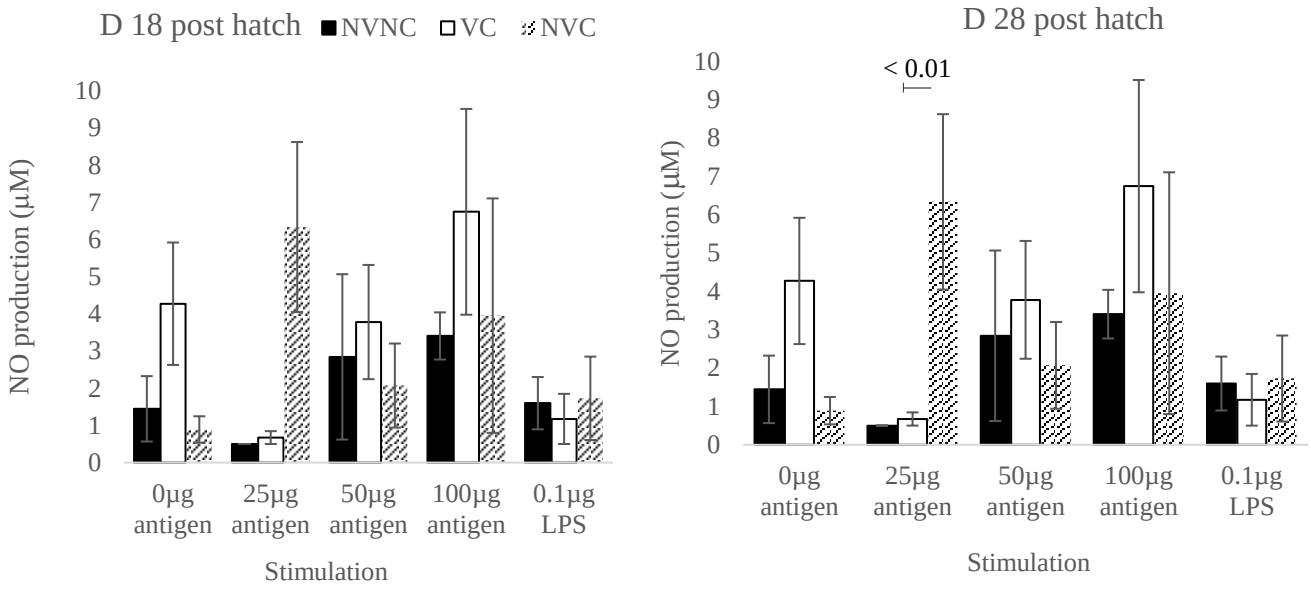

Figure 5.7. Effect of vaccination and challenge on nitric oxide production from spleen mononuclear cells of broiler birds. Chitosan-nanoparticles were synthesized and entrapped with *C. perfringens* extracellular proteins and Salmonella Enteritidis flagellar proteins. At day 0 (day of hatch), 3, and 14 post-hatch, each broiler chick in the non-vaccinated non-challenged (NVNC), vaccinated challenged (VC) and non-vaccinated challenged (NVC) groups were orally gavaged with 0.5ml PBS, 0.5µg chitosan nanoparticles, and 0.5ml PBS respectively. At day 14 post-hatch, chicks in the VC and NVC groups were orally infected with 5,000 oocysts of E. maxima, followed by an oral gavage with log 8 cfu/ml *C. perfringens* on d 19, 20 and 21 post-hatch. On d 18 and d 28 post-hatch, splenocyte mononuclear cells were stimulated with 0, 0.05, 0.1, 0.25 or 0.5 µg ECP for 48 hrs. Nitric oxide production was measured using Greiss reagent and standard curve analysis of Na nitrate reduction. Mean ± SEM of 6 replicates (n = 6).

