A natural model of immunomodulation for necrotic enteritis in poultry

by

Wanwei He

A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

in

Physiology, Cell & Development Biology

Department of Biological Sciences
University of Alberta

Abstract

Necrotic enteritis (NE) is an economically important disease in poultry, caused by the opportunistic pathogen *Clostridium perfringens*. Well-known as a multifactorial disease, NE development is under the influence of a wide range of environmental risk factors. Current *in vivo* NE challenge models typically incorporate pre-exposure to disease risk factors, in combination to inoculation of *C. perfringens* culture to obtain a clinical NE outcome. My first goal was to establish a model based on the natural uptake of *C. perfringens* from the barn environment to produce subclinical infection. We incorporated access to litter, coccidial exposure, feed composition, and feed withdrawal stress to achieve the commonly observed subclinical NE infection peak at 3 weeks post hatch.

Antibiotics were traditionally used to control infectious diseases in poultry but has aroused public concern with the emergence of resistant microbes. As reduction and removal of antibiotics become the trend in poultry farming, my second goal was to understand the impact of antibiotic removal during a naturally-occuring NE condition. My data shows that drug-free animals showed short-term body weight decline during NE induction, though the overall feed intake, weight gain, feed conversion, and carcass traits were not affected. Surprisingly, medication treatment increased ileal pH and cecal pathogenic *C. perfringens* load. These findings suggest an urgent need for drug-free alternatives to combat NE infection as the industry is moving away from antibiotic use.

Colonization by *C. perfringens* occurs early after hatch and induces host immune tolerance that allows it to persist as part of the bird's commensal flora. β-glucan, a yeast cell wall component, is well characterized for its immunomodulation capacity and has recently been identified as a primary driver of trained immunity. The last goal of the project was to assess if the context of

early-life exposure to *C. perfringens* may affect NE infection outcomes. β-glucan was co-administered with *C. perfringens* and the impact on disease severity and bird performance was subsequently determined. Intra-abdominal injection of β-glucan post-hatch improved ileal morphology, prevented organ weight decline, and avoided NE-induced increase in feed conversion ratio. Further analysis revealed metabolic and functional changes of abdominal leukocytes following the initial stimulation, including increased glycolysis, proinflammatory cytokine expression, and ROS production. These results suggest that β-glucan can reduce the negative impacts of NE by influencing the context in which *C. perfringens* is first encountered.

Preface

This thesis is an original work by Wanwei He. This work received ethical approval from the University of Alberta Research Ethics Board and was funded through Alberta Agricultural and Forestry grants awarded to Dr. Daniel R. Barreda. Parts of the research conducted in this thesis are in process of publication in peer-reviewed journals. Details of these publications are below:

Chapter 3:

He W, Gose E, Wakaruk J, Barreda DR, Korver DR. A poultry necrotic enteritis disease model based on natural *Clostridium perfringens* uptake reveals the influence of coccidial exposure and housing conditions on disease severity. Frontier in Physiology. In progress. I was responsible for the experimental design, data collection and analysis, and manuscript preparation. E Gose and J Wakaruk assisted with data collection. DR Korver and DR Barreda were the supervisory authors, contributed with the experimental design and manuscript editing.

Chapter 5:

He W, Kamely M, Wakaruk J, Korver DR, Barreda DR. Environmental context during early-life *Clostridium perfringens* exposure affected disease resistance of broiler chicken to a natural necrotic enteritis challenge. Frontier in Physiology. In progress. I was responsible for the experimental design, data collection and analysis, and manuscript preparation. M Kamely assisted with data collection and manuscript editing. DR Barreda and DR Korver were the supervisory authors, contributed with the experimental design and manuscript editing.

Acknowledgement

I would like to express my sincere gratitude to my supervisor Dr. Daniel R. Barreda. Dan is always supportive by bringing in resources and encouraging us to become an expert in our special field. This has gradually built up my confidence in conducting research independently. His passion in science and willingness to maximize the value from the available resources has influenced me deeply and will keep inspiring me.

I would also like to thank my committee member Dr. Doug Korver, who had provided supervision and guidance from the perspective of Poultry Science. The on-farm experience working with him was unforgettable. I am grateful to be able to learn so much from his expertise and experience. Dr. Richard Uwiera, as the external examiner of my final examination, provided valuable thoughts and comments which are highly helpful to improve the quality of this project.

The University of Alberta is an incredible institution that allowed me to work in collaboration with scientists from other departments. My work on bacterial culture and quantification was supported by Dr. Lynn McMullen and Dr. Leluo Guan. I am very grateful for the experience in the Agricultural Genomics and Proteomics Unit and Agri-Food Discovery Place. The training provided by M.Sc. Yanhong Chen and M.Sc. Ross Lowe is highly appreciated.

I still remember how thrilled I was being welcomed to the lab in the summer of 2018. The lab members had turned out to be my strong support during my study. Every farm trial went smoothly thanks to the arrangement of Lab manager Jeremy Wakaruk. Special thanks to current lab member, Dr. Mohammad Kamely, Ph.D. Candidate Amro Sherif, Farah Haddad, Emilie Albers, and Matthew Michnik, who assisted in data collection and analysis. Dr. Juan More

Bayona and Dr. Débora Torrealba shared valuable experience and helped me to build a solid start of my study.

The on-farm data collection could not be achieved without the unconditional help from Ph.D. Candidate Emanuele Goes, Mauricio Sanabria Bernal, Taresa Chieng, M.Sc. Emmanuel Opoku Yeboah, M.Sc. Kim Thorsteinson and M.Sc. Nicole Zukiwsky. The enthusiastic staff in the Poultry Research Center, Kerry Nadeau, Shawn Rankin, Giles Hinse, Chris Ouellette, were always there to maintain a professional research environment.

And finally, to the city of Edmonton, thank you for offering a festival-packed summer full of joy, and a snow-covering winter with tranquility and peace.

Table of Contents

Chapter 1: Introduction and literature review 1
1.1 Introduction 1
1.2 Thesis objectives 2
1.3 Thesis outline 3
1.4. Literature review 3
1.4.1 Necrotic enteritis in poultry 3
1.4.1.1 Introduction 3
1.4.1.2 Disease prevalence 4
1.4.1.3 Impact on the economy and public health 5
1.4.1.4 Clinical signs and pathological changes 6
1.4.1.5 Pathogenesis 7
1.4.1.6 Risk factors contributing to disease susceptibility 9
1.4.1.7 Prevention and control strategies 10
1.4.1.8 Experimental disease models 11
1.4.2 The avian immune system and its role against microbial infection 12
1.4.2.1 Pattern recognition receptors (PRRs) 13
1.4.2.2 Cellular mediators 14
1.4.2.2.1 Heterophils 15
1.4.2.2.2 Monocytes and macrophages 16
1.4.2.2.3 Lymphocytes 17
1.4.2.3 Cytokines and chemokines 19
1.4.2.3.1 IL-1β 19
1.4.2.3.2 IL-6 20
1.4.2.3.3 TNF-α 21
1.4.2.3.4 IL-8 23
1.4.2.4 Reactive oxygen and nitrogen species 24
1.4.2.5 Organs 25
1.4.2.5.1 Liver 26
1.4.2.5.2 Spleen 27
1.4.2.5.3 Intestine 28
1.4.2.5.4 Bursa of Fabricius 29
1.4.2.6 Host immunity in *C. perfringens* colonization 30
1.4.2.7 Host immunity in NE infection 31
1.4.3 Immune memory 33
1.4.3.1 Epigenetic regulation of innate immune memory 34

1.4.3.2 Metabolic regulation of innate immune memory 34
1.4.3.3 Hematopoiesis regulation of innate immune memory 36
1.4.4 Contribution of innate immunity to vaccine immunogenicity 36
Chapter 2: Materials and methods 37
2.1 Animals and establishment of a natural NE challenge 38
2.2 Growth performance and sampling procedure 39
2.3 Intestine lesion scoring 40
2.4 Bacterial quantification 41
2.4.1 DNA extraction and purification 41
2.4.2 Quantitative real-time PCR (qRT-PCR) 41
2.5 Intestine histology 42
2.6 Circulatory leukocyte profiles 43
2.7 Immune priming 43
2.7.1 Preparation of heat-killed *C. perfringens* 43
2.7.2 Intra-abdominal injection 43
2.8 Gene expression 44
2.9 Leukocyte infiltration and respiratory burst 44
2.9.1 Cell collection and staining 44
2.9.2 Imaging Flow cytometry 45
2.10 Statistical analysis 45
Chapter 3: Optimization of a natural NE challenge model based on *C. perfringens* uptake from the farming environment 52
3.1 Introduction 52
3.2 Results 53
3.2.1 Mortality and clinical signs 53
3.2.2 Detection and quantification of *C. perfringens* 54
3.2.3 Gross examination of intestine lesion 54
3.2.4 Microscopic examination of intestine lesion 55
3.3 Discussion 56
Chapter 4: The impact of NE infection on growth performance and disease severity under drug-free conditions 62
4.1 Introduction 62
4.2 Results 63
4.2.1 Medication reduced body weight decline in NE challenged animals 63
4.2.2 Medicated animal showed altered empty intestine weight and ileal pH 64
4.2.3 Medication treatment caused higher abundance of cecal pathogenic *C. perfringens* 64
4.2.4 Medication treatment did not improved production performance or gut lesion status 64