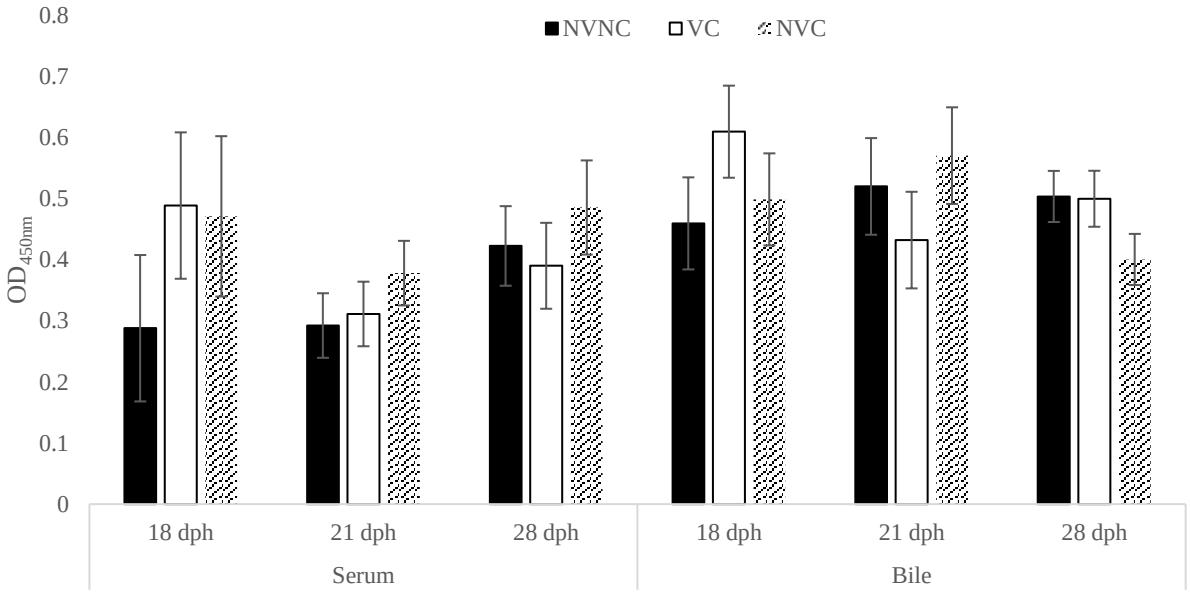

Figure 5.8. Anti-ECP-specific serum IgG and bile IgA

nanoparticles were synthesized and entrapped with *C. perfringens* extracellular proteins andb Salmonella Enteritidis flagellar proteins. At day 0 (day of hatch), 3, and 14 post-hatches, each broiler chick in the non-vaccinated non-challenged (NVNC), vaccinated challenged (VC) and non-vaccinated challenged (NVC) groups were orally gavaged with 0.5ml PBS, 0.5µg chitosan nanoparticles, and 0.5ml PBS respectively. At day 14 post-hatch, chicks in the VC and NVC groups were orally infected with 5,000 oocysts of E. maxima, followed by an oral gavage with log 8 cfu/ml *C. perfringens* on d 19, 20 and 21 post-hatch. Serum and bile for anti-ECP Serum