4.3 Discussion 65
Chapter 5: The effect of immunomodulation by β-glucan against NE infection 70
5.1 Introduction 70
5.2 Results 71
5.2.1 β-glucan prevented reduction in feed efficiency caused by CP exposure 71
5.2.2 β-glucan exposure improved ileal morphology on day 21 72
5.2.3 Gut lesion score and *C. perfringens* abundance was not affected by early-life immune priming 72
5.2.4 Spleen and liver weight decline was avoided by β-glucan co-exposure 73
5.2.5 β-glucan exposure increased circulatory heterophil percentage on day 21 73
5.2.6 β-glucan enhanced glycolysis and cytokine expression in abdominal leukocytes 73
5.2.7 β-glucan induced significant leukocyte infiltration at the priming site 74
5.2.8 β-glucan enhanced ROS production in abdominal macrophages 75
5.3 Discussion 75
Chapter 6: General discussion 89
6.1 Introduction 89
6.2 Summary of findings 90
6.2.1 Appropriate combination of risk factors induced NE mimicking the field cases 90
6.2.2 Antibiotic inclusion prevented short-term body weight decline but negatively impacted gut health 92
6.2.3 β-glucan improved animal health by influencing the initial responses against *C. perfringens* 92
6.3 Future directions 94
References 95

List of Abbreviations

ALDOB: fructose-bisphosphate B

BCG: Bacillus Calmette-Guérin

CLR: C-type lectin receptor

CP: *Clostridium perfringens*

FCR: feed conversion ratio

GALT: gut-associated lymphoid tissue

HIF: hypoxia-inducible factor

IEL: intraepithelial lymphocytes

IFN: interferon

IL: interleukin

iNOS: inducible nitric oxide synthase

LPS: lipopolysaccharide

LTi cell: lymphoid tissue inducer cell

MAPK: mitogen-activated protein kinase

NE: necrotic enteritis

NF-κB: nuclear factor kappa B

NK cell: natural killer cell

NLR: nod-like receptors

NO: nitric oxide

oxLDL: low-density lipoprotein

PALS: periarteriolar lymphoid sheaths

PAMP: pathogen-associated molecular patterns

PRR: pattern recognition receptor

PWP: periellipsoidal white pulp

SCFA: short chain fatty acid

TCA: tricarboxylic acid

TGF: transforming growth factor

Treg: regulatory T cell

RNS: reactive nitrogen species

ROS: reactive oxygen species

TIbV: trained immunity-based vaccines

TLR: toll-like receptor

TNF: tumor necrosis factor

List of Tables

Table 1. Flock size, housing type, and coccidial challenge dosage in 3 experimental flocks

Table 2. Ingredient and calculated nutrient composition of experimental diets for birds during starter, grower, and finisher stage

Table 3. Sequences of primers used in quantification of *C. perfringens* by qPCR

Table 4. Primers used for the qPCR analysis of chicken mRNAs

Table 5. Mortality and overall lesion prevalence in 3 experimental flocks

List of Figures

Figure 1. Induction of subclinical NE in broiler chicken by application of disease predisposing factors.

Figure 2. A wide range of environmental factors determine the manifestation of necrotic enteritis caused by *C. perfringens*

Figure 3. Quantification of *C. perfringens* and intestine lesion confirmed induction of subclinical necrotic enteritis using the natural infection model

Figure 4. Effect of medication treatment on body weight decline, intestinal parameters, and bacterial density in chicken affected by necrotic enteritis

Figure 5. Gut lesion profile and production performance of medicated and drug-free animals

Figure 6. Impact of early-life immune priming by PBS, CP, and β-glucan+CP on (A) Feed conversion ratio, (B) Weight gain, and (C) Feed intake

Figure 7. Impact of early-life immune priming by PBS, CP, and β-glucan+CP on ileal morphology following necrotic enteritis challenge

Figure 8. Impact of early-life immune priming by PBS, CP, and β-glucan+CP on the prevalence of animals with necrotic lesions

Figure 9. Impact of early-life immune priming by PBS, CP, and β-glucan+CP on (A) Lesion score, (B) Total *C. perfringens* density, and (C) *NetB* abundance

Figure 10. Impact of early-life immune priming by PBS, CP, and β-glucan+CP on relative organ weight following necrotic enteritis challenge

Figure 11. Impact of early-life immune priming by PBS, CP, and β-glucan+CP on circulatory leukocyte profile following necrotic enteritis challenge

Figure 12. Changes in metabolic gene and inflammatory cytokine expression following initial *C. perfringens* exposure with and without β-glucan costimulation

Figure 13. Impact of *C. perfringens* concentration on leukocyte infiltration with and without β-glucan inclusion

Figure 14. Changes in infiltrating leukocyte composition and function following initial *C. perfringens* exposure with and without β-glucan costimulation.

Chapter 1: Introduction and literature review

1.1 Introduction

Necrotic enteritis (NE) in poultry is caused by *Clostridium perfringens*, a commensal, gram-positive, spore-forming anaerobe. *C. perfringens* comprises part of the gut microbiota of healthy chickens, with a high diversity of strains representing the total *C. perfringens* population. One key step in NE pathogenesis is the replacement of non-pathogenic *C. perfringens* by the pathogenic strains, which subsequently lead to increased expression of virulence factors. A number of risk factors have been identified to increase animals' susceptibility to NE infection, which are put into consideration for experimental NE reproduction as well as for disease prevention in practical production.

There is a need for effective control strategy of *C. perfringens* owing to the increased NE incidence and its cost to the poultry industry. Immunomodulation in chicken is a promising strategy to enhance host resistance against this disease. Vaccines are well-established immunomodulators that take advantage of protective adaptive immune memory. Development of commercial NE vaccines has proven challenging. In the lab, most effective strategies to date still rely on multiple-dosage vaccination regimens which, unfortunately, have limited practical use in the field (Mot et al., 2013 and 2014). A single-dose administration at hatch, when most other vaccines are administered, would be the most amenable for current commercial broiler chicken producers.

Antimicrobial responses in young chicks rely heavily on the innate immune system via recognition of pathogen structurally conserved molecules by innate immune cells (Alkie et al.,

2019). Recent studies have revealed that these innate leukocytes undergo functional changes after primary stimulation that contribute to subsequent defenses (Gourbal et al., 2018; Netea et al., 2016). These phenotypic changes have been cumulatively termed innate immune memory or trained immunity, which have opened a new avenue in immune modulation to enhance disease resistance in food animals (Byrne et al. 2020).

1.2 Thesis objectives

My thesis aims to demonstrate the protective effect of immunomodulation by β-glucan against NE infection. Colonization by *C. perfringens* occurs early after hatch and induces host immune tolerance that allows it to persist as part of the microbiota. As shown in mammals, the metabolites and structural components of *Clostridium spp.* trigger the development of tolerized phenotype in immune cells (Atarashi et al., 2011; Nagano et al., 2012). Recent evidence suggests immune responsiveness against a recurring pathogen can be modified by the context during the initial exposure. β-glucan, a primary driver of trained immunity, was shown to revert tolerized phenotype of innate immune cells, enhancing responsiveness against subsequent infection (Gourbal et al., 2018; Netea et al., 2016). In this project, a natural NE infection model was used to examine the effectiveness of β-glucan to prevent immune tolerance against *C. perfringens*. Current *in vivo* NE challenge models typically incorporate risk factor pre-exposure, in combination with exogenous *C. perfringens* inoculation. Our goal was to establish a model that more accurately mimicked typical farming conditions and the natural uptake of *C. perfringens* from the barn environment. Specific objectives of this thesis are (1) Optimization of a natural NE challenge based on current understanding in NE pathogenesis; (2) The impact of antibiotic removal on growth performance and disease severity during the naturally-occurring NE challenge; (3) Protective effect of immunomodulation by β-glucan against NE infection.

1.3 Thesis outline

This thesis is made up of six chapters. The introduction and literature review chapter provides a general introduction of NE disease and avian immunity in antimicrobial responses. It describes the etiology, epidemiology, pathology, experimental model, and prevention strategy of NE disease. The review on avian immunology focuses on the innate arm of immune responses, introducing the molecules, cells, and organs involved in innate response against microbial pathogens. This section then introduces the host immune tolerance to commensal bacteria and the role of trained immunity in redirecting host immunity from tolerized to active status. Chapter 2 describes methods, materials, and protocols used throughout this thesis. In Chapter 3, I present a novel natural NE disease model and evaluate the infection outcome using multiple diagnosing techniques. Chapter 4 demonstrates the impact of removing antibiotics and anticoccidials in poultry production under a NE challenge. Chapter 5 describes the effectiveness of immune modulation by β-glucan against *C. perfringens* tolerance. Chapter 6 offers an integrative discussion of my results and places them in the context of established knowledge, recent salient findings in the field, and relevant issues in the poultry industry.

1.4. Literature review

1.4.1 Necrotic enteritis in poultry

1.4.1.1 Introduction

Necrotic enteritis (NE) in poultry significantly impacts welfare, profitability, and the food safety aspects of broiler chicken production. Acute and severe *C. perfringens* infections induce clinical disease are manifested as a sudden increase in flock mortality with or without prodromal signs

(early symptoms of NE infection) (Timbermont et al., 2011). Subclinical form of NE describes a mild infection without noticeable disease symptoms. It is the chronic, subclinical infection that often goes undetected, hampering growth and feed efficiency, as well as carcass quality (Wade and Keyburn, 2015).

NE is well-characterized for its multifactorial nature, and many risk factors have been identified to promote the development of pathogenic *C. perfringens.* With the background of reduced antibiotic use in poultry production, NE control strategies have focused on avoiding risk factors and promoting birds' resistance against the pathogen. The need to understand NE and investigate disease control strategy has necessitated a reliable and reproducible disease model.

1.4.1.2 Disease prevalence

In broilers, outbreaks of NE have been reported around the world, periodically causing serious problems (Johansson, 2006). Estimates of the prevalence of NE vary widely, ranging from 1% to 40% of commercial broiler flocks being affected in North America and the EU (McDevitt et al., 2006). Although NE has been characterized for more than half a century (Parish, 1961), only a few studies have directly measured the incidence of NE. In the last century, research groups from Canada and Europe have reported NE frequencies based on evaluating animals submitted to diagnostic laboratories (Long, 1973; Bernier et al., 1974; Kaldhusdal & Skjerve, 1996). This approach provided validated results from the examined animals but may not reflect the total population that was at risk. Hermans and Morgan (2007) conducted a large-scale survey involving 857 farms in the UK and reported 32.8% of the respondents recognized the presence of NE during 2001. However, as noted by the author, the data collected was vulnerable to misclassification, as it relied on the farmer's ability to report NE cases. Løvland and Kaldhusdal (1999) proposed liver lesion measurement in slaughtered animals as an indicator to determine

NE occurrence. Using this indirect approach, Kaldhusdal et al., (2016) reported up to 25% of Norwegian broiler flocks were affected by NE during the years from 1978 to 2015.

1.4.1.3 Impact on the economy and public health

In the past decade, subclinical NE infection has become more prevalent than the clinical form (Timbermont et al., 2011). Infection can reduce the weight gain of broilers due to decreased feed intake (Rumes et al., 2014), increase metabolic rate due to inflammation (Cao et al., 2012), and reduce nutrient absorption due to gut damage (Hoerr, 1998). This is associated with a hidden economic loss from birds surviving subclinical infection. Compared to healthy animals, flocks affected by subclinical NE suffer from a 12% decrease in body weight and a 10.9% increase in FCR (Skinner et al., 2010). Flocks with frequent NE occurrence suffered from a 33% loss in profit compared to NE-free flocks (Løvland and Kaldhusdal, 2001).

Before the 21 century, costs of NE were usually underestimated because only the impact of clinical NE was considered (Wade and Keyburn, 2015). In the early 2000s, NE cost the international poultry industry over $2 billion (USD) per year, largely associated with medical treatments, impaired growth, and carcass discard (Hofacre, 2001). The economic impact steadily increased to $6 billion (USD) in 2015 as the world poultry production expanded (Wade and Keyburn, 2015). The economic cost of this disease is already significant, while the changes in the availability of certain feed ingredients and therapeutics may worsen the future cost of NE (McDevitt et al., 2006). This can be seen in the European experience, where the three epidemics of NE in the last century were associated with a change of feed cereal types and abolishment of antibiotic growth promoters (Kaldhusdal et al., 2016).

The subclinical form of NE has aroused public health concern as the accumulation of *C. perfringens* in poultry constitutes a risk for food poisoning in humans. *C. perfringens* carrying enterotoxin genes was detected in retail chicken from Canada and Japan (Nowell et al., 2010; Miki et al., 2008). And outbreaks of food poisoning due to *C. perfringens* have been traced back to the consumption of chicken meat (Immerseel et al., 2004). According to the Centers for Disease Control in the United State, *C. perfringens* is the fourth-leading cause of bacterial-induced foodborne illnesses behind *Salmonella*, *Campylobacter*, and *Escherichia coli* (CDC, 2014).

In conclusion, though clinical NE outbreak causes intense mortality, the subclinical form of the disease is more important as it is associated with a high economic loss and risk of pathogen transfer to the food chain (Olkowski et al., 2008).

1.4.1.4 Clinical signs and pathological changes

The clinical form of NE is mostly seen in 3 to 4-week-old animals; symptoms include diarrhea, depression, decreased appetite, and ruffled feathers (Paiva et al., 2014; Timbermont et al., 2011). Symptoms are short-lived because affected birds die quickly within hours. Subclinical NE is usually mild with no clinical signs or sudden increase in mortality. In the subclinical cases, pathological changes in the small intestine and liver are considered to be sensitive disease indicators (Kaldhusdal et al., 2016; Williams et al. 2003). The typical intestinal lesion found in NE-affected birds is caused by proteolytic activities and toxins production by *C. perfringens*, which lead to disorganization of extracellular structure and cell lysis (Martin and Smyth, 2010; Olkowski et al., 2008). During the gross examination, one may find roughness, ulceration, hemorrhage, and fibrin accumulation at the impacted intestine mucosa (Shojadoost et al., 2012). Lesions under microscopic examination are typically characterized by fibrin-like cellular debris

covering the necrotic mucosa. As the disease progresses, *C. perfringens* can reach the liver via the bile duct and portal circulation (Timbermont et al., 2011). This results in cholangiohepatitis or liver enlargement, sometimes with focal lesions in the organ. In commercial farming, pathological changes due to subclinical NE are often not detected until chickens are slaughtered, subsequently causing carcass condemnation.

1.4.1.5 Pathogenesis

C. perfringens is ubiquitously distributed in nature and comprises part of the gut microbiota of healthy chickens. There is a high diversity of strains representing the total *C. perfringens* population, and pathogenic strains of *C. perfringens* only account for an insignificant proportion (Abildgaard et al., 2010b). The NE-causing strains are characterized by a capacity to produce necrotic enteritis toxin B (NetB) (Keyburn et al., 2008; Lacey et al., 2018), and harbor a collection of genes that function to enhance their proliferation, maintenance, and virulence, including antibiotic resistance genes, adhesins, catabolic enzymes, toxin and bacteriocins (Bannam et al., 2011; Freedman et al., 2015; Parreira et al., 2012).

Toxins are the main virulence factors of NE-causing *C. perfringens*. NetB, a β-pore-forming toxin, was shown to fulfill the molecular Koch's postulates and is essential for NE induction (Rood et al., 2016). *NetB* mutation in the virulent *C. perfringens* leads to loss of cytotoxicity, with virulence being restored by complementation with the wild-type gene (Keyburn et al., 2008). This is in agreement with epidemiology findings where *netB* is more prevalent in pathogenic strains than non-pathogenic strains (Lacey et al., 2018). *NetB* is located on a NE-associated pathogenicity locus (NELoc-1) encoded on a large conjugative plasmid (Lepp et al., 2010). NELoc-1 composes 36 additional genes, including those predicted to encode leukocidins

and internalin-like protein. *NetB* alone, in the absence of other NELoc-1 genes, was unable to restore full virulence *in vivo*, suggesting the cooperative role of numerous virulence factors during NE pathogenesis (Zhou et al., 2017).

Collagenolytic and proteolytic enzymes produced by *C. perfringens* destroy basal lamina and lateral domains of enterocytes cells, which are responsible for the pathological change in the impact tissue (Olkowski et al., 2008). The catabolic activities and toxins secretion by *C. perfringens* lead to exposure of a variety of extracellular matrix molecules, which can be bound more strongly by pathogenic *C. perfringens* compared to non-pathogenic strains (Martin and Smyth, 2010; Wade et al., 2015). The adhesin-encoding gene *cnaA* and downstream genes, located in the chromosomal VR–10B locus, are responsible for adherence to collagen types IV and V and gelatin (Wade et al., 2016). A *cnaA* mutant in pathogenic *C. perfringens* significantly reduced colonization of the bacterium and abolished its NE-causing ability. Lepp et al. (2013) reported the presence of VR-10B locus is more prevalent in *netB*-positive isolates (87%) compared to *netB*-negative strains (42%). This evidence suggests the adherence and retention advantage of NE-causing strains in the gastrointestinal tract.

The replacement of commensal *C. perfringens* by pathogenic strains represents one key step in NE pathogenesis (Barbara et al., 2008). The genetic diversity of *C. perfringens* in healthy animals is generally high and much lower in NE-affected animals, usually dominated by one or two virulent clones solely (Engström et al., 2003; Nauerby et al., 2003; Gholamiandekhordi et al., 2006). Diseased flocks often harbored a decreased genotypic richness, suggesting the selective proliferation of the NE-related strains (Gaucher et al., 2017; Lacey et al., 2016). Inter-strain growth-inhibition mediated by bacteriocin secretion is one strategy of pathogenic *C. perfringens* during the pathogen-commensal competition (Barbara et al. 2008; Timbermont et al.