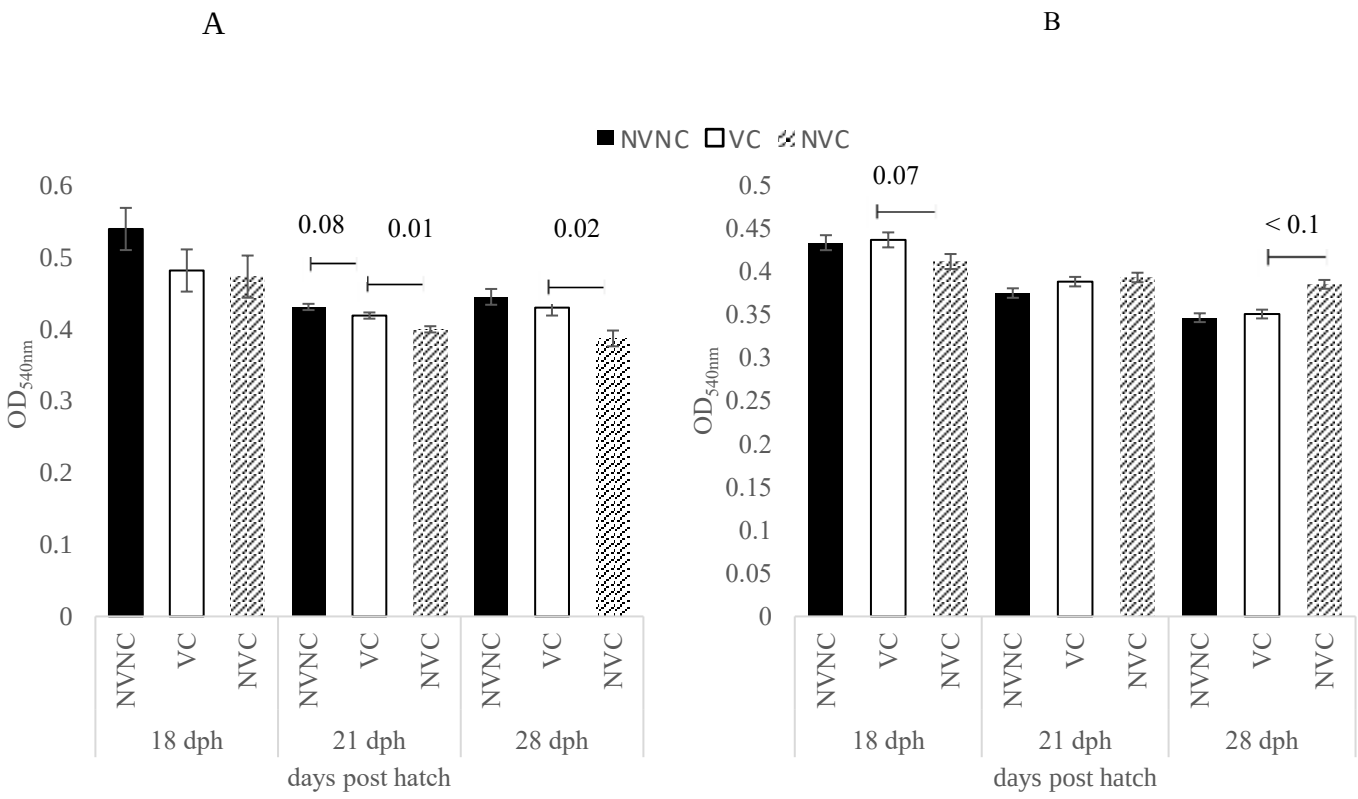

Figure 5.9. Effect of vaccination and challenge on ECP neutralization. Chitosan-nanoparticles were synthesized and entrapped with *C. perfringens* extracellular proteins and Salmonella Enteritidis flagellar proteins. At day 0 (day of hatch), 3, and 14 post-hatches, each broiler chick in the non-vaccinated non-challenged (NVNC), vaccinated challenged (VC) and non-vaccinated challenged (NVC) groups were orally gavaged with 0.5ml PBS, 0.5µg chitosan nanoparticles, and 0.5ml PBS respectively. At day 14 post-hatch, chicks in the VC and NVC groups were orally infected with 5,000 oocysts of E. maxima, followed by an oral gavage with log 8 cfu/ml *C. perfringens* on d 19, 20 and 21 post-hatch. Serum (Panel A) and bile (Panel B) were collected at d 18, 21, and d 28 post-hatch and incubated with 825 µg ECP to neutralize ECP. LMH cells were incubated with the above neutralizing solution for 8 hours. Cytotoxicity

was measured by LDH release and values reported as Optical Density (OD) values. Mean ± SEM. n = 4 replicates. $P < 0.05$.

CHAPTER 6

Conclusions

The long-term goal of this research is to design a safe and efficacious oral vaccine against necrotic enteritis in poultry production. The overall specific aim of this study to assess the physicochemical properties, *invitro* and *in vivo* safety, immunogenicity and finally, protective efficacy against an experimental challenge of necrotic enteritis of chitosan nanoparticles loaded with native or inactivated extracellular proteins of *C. perfringens* and *Salmonella* flagellar proteins. Results from the first aim indicated that both nanoparticle formulations were safe in red blood cells, releasing their antigen slowly over time. Both formulations were also stable over a wide range of pH. The zeta potential for both nanoparticles indicate that they are likely to remain poly dispersed in neutral solution. The next logical step was to assess the induction of measurable cellular and humoral responses in broilers by the synthesized nanoparticles when administered by oral gavage in broilers. Overall, chitosan nanoparticles loaded with native extracellular proteins of *C. perfringens* and *Salmonella* flagellar proteins was just as safe as, and more immunogenic than chitosan nanoparticles loaded with inactivated extracellular proteins of *C. perfringens* and *Salmonella* flagellar proteins. In the final aim of this study, chitosan nanoparticles loaded with extracellular proteins of *C. perfringens* and *Salmonella* flagellar proteins were immungenic, decreased feed conversion ratio, and increased feed conversion ratio of broilers. There were also numerical decreases in gut necrotic lesions, and mortality of vaccinated birds.

For the limitations, the proteins used for the entire study consisted of a crude mix of extracellular proteins. The extraction protocol was not repeated to account for batch-to-batch variations. Some factors that can affect the quality of extracted proteins include strain of *Clostridium*, incubation conditions and purification protocol. Microbiological tools such as differential and/or selective media to differentiate challenge *Clostridium* strain from commensal *Clostridium* and other organisms, wiould make assessing vaccine efficacy in reducing gut colonization more feasible. ELISA results were hampered by lack of pure, recombinant proteins that are unique to the challenge strain and maternal antibodies.

For future directions, refining the crude antigen to include the most immunogenic fractions of *Clostridium* extracellular protein Including selected *Eimeria* proteins and can increase the nanoparticle vaccine efficacy. Antigen refinement may involve either or both of native and recombinant proteins. The *in vitro* and *in vivo* mucoadhesive properties, zeta potential and antigen release of different formulations of nanoparticle vaccine should be assessed to measure batch to batch variation. Further studies should also assay protection in homologous vs heterologous challenge, vaccine-induced NE, and using different routes of vaccine administration. The parameters to assay protection can be expanded to include oocyst shedding, shifts in microbial population and changes in barrier function. Finally, the authors strongly recommend that more ‘omic’ resources are dedicated to understanding the basic immunology of necrotic enteritis. This will aid in the the development of molecular tools, the identification of markers of protection, and the development of targeted intervention strategies.