2009). Perfrin is an important bacteriocin produced by *C. perfringens*, which causes growth inhibition in strains without perfrin (Timbermont et al., 2014). Interestingly, this bacteriocin is found only in *netB*-positive *C. perfringens* strains. Perfrin and netB may function cooperatively in the NE pathogenesis cycle, in which the bacteriocin suppresses competitors for nutrient access thus explosive proliferation, while the increased toxin secretion damages tissue thus releasing more nutrients (Timbermont et al., 2009 and 2014).

1.4.1.6 Risk factors contributing to disease susceptibility

Both field experience and efforts to reproduce experimental infection have shown that NE induction requires specific predisposing factors (Stanley et al., 2014; Van Immerseel et al., 2009). A number of risk factors have been identified to promote pathogenic *C. perfringens* development at the expense of commensal strains. Exposure to coccidial parasites remains the best studied for its strong link to NE development (Stanley et al., 2014; Wilson et al., 2018). Coccidiosis-induced epithelial extracellular matrix disruption, plasma protein leakage, and mucus production provide an extra selective advantage for pathogenic *C. perfringens*, which possess the stronger binding ability and mucolytic activity (Collier et al., 2008; Martin and Smyth, 2010). Diet components also constitute relevant key risk factors associated with NE development. Feeds rich in water-soluble non-starch polysaccharides, such as wheat-based diets increase digesta viscosity, prolong transit time, thus promote pathogen retention (Annett et al., 2002; Shojadoost et al., 2012). The non-digestible fructans in wheat can also interact with glycoproteins on the epithelial surface leading to increased mucin production (Kleessen et al., 2003), which could be utilized by *C. perfringens* as a nutrient source (Lepp et al., 2010). Specific additives such as fishmeal can supply abundant glycine and methionine, which enhances *C.*

perfringens proliferation and toxin production (Dahiya et al., 2007; Shojadoost et al., 2012; Wilkie et al., 2005).

Other risk factors associated with increased NE occurrence are colder months (reduced ventilation/increased litter moisture) and poor husbandry management (Hofacre et al., 2018), such as food deprivation, heat/cold stress, inadequate hygiene routines, and overcrowding. NE has traditionally been linked to specific housing conditions where birds are kept on litter (Goossens et al., 2020), which is associated with the easy spread of excreted gut pathogens (Mot et al., 2014).

1.4.1.7 Prevention and control strategies

For decades, poultry producers have prevented and treated NE by routine application of antimicrobial growth promoters, such as bacitracin, lincomycin, amoxicillin, and tylosin (Saleem, 2012). Under the public concern of antibiotic resistance, reduction and removal of antibiotics have become the future trend for poultry production. Better farm management, including biosecurity measures, feed quality, and housing conditions are relevant for NE prevention (Mot et al., 2014). However, evidence has suggested the pathogen survived both bird-free period and disinfection procedures (Engström et al., 2012). An empty broiler house that has been disinfected was found to retain *C. perfringens*, where *netB*-carrying strains accounted for 45% among all isolates. Enhanced disease resistance from the animals' aspect thus represents an important counterpart in NE control, especially under the modern practice of high-density production conditions.

Genetic selection for disease-resistance breeds is a potential strategy to be incorporated in NE control programs (Swaggerty et al., 2016; Truong et al., 2015). Selection markers including

elevated immune-related genes and antimicrobial mediators allow for the identification of chicken breeds with stronger resistance against *C. perfringens* infection. Immune modulation in chicken is another promising strategy to enhance resistance against NE infection. Vaccines are well-established immunomodulators that depend on adaptive immune memory. However, the most effective strategies to date depend on multiple-dose regimens which have limited relevance in the field, where a single application at hatch is more practical (Mot et al., 2013 and 2014). Immune modulation by feed additives, including probiotics, organic acids, essential oils, and microbial products, have been tested *in vivo* and shown to be at least partially protective (Khalique et al., 2020).

1.4.1.8 Experimental disease models

A consistent experimental induction of NE, especially the subclinical form, has proved challenging. The disease outcome was not always predictable even when the same research team used the same method (McDevitt et al., 2006). Current experimental models typically induce the clinical disease driven by inoculation of *C. perfringens* culture. One problem is there are a variety of methods employed by different researchers which may lead to inconsistency among experiments (Bortoluzzi et al., 2019; Latorre et al., 2018; Lee et al., 2011). *C. perfringens* inoculation has been accomplished by various administration routes (oral gavage, in-feed application, etc.), bacteria dosage, and exposure durations. Johansson (2006) summarized that NE induction via oral *C. perfringens* inoculation has produced varying results. It was also pointed out by the author that the *C. perfringens* already existed in the natural flora, not the inoculated strain, might be responsible for causing lesions.

In recent years, a novel approach to induce NE infection has gathered increasing attention, which is based on animals' natural uptake of *C. perfringens* from the environment. Multiple research groups have reported that exposure to NE-related risk factors, without inoculation of bacterial culture, is sufficient to produce subclinical NE in a flock (Abildgaard et al., 2010a; Calik et al., 2019; Emami et al., 2019 and 2021; Fernando et al., 2011; Løvland et al., 2003; Palliyeguru et al., 2010 and 2011). This approach allows the natural development of pathogens comprising part of the gut flora, thus mimicking the complex NE pathogenesis and disease outcome similar to field conditions. Although the published natural models are successful in terms of replicating subclinical disease, there are inconsistencies among studies in certain disease outcomes, such as lesion intensity and timing of NE outbreak. This suggests future optimization is needed to develop a reliable natural infection model.

1.4.2 The avian immune system and its role against microbial infection

The avian immune system comprises a repertoire of organs, cells, molecules, and genes that contribute to antimicrobial response during NE infection. Remarkably, innate immunity is of crucial and controlling importance, for its role in an initial response to pathogens, which limits infection and determines the subsequent responses by long-term immunity mediated by the adaptive systems (Kaiser, 2010). The inflammatory response is a critical defense mechanism of innate immunity against infection, which describes a series of local and systemic responses facilitated by cellular and molecular mediators.

Classically, innate immunity was differentiated from adaptive immunity by several characteristics, including rapid and non-specific response, and incapable of generating long-term memory (Juul-Madsen et al., 2014). However, the classic distinction between innate and adaptive

immunity was blurred since the discovery of pattern recognition receptors (PRRs), which allow innate immune cells to recognize microorganisms specifically at some level (Janeway et al., 2002). In the past years, a growing body of evidence has revealed the existence of memory in innate immunity, which is considered an important feature of innate defense in parallel with the classical T/B cell-mediated adaptive memory (Netea et al., 2016).

1.4.2.1 Pattern recognition receptors (PRRs)

The innate immune system recognizes microbial components, known as pathogen-associated molecular patterns (PAMPs), via germline-encoded PRRs (Juul-Madsen et al., 2014). There are up to 100 types of PRRs expressed in multiple cell types, which recognize different PAMPs, activate specific signaling pathways, and induce distinct antimicrobial responses. Studies have reported that many types of PRRs are involved in the inflammatory responses induced by *C. perfringens*, such as toll-like receptors (TLRs) and nod-like receptors (NLRs) (Guo et al., 2015).

TLRs are the best-characterized family of PRRs in vertebrates. TLRs are expressed by both immune cells, such as macrophages and dendritic cells, as well as non-immune cells such as epithelial cells. In chickens, 10 TLRs have been discovered (Alkie et al., 2019). Generally, TLR1, TLR2, TLR4, and TLR6 recognize lipids/lipopeptides; TLR5 recognizes bacterial flagellin; TLR3, TLR7, and TLR21 recognize nucleic acids; TLR15 recognizes bacterial and fungal proteases. Dimerization of TLRs can increase the range of PAMPs that can be recognized by these TLRs in combination. TLR1 and TLR6, at least, have been shown to form functional heterodimers with TLR2 (Kaiser, 2010). TLR activation by cognate ligands leads to recruitment of adaptor proteins (e.g., MyD88, TRAM, TIRAP, and TRIF) that trigger distinct signaling pathways, culminating in nuclear factor kappa B (NF-κB) and mitogen-activated protein kinase

(MAPK) activation (Broom, 2019). Consequently, these result in a diverse spectrum of responses, such as cytokine and chemokine production, cell maturation, secretion of antimicrobial peptides, etc. (Juul-Madsen et al, 2014). In chickens, *C. perfringens* infection was shown to significantly increase the mRNA expression of TLR1, TLR4 and TLR15, as well as MyD88 and NF-κB (Wang et al., 2020).

C-type lectin receptors (CLRs) are important for fungi recognition and are considered to complement TLR ligand binding and downstream responses of innate immune cells (Nerren and Kogut, 2009). One important CLR, known as dectin-1, is the primary sensor for β-glucan, a component of fungal and bacterial cell walls. Dectin-1 is predominantly expressed on myeloid cells such as macrophage/monocyte and neutrophils. Upon activation, dectin-1 triggers intracellular signaling involving CARD9, Syk kinase, and Raf-1, inducing the production of several cytokines, such as Tumor necrosis factor α (TNFα), interleukin (IL) -2, IL-6, and IL-23.

Nod-like receptors (NLRs), including the NODs and the NALP subfamilies, are cytoplasmic PRRs that interact with intracellular PAMPs (Kaiser, 2010). After ligand recognition, NLRs trigger inflammatory caspases, leading to the activation of pro-inflammatory cytokines and/or NF-κB pathway. NODs recognize bacterial peptidoglycan, while the exact ligands for NALPs are not fully understood.

1.4.2.2 Cellular mediators

The cellular components of the innate immune system range from epithelial and stromal cells, to more classically defined immune cell types (Juul-Madsen et al., 2014). These include myeloid origin cell types such as monocytes, macrophages, and granulocytes, and those derived from lymphoid progenitors, such as natural killer cells. The myeloid phagocytic cells, including

monocyte/macrophages, heterophils, basophils, and eosinophils, are known as professional phagocytes (Riera et al., 2016). Upon activation via PRRs, these cells phagocytose and destroy pathogens, release cytokines and other soluble mediators like histamine, reactive oxygen species (ROS), reactive nitrogen species (RNS), lysozyme, and antimicrobial peptides. The phagocytosed antigens are subsequently presented to B or T cells, contributing to the activation of the adaptive immune response.

1.4.2.2.1 Heterophils

Avian heterophils, the counterpart to mammalian neutrophils, provide the first line of defense against bacterial pathogens by releasing a repertoire of microbicidal agents (Genovese et al., 2013). Heterophils are the most common circulatory leukocyte in the majority of avian species (Claver and Quaglia, 2009). During pathogen invasion, heterophils migrate to the infection site through chemotaxis, a process where they are attracted via chemokines produced by resident leukocytes. The heterophil activation via PRRs, such as TLRs and dectin-1, then results in pathogen killing by oxidative burst, production of extracellular traps (containing DNA and histones), phagocytosis, and cellular degranulation (Genovese et al., 2013). A variety of antimicrobial peptides, adhesion molecules, enzymes, and other toxic mediators in the cytoplasmic granules are released during heterophil degranulation to fight the invading agents.

Heterophils in neonatal chicks display impaired phagocytosis, degranulation, and oxidative burst, which is correlated with susceptibility to bacterial infection (Alkie et al., 2018). This inefficiency in function continues until approximately 21 days of age, overlapping the period where NE is prone to occur. Compared to mature birds, the newly-hatched chicks can express the same spectrum of TLRs. Stimulation with TLR agonists was shown to enhance phagocytosis and

degranulation in young chicks challenged with bacterial infection (Mackinnon et al., 2009). In response to dectin-1 stimulation, chicken heterophils showed significantly increased production of ROS (Nerren and Kogut, 2009). This indicates the innate responses of heterophils in young birds can be boosted to the adult level and contribute to disease resistance at a susceptible age.

1.4.2.2.2 Monocytes and macrophages

Macrophages originate from hematopoietic stem cells in the bone marrow by differentiating stepwise into monocytes, which enter the bloodstream and subsequently migrate into various tissues as resident macrophages (Qureshi et al., 2000). Alternatively, tissue-resident macrophages are also capable of self-renewing *in situ* (Munro and Hughes, 2017). Monocytes serve as a major phagocytic cell type in chicken circulation, while tissue-resident macrophages are widely distributed and play a central role in antimicrobial defense and maintenance of homeostasis (Kaspers and Kaiser, 2014). Some of the classical macrophage functions include phagocytosis, respiratory burst, cytokine, and chemokine responses. Macrophage phagocytosis is mediated by specific cell surface receptors, such as CLRs, scavenger receptors, Fc receptors, and complement receptors. The recognized pathogens are internalized into phagosomes which are subsequently fused with lysosomes, and destroyed by antimicrobial peptides and enzymes. Upon pathogen detection, resident macrophages also induce the secretion of signaling molecules that activate and amplify the antimicrobial responses. One example is the release of ROS, which not only contributes to direct pathogen killing but activates immune signaling pathways, such as NF-κB and MAPK pathways (Iles and Forman, 2002). Another example is cytokine production that greatly influences the local and systemic immune response.

Macrophages display diverse and plastic functional phenotypes in response to various environmental cues (e.g., microbial products, damaged cells, activated lymphocytes). The M1/M2 polarity represents two extreme states of macrophage activation, and imbalances of macrophage polarization are frequently associated with disease conditions (Wang et al., 2014). The M1 macrophages are associated with pathogen killing, characterized as increased production of ROS, nitric oxide (NO), and proinflammatory cytokines. In contrast, the M2 macrophages are important for homeostasis maintenance, generating anti-inflammatory cytokines, such as IL-10, and a very low level of pro-inflammatory cytokines.

Chicken macrophages are highly responsive to several immune stimulants, such as LPS and β-glucan (Qureshi, 2003). Modulation of macrophage function is therefore considered an important approach when dealing with poultry diseases, which can be achieved by genetic selection, dietary manipulations, and new vaccine strategies (i.e., use of novel adjuvants).

1.4.2.2.3 Lymphocytes

Lymphocytes are classically related to adaptive immunity, while recent insights have revealed their role in the innate response during the early stages of infection. Multiple innate lymphocyte subsets, in mammals, are known to coordinate protective responses through direct cytotoxicity, secretion of tissue-protective factors, and production of cytokines (Gasteiger and Rudensky, 2014). Like helper T (Th) cell subsets in adaptive immunity, natural killer (NK) cells, Th2-type innate lymphocytes, and RORγ+ lymphoid tissue inducer-related (LTi) cells play distinct roles in innate immunity by producing Th1, Th2, and Th17, cytokines, respectively (Koyasu and Moro, 2012). NK cells are the primary early source of Th1 cell-associated cytokines like Interferon-γ (IFNγ) and TNFα (Meijerink et al., 2021). Chicken NK cells are characterized as large granular

lymphocytes, expressing CD8 but lacking both B cell and T cell receptors. About 50% of the total NK cells in chickens are found in the intestinal epithelium (Abdolmaleki et al., 2018). These cytotoxic lymphocytes are capable of destroying infected cells during the early phase of infection and amplifying the protective response by other leukocyte populations via cytokine secretion. Th2 cells control immunity to extracellular parasites and allergic inflammatory responses. In mice, the recently defined natural helper cells, also termed nuocytes, are innate lymphocytes associated with Th2 responses by producing IL-5 and IL-13 (Koyasu and Moro, 2012). Th17 responses are critical for neutrophil-mediated inflammatory responses, which contribute to antibacterial and anti-coccidial defense in chickens (Kim et al., 2019; Tang et al., 2018). The signature cytokine, IL-17, triggers neutrophil recruitment, antimicrobial peptide production, as well as cytokines and chemokines secretion, in particular IL-6, IL-8, and Granulocyte-macrophage colony-stimulating factor. However, the avian Th2 and Th17-type innate lymphocyte populations remain to be identified.

Another important helper T cell lineage described in adaptive immunity is regulatory T cell (Treg). Tregs inhibit Th1, Th2, and Th17 cells via direct contact and releasing cytokines such as IL-10 and transforming growth factor β (TGF-β) to maintain immune tolerances (Huang and Chen, 2016). Recent evidence suggests Tregs also interact with innate cells including macrophages, neutrophils, and NK cells (Okeke and Uzonna, 2019). Tregs can direct macrophage differentiation to the M2 phenotype via IL-10 and TGF-β dependent mechanisms. This has indicated the importance of the cross-talk between Tregs and innate immune cells for the regulation of inflammation and maintenance of immune tolerance.

1.4.2.3 Cytokines and chemokines

Cytokines are regulatory peptides that act as extracellular signals between cells during immune response as well as in immunological development (Kaiser and Stäheli, 2014). These low molecular weight proteins, typically less than 30 kDa, are widely produced by various cell types, having pleiotropic effects contributing to immune regulation. The acute inflammatory reaction initiates the production of a number of pro-inflammatory cytokines, such as IL-1β, IL-6, and TNF-α (Al-Khalaifah and Al-Nasser, 2018). These cytokines are produced by both leukocyte and non-immune cells, such as epithelial cells and fibroblasts at the infection site. A further group of regulatory molecules during inflammatory response is the chemokine, which regulates leukocyte traffic in a process termed chemotaxis. *C. perfringens* infection in chickens significantly upregulated the mRNA expression of inflammatory cytokines, such as IL-6, IL-8, and TNF-α (Guo et al., 2015; Wang et al., 2020).

1.4.2.3.1 IL-1β

As of today, 4 IL-1 family members have been characterized in chicken, including IL-1β, IL-1 receptor antagonist (RN), IL-36RN, and IL-18 (Gibson et al., 2014). Their biological effects on target cells are mediated by the IL-1 receptor (IL-1R) family, which is part of a wider superfamily of Toll/IL1-R (TIR) domain-containing receptors. TIR domains interact with different adapter proteins, such as MyD88, TIRAP, and TRIF (Karnati et al., 2015). MyD88 and TIRAP trigger activation of NF-κB and the MAPK signaling cascade, activating a series of chemokines and cytokines that contribute to inflammation responses. TRIF activates interferon regulatory factors and induces IFN production, and it also activates NFκB and MAPK pathways. IL-1-mediated inflammation can be inhibited by IL-1RN, a naturally occurring receptor antagonist.

IL-1β was the first chicken IL-1 ligand identified and its roles in antimicrobial defense are well described (Gibson et al., 2014). As a rapidly-induced proinflammatory mediator, chicken IL-1β was shown to be upregulated in response to bacterial, viral, and parasite challenges. IL-1β is synthesized as a biologically inactive precursor and needs to be cleaved by caspase-1 to generate the functional cytokine (Mills and Dunne, 2009). Caspase-1 is activated following the assembly of the inflammasome complex, a process triggered by members of the NLR receptor family upon sensing danger signals, such as microbial components (Pétrilli et al., 2007). Caspase-1 also processes IL-18, which subsequently stimulates IFN-γ secretion (Göbel et al., 2003). IL-1β binding to the target cells triggers activation of a range of proinflammatory cytokines, including IL-6, IL-8, TNF-α, and IL-1β itself.

1.4.2.3.2 IL-6

IL-6 is a multifunctional cytokine that regulates inflammatory responses, acute phase reactions, and hematopoiesis (Schneider et al., 2001). A wide spectrum of cells produce IL-6, including fibroblasts, endothelial cells, neuronal cells, macrophages, and T cells (Rincón et al., 1997). The synthesis and secretion of IL-6 are induced during inflammatory conditions via TLR activation or stimulation by IL-1 or TNF-α. Upon binding to its target cells, the IL-6/IL-6R complex interacts with gp130, a transmembrane protein, to initiate intracellular signaling termed classic IL-6 signaling (Rose-John, 2012). IL-6R is only present on a few types of cells, including hepatocytes and some leukocytes, while gp130 is expressed on all cells. During the inflammatory response, membrane-bound IL-6R undergoes ectodomain shedding, leading to the generation of soluble IL-6R (sIL-6R). Cells that do not express IL-6R can respond to the IL-6/sIL-6R complex via gp130 activation, a process termed IL-6 trans-signaling. It was established that IL-6R

shedding from neutrophils, the first cells to arrive at the infection site, leads to the stimulation of endothelial cells, which lack membrane IL-6R.

Acute phase responses describe a series of systemic and metabolic changes under infection or inflammation (Juul-Madsen et al., 2014). IL-6 is the major inducer of the hepatic acute phase responses via classic IL-6 signaling (Schmidt-Arras et al., 2016). This leads to the secretion of acute phase proteins, which function as protease inhibitors, transport proteins, as well as components of the complement system, inflammatory responses, and the coagulation cascade. IL-6 trans-signaling mediated by sIL-6R controls the pro-inflammatory infiltrate by regulating leukocyte apoptosis as well as the expression of chemokines and adhesion molecules (McLoughlin et al., 2005). Evidence has also shown that IL-6 activates macrophage hematopoiesis, and directs lymphocyte differentiation towards Th1/Th17 responses while inhibiting the TGF-β induction of Tregs (Chomarat et al., 2000; Maes et al., 2014).

1.4.2.3.3 TNF-α

TNF-α plays an important role in systemic inflammation, cytotoxicity to tumor cells, and apoptosis (Al-Khalaifah and Al-Nasser, 2018). Its roles in the propagation of inflammation include activation and recruitment of immune cells, induction of respiratory burst by activating ROS and RNS producing enzymes.

TNF-α is produced predominantly by activated macrophages and T lymphocytes as pro-TNF, which is expressed on the plasma membrane, where it can be cleaved and released in a soluble form (Bradley, 2008). Both transmembrane and soluble TNFs are involved in inflammation and exert their biological function in a cell-to-cell contact fashion or act at remote sites, respectively (Horiuchi et al., 2010). TNF-α converting enzyme mediates release of TNF-α from the cell

membrane, and this enzyme is also involved in processing several surface proteins, including TNF receptors and IL-6 receptors (Gooz, 2010). Responses to TNF-α are triggered by binding to one of two distinct receptors, TNFR1 and TNFR2, both involved in NF-κB activation. It was shown that over 200 molecular associations are involved in the modulation of the TNF–NF-κB pathway (Bouwmeester et al., 2004). Interaction with both identical and unrelated molecules by TNFR1 and TNFR2 cause distinct yet shared biological responses. Ligation of TNFR1 is necessary and sufficient to induce cytotoxic and pro-inflammatory responses, whereas TNFR2 is more related to promotion of cell activation, migration, or proliferation (Bradley, 2008). Under certain circumstances, TNFR2 captures TNF-α and passes it to TNFR1, thus contributing to TNFR1 responses. This may be mediated by the ligand-induced formation of TNF receptor heterocomplexes. In response to TNF-α, vascular endothelial cells undergo a range of pro-inflammatory changes, which increase leukocyte adhesion, transendothelial migration, and vascular leak (Bradley, 2008). In combination with the release of chemokines, these responses result in the robust recruitment of different leukocyte populations.

Recent work by Rohde et al. (2018) led to the identification and functional characterization of the chicken TNF-α. Both the full-length cytokine and the extracellular domain rapidly induced an NFκB-luciferase reporter in CEC-32 reporter cells. Before that, the LPS-induced TNF-α factor (LITAF) was considered as TNF-α in many studies, which had created confusion (Elleder and Kaspers, 2019). LITAF is a transcription factor regulating the TNF superfamily and plays a role in the antibacterial and anticoccidial activity of chicken macrophages (Hong et al., 2006). In response to LPS stimulation, mammalian macrophages induce expression of LITAF, which binds the TNF-α gene promoter and activates transcription of the cytokine.

1.4.2.3.4 IL-8

IL-8, also known as CXCL-8, is a member of the inflammatory chemokine family that functions as a leukocyte chemoattractant and potent angiogenic factor (Kim et al., 2017). In response to appropriate stimulation, IL8 can be released by a wide variety of cells, including monocytes, macrophages, endothelial cells, epithelial cells, T lymphocytes, and fibroblasts (Russo et al., 2014). In mammals, IL-8 acts on CXCR1 and CXCR2 receptors, which are expressed on numerous leukocyte subsets, including neutrophils, monocytes, CD8+ T cells, and NK cells. Activation of either receptor on leukocytes induces chemotaxis and calcium flux. In neutrophils, IL-8 ligation stimulates ROS production and the release of granule enzymes. Other cell types possessing CXCR1/CXCR2 include epithelial, endothelial, fibroblasts, and neurons, contributing to the biological effects of IL8, such as angiogenesis.

The chicken appears to have evolved two IL-8-like chemokines, CXCLi1 and CXCLi2, both binding chicken CXCR1 (Poh et al., 2008). CXCLi2 is more similar in sequence to human IL-8 and was first named chemotactic and angiogenic factor (CAF). As its name suggests, it is chemotactic for monocyte/macrophages and lymphocytes, and also stimulates sprouting and growth of blood vessels at high concentrations. CXCLi1, also known as K60, was later identified in the LPS-stimulated chicken macrophage cell line HD11. Both CXCLi1 and CXCLi2 play a role in inflammatory responses against bacterial and viral infection in chicken (Qi et al., 2017; Smith et al., 2008). The adjacent genomic location of CXCLi1 and CXCLi2, and their high sequence similarity suggest they arise as a duplication of an ancestral IL8 gene in the avian (Poh et al., 2008).

1.4.2.4 Reactive oxygen and nitrogen species

The release of reactive oxygen and nitrogen intermediates represents an important antimicrobial mechanism of activated macrophages and heterophils. It is well established that chicken macrophages are activated to produce reactive oxygen species (ROS) and nitric oxide (NO) in response to microbial stimulation (He et al., 2011). This antimicrobial response is under the regulation of multiple cytokines, such as IFN-γ and IL-4.

The NADPH oxidase pathway and inducible nitric oxide synthase (iNOS) pathways, two parallel yet connected pathways, are responsible for the generation of superoxide ($O_2^{-}\cdot$) and $NO\cdot$ radicals, respectively (Fang, 2004). $O_2^{-}\cdot$ and other oxygen-derived intermediates are known as ROS, and $NO\cdot$ and its derivatives are collectively referred to as reactive nitrogen species (RNS). The components of the NADPH oxidase complex comprise a number of membrane and cytosolic proteins (Vignais, 2002). Cytokines, including TNF-α and IFN-γ, as well as microbial products can modulate the transcription of the genes encoding oxidase components (Fang, 2004). iNOS is not constitutively expressed but increases transcription in response to PRR stimulation and pro-inflammatory cytokine signaling, such as IL-1β and TNF-α. There are two more isoforms of NOS: neuronal nitric oxide synthase (nNOS) and endothelial NOS (eNOS), which are constitutively expressed to produce NO, functioning in different physiological conditions (Predonzani et al., 2015).

In the chicken, production of ROS and NO is an important antimicrobial effector against *C. perfringens* infection (Mora et al., 2020; Zhang et al., 2017). ROS and RNS can damage many microbial components, such as lipids, proteins, and nucleic acids (Fang, 2004). During phagocytosis of pathogens, ROS are generated at the phagosome membrane and released directly

into the phagosome (Fialkow et al., 2007). Additionally, macrophages, neutrophils, and heterophils can release extracellular ROS to attack pathogens in the surrounding environment (Dupré-Crochet et al, 2013; Han et al., 2019). Soluble stimuli, such as chemotactic peptides, were shown to trigger oxidase assembly in the plasma membrane leading to extracellular ROS secretion.

ROS and RNS are critical in antimicrobial defense not only because they can destroy pathogens, but also because of their role in immune regulation (Nathan et al., 2000). ROS are produced by nearly all organisms and cells, and its role as signaling molecules in physiological and immunological responses has been well characterized (Herb and Schramm, 2021). ROS were shown to regulate the induction of inflammasomes and cytokine secretion via redox-regulation of immune signalings, such as the NF-κB pathway. These signaling effects are not limited to the ROS-producing cells. The cross-membrane diffusion of extracellular ROS, such as H_2O_2, can subsequently enable ROS signaling in neighboring cells (Dupré-Crochet et al, 2013). It is considered that a less amount of ROS is required in signaling transduction than in pathogen killing. As for RNS, their roles as a master regulator for the activity of macrophages, T lymphocytes, NK cells, and dendritic cells are well documented (Predonzani et al., 2015). RNS were shown to inhibit G proteins, activate or inhibit kinases, caspases, metalloproteases, transcription factors, and DNA methyltransferase (Dupré-Crochet et al, 2013), contributing to the regulation of immune responses.

1.4.2.5 Organs

1.4.2.5.1 Liver

The liver is the largest solid organ in the body of the vertebrates, which plays a central role in the innate immune response to infection (Gao et al., 2008). It has been well characterized as a key mediator in acute phase responses during *C. perfringens* infection (Ruhnke et al., 2017; Saleem, 2012).

Cells within the liver are subject to persistent signaling from the hepatic blood supply and subsequently contribute to the regulation of immune activation and homeostasis maintenance. 80% of the liver volume is made up of hepatocytes which fulfill both metabolic and detoxifying functions of the body (Gao et al., 2008). Hepatocytes are critical for systemic innate responses via the production of secreted proteins involved in host defense (Li et al., 2007). During infection, the increased pro-inflammatory cytokines produced at the challenge site, especially IL-6, IL-1, and TNF-α, can reach the liver through circulation, and trigger hepatocytes to produce antimicrobial peptides, complements, and secreted PRRs. In chickens, liver-derived antimicrobial peptides were shown to play a role in defense against both Gram-positive and Gram-negative bacteria. Hepatocytes are the major sources for the production of secreted PRRs, which function in complement activation and microbial cell opsonization for phagocytosis (Gao et al., 2008). The liver is also the primary producer of many other acute phase proteins, contributing to innate defenses against infection, as well as reducing tissue damage through the inactivation of proteinases released by pathogens and damaged cells.

The nonparenchymal cells in the liver are represented by endothelial cells, stellate cells, and an abundant reservoir of immune cells (Robinson et al., 2016). The liver-resident macrophages,

known as Kupffer cells, account for over 80% of the total population of tissue-resident macrophages, which are responsible for the elimination of insoluble waste by phagocytosis (Gao et al., 2008). These cells recognize and destroy microbial components present in the blood circulating from the intestine, an environment rich in bacterial products, toxins, and food antigens. Other hepatic immune cell types, such as dendritic cells, neutrophils, and NK cells, also play a role in antimicrobial responses (Freitas-Lopes et al., 2017).

Besides expressing strong innate defense mechanisms, the liver is also a major site of inducing immune tolerance. The dendritic cells, endothelial cells, Kupffer cells, and stellate cells, are involved in mediating immunosuppression by secretion of anti-inflammatory cytokines such as IL-10 and TGF-β (Tiegs and Lohse, 2010). Kupffer cells were also shown to interact with hepatic Tregs to create a local suppressive microenvironment, leading to systemic T cell tolerance (Breous et al., 2009).

1.4.2.5.2 Spleen

The spleen mounts both the innate and adaptive responses in a uniquely organized way, making it an important organ for immune regulation (Mebius and Kraal, 2005). The spleen consists of the blood-containing red pulp, and the white pulp, which is full of lymphoid cells. The red pulp is a blood-filtering system, in which pathogens, cell debris, and aging erythrocytes are efficiently removed by splenic macrophages. The white pulp is a highly organized lymphoid region that can initiate adaptive immune responses. The chicken white pulp contains periarteriolar lymphoid sheaths (PALS), surrounding the central arteries, and the periellipsoidal white pulp (PWP) that surrounds the penicillary capillaries (Oláh et al., 2014). The PALS is a dense sheath of T lymphocytes, which are most involved in adaptive immunity. The PWP is described as

analogous to the mammalian marginal zone, which bridges between innate and adaptive immunity (Mebius and Kraal, 2005). This region contains discrete subsets of B cells and macrophages, and the latter can recognize and take up pathogens via PRRs expressed on the surface. The B cells subset can be activated by the macrophages, or directly react to the pathogens, after which they become antigen-presenting cells or plasma cells.

1.4.2.5.3 Intestine

The intestinal epithelium comprises various cell lineages, derived from a common stem cell progenitor (Santaolalla and Abreu, 2012). These are absorptive enterocytes, mucus-producing goblet cells, hormone-producing enteroendocrine cells, and Paneth cells which produce antimicrobial peptides. The lamina propria located beneath the epithelium contains a mixture of immune cell subsets, including macrophages, granulocytes, and lymphocytes. In response to microbial infection, the epithelial cells and immune cells recognize the pathogens by PRRs, which trigger the initiation of the innate immune response, characterized by NF-κB activation, cytokine production, and chemokine-mediated leukocyte recruitment. The epithelial cells express a diverse set of antimicrobial molecules (Eckmann, 2004), which possess broad-spectrum defense activities via direct binding and lysis of microbial membranes, as well as neutralizing bacterial endotoxins (Shao et al., 2016). It was demonstrated that chicken intestinal epithelium expresses β-defensin and cathelicidins which play a role in immunoprotection against *Salmonella Enteritidis* (Derache et al., 2009; Shao et al., 2016).

The intestinal epithelium is constantly exposed to both pathogenic and commensal microbes, thus it is important for the host to mount effective antimicrobial defenses while maintaining intestinal homeostasis. Gut phagocytes, such as macrophages and dendritic cells, have pivotal

roles in the maintenance of gut homeostasis (Aychek and Jung, 2014; Chistiakov et al., 2015; Jiao et al., 2020). The tolerogenic phenotypes of these cells are modulated by complex signals, including commensal microbiota, apoptotic cells, as well as TGF-β and IL-10 produced by enterocytes. Under appropriate stimulation, macrophages and dendritic cells trigger downstream responses of innate lymphoid cells via cytokine production, which promote Treg generation and immune suppression.

Innate responses can initiate downstream adaptive responses against the encountered pathogens or commensals (Brisbin et al., 2008). The Th-dependent responses to pathogens elicit antibody responses of high affinity and specificity, while responses to commensal bacteria are of broader specificity and lower affinity. Chickens lack encapsulated lymph nodes like those in mammals, but diffuse lymphoid tissue is well developed (Oláh et al., 2014). The gut-associated lymphoid tissue (GALT) is the main site for the induction of humoral responses towards gut flora. The GALT consists of organized lymphoid tissues and dispersed lymphocytes. The organized lymphoid tissues are made up of cecal tonsils, Peyer's patches, Meckel's diverticulum, the bursa of Fabricius, and various lymphoid aggregates located along the intestinal tract (Brisbin et al., 2008). The dispersed intraepithelial lymphocytes (IELs) are part of the intestinal mucosal barrier along with enterocytes, goblet cells, and Paneth cells. IELs comprise a diverse population of lymphocytes such as NK cells, T cells, and B cells. Intestinal IgA production by B cells is an important adaptive mechanism to prevent the entry of bacteria into the subepithelium.

1.4.2.5.4 Bursa of Fabricius

The bursa of Fabricius is the primary lymphoid organ for B cell development uniquely found in birds. It provides the environment for B cell lineage specification and commitment, thus plays an

important role in antibody response against microbial infection (Sun et al., 2015). During embryonic development, B cell precursors colonize the bursa anlage, where they differentiate and expand their B cell receptor repertoire. At hatch, these diversified B cells exit the bursa of Fabricius and populate peripheral lymphoid organs, where they can encounter antigen and differentiate into antibody-producing cells (Nagy et al., 2020). It was shown that removal of the bursa in rapid-growing chicken diminished the antibody response to *Salmonella* infection (Taylor and McCorkle, 2009). Appropriate immunomodulation can stimulate the bursa, increasing the lymphocyte count per follicle, resulting in higher serum IgA levels and stronger resistance against bacterial infection (Villagrán-de la Mora et al., 2020).

1.4.2.6 Host immunity in *C. perfringens* colonization

C. perfringens is widely distributed in animal production environments such as housing structures, litter, contaminated feed, and water. This bacterium colonizes the chicken gut flora early in life during hatch. Evidence has indicated the ability of *Clostridium spp.* to induce immune tolerance as a survival strategy to persist as part of the gut microbiota. The main cell wall component of *C. perfringens*, peptidoglycan, is capable to induce unresponsiveness of immune cells via repeating TLR/NOD stimulation (Fu et al., 2012; Guo et al., 2015; Hedl et al., 2007; Ifrim et al., 2014). Furthermore, metabolic regulation and Treg-mediated immune tolerance in the host intestinal epithelium are also known to be involved.

Chickens are hatched with a sterile intestinal tract, and epithelial cells rely on glucose from the circulation as an energy source (Volf et al., 2016). Birds at this age possess highly expressed glycolytic enzymes, such as fructose-bisphosphate B (ALDOB). As the gut flora develop and start to produce short chain fatty acids (SCFAs), the enterocytes decrease ALDOB expression

and switch from glucose to SCFA metabolism. By serving as donor substrates for generating acetyl-CoA, SCFAs provide metabolic input which modulates the histone acetylation and hence gene expression (Luu et al., 2019). *Clostridium spp.* fuel enterocytes by releasing SCFAs, mostly butyrate, which inhibits the activation of NF-kB (Lopetuso et al., 2013). Butyrate also modifies cytokine expression through its action as a non-competitive inhibitor of histone deacetylases, leading to an anti-inflammatory effect. It was shown in newly-hatched chicks that the intestinal tract shows alteration in cytokine level as the gut microbiota develop (Crhanova et al., 2011).

Uptake of *Clostridium* antigens by lamina propria dendritic cells leads to the production of retinoic acid and other regulatory molecules, which activates naïve CD4+ T cell differentiation into Treg cells (Nagano et al., 2012). In mice, colonization of human commensal *Clostridium spp.* developed an IL-10 and TGF-β-rich environment, which supports induction, proliferation, and expansion of Tregs (Atarashi et al., 2011). It has been reported that intestinal IL-10 mRNA was significantly upregulated in *C. perfringens* infected chicken (Collier et al., 2008). IL-10, IL-4, IFN-γ are also correlated with changes in bacterial communities during the post-hatch period (Kumar et al., 2018).

1.4.2.7 Host immunity in NE infection

In response to acute NE infection, the chicken body generates systemic responses involving multiple organs, such as the intestine, spleen, and liver. Our current understanding of host responses against this disease is predominantly based on experimental NE induced by *Eimeria*/*C. Perfringen*s coinfection. Intestinal cytokine expression following the NE challenge suggests a complex response involving proinflammatory (IFN-α, IL-1β, IL-6, IL-8, IL-17, LITAF) and anti-inflammatory (TGF-β4) cytokines, as well as Th1 (IFN-γ, IL-2, IL-10, IL-12, IL-15, IL-16) and

Th2 (IL-13) cytokines (Park et al., 2008). Kim et al. (2014), using a microarray hybridization approach, reported 1,049 transcripts of chicken intestinal lymphocytes that were altered in response to NE. Biological functions and pathways were identified from the differentially regulated genes, including leukocyte trafficking, LPS/IL-1 mediated inhibition of retinoid X receptor functions, hepatic stellate cell activation, tight junction signaling, pyruvate metabolism, and HIF-1 signaling, all of which were related to host immunity and inflammation. In the spleen, inflammation-related genes were shown upregulated during NE infection, such as IL-1β, IL-6, TNFSF15, galectin 3, and IFNAR1 (Sarson et al. 2009; Zahoor et al., 2018). Liver enlargement typically found in NE-affected animals is associated with robust systemic inflammation (Xue et al., 2018). Enhanced production of acute phase proteins has been well characterized in NE-affected animals. It was reported that the level of serum α-1 glycoprotein (Ruhnke et al., 2017) and ceruloplasmin (Saleem, 2012) were significantly increased at 1 day post-infection. Hong et al. (2014) described the role of intestinal and splenic microRNAs in NE resistance by post-transcriptional regulation of their target genes. This includes modulation in several inflammatory genes, such as suppressor of cytokine signaling 3 (SOCS3), serpin peptidase inhibitor member 1 (SERPINF1), TNF receptor-associated factor 3 (TRAF3), TNFSF11β, TNFRSF21, and CXCL14.

The activation of innate immunity and complex downstream signaling eventually lead to the development of adaptive immunity. This process involves the development of intraepithelial CD8+ cytotoxic and CD4+ T helper populations, which increase in a time-dependent manner through day 10 to16 post-infection (Ruhnke et al., 2017). Generally, adaptive immune defense at the mucosal surface is mediated by lymphocyte activation and local secretion of IgA (Mot et al., 2014). IgY also plays a role in the passive protection of young chickens as it is the major

transferred maternal antibody. However, maternal antibodies decline by around 3 weeks of age, which could explain why NE outbreak typically occurs around this time (Mot et al., 2014).

1.4.3 Immune memory

Immune memory is defined as the ability of the host to recognize and generate robust secondary responses against previously encountered pathogens (Sharrock and Sun, 2020). Historically, memory was described as the hallmark of adaptive immunity, exclusively owned by memory B and T cells. The newly defined innate immune memory describes a phenotypically functional change in innate cell types, which does not involve the generation of memory cells or clonal expansion. Innate memory can be expressed as trained immunity or innate immune tolerance, where the former leads to enhanced responses while the latter results in unresponsiveness towards secondary stimulation (Netea et al., 2016).

Studies investigating innate memory were carried out mostly focused on macrophages and monocytes. Pre-exposure to β-glucan, BCG, or oxidized low-density lipoprotein (oxLDL) was demonstrated to enhance cytokine responses upon secondary infection with non-related stimuli (Bekkering et al., 2016). In contrast to β-glucan or BCG-induced trained immunity, the priming of immune cells with repeating or large dosage of LPS has long been known to induce tolerance, resulting in quiescence towards secondary stimulation (Fan et al., 2004). It is getting clear that modulation of innate memory involves reprogramming of the intracellular immune signaling and metabolic pathways, as well as systemic hematopoiesis at the bone marrow level.
Ifrim et al. (2014) identified the signaling pattern responsible for the induction of trained immunity and tolerance, suggesting that both the nature and concentration of the PAMPs encountered determine the functional fate of the cells.

1.4.3.1 Epigenetic regulation of innate immune memory

Following PRR activation, monocytes and macrophages were shown to undergo multiple levels of epigenetic modification, preparing them for subsequent challenges (Saeed et al., 2014). These changes include DNA and histone modifications, as well as chromatin remodeling. In quiescent myeloid cells, most of the proinflammatory gene loci are in a repressed configuration. As a consequence of the initial exposure, histone-modifying complexes reshape the coding loci from repressed configuration to an open structure, thus increasing transcription accessibility (Ramirez-Carrozzi et al., 2006). Part of these histone modifications is retained after clearance of the primary stimulus, which affects the transcription of corresponding genes during the subsequent challenge (Quintin et al., 2012; Novakovic et al., 2016). For example, histone H3 with a single methyl group on lysine 4 of its N-terminal tail (H3K4me1) was identified as an activated enhancer (Smale et al., 2014). In trained NK cells, H3K4me1 was shown to be enriched in the *ifng* locus, which is crucial for IFN-γ transcription (Rasid et al., 2019). Other histone modifications involved in innate memory include H3K4 trimethylation and H3K27 acetylation, both being active marks, and H3K9 trimethylation, being a repressive mark (Moorlag et al., 2018).

1.4.3.2 Metabolic regulation of innate immune memory

A metabolic shift not only meets the divergent energetic demand in trained immunity but also contributes to the regulation of immune pathways. Studies integrating transcriptomics and metabolomics data have identified glycolysis, glutaminolysis, and cholesterol synthesis as crucial in establishing β-glucan-induced trained immunity (Cheng et al., 2014; Domínguez-Andrés et al., 2019a).

In glycolysis, glucose is converted into pyruvate in the cytoplasm, which can be turned into lactate or acetyl-CoA. Acetyl-CoA can enter the tricarboxylic acid (TCA) cycle, transferring its energy to ATP via oxidative phosphorylation. β-glucan-trained monocytes tend to divert pyruvate into lactate production, a process mediated by the dectin-1–Akt–mTOR–HIF-1α pathway (Cheng et al., 2014). Upregulation of glycolysis is common during immune cell activation and can be found in activated T cells and proinflammatory macrophages (Wang et al., 2017; Menk et al., 2018). Compared with oxidative phosphorylation, glycolysis can produce ATP in a faster though less efficient manner. It was demonstrated that lactate inhibits histone deacetylase activity thus modulating gene accessibility to transcription machines (Latham et al., 2012). A switch toward glycolysis also results in higher intracellular ratios of NAD+/NADH, which act with sirtuin deacetylases to modulate monocyte function through chromatin modification (Jang et al., 2012; Cheng et al., 2014). The components in glycolysis can also affect immune function independently from histone modification. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a glycolytic enzyme that can reduce IFN-γ and TNF-α production by binding the 3' UTR of RNA, is utilized at a higher rate during enhanced glycolysis, thus allowing higher cytokine production (Chang et al., 2013; Millet et al., 2016).

Amino acid metabolism, closely connected with glucose metabolism, is also part of the regulation network of trained immunity. Glutamine metabolism fuels the TCA cycle through replenishing a-ketoglutarate and succinate. In β-glucan-trained monocytes, metabolism of glutamine was enhanced accompanied by an increased level of succinate (Arts et al., 2016). Cholesterol biosynthesis is connected to glucose metabolism via acetyl-CoA. Mevalonate, metabolites in the proximal step of the cholesterol synthesis, was shown to activate the mTOR–HIF-1α pathway and subsequently induce epigenetic changes (Bekkering et al., 2018).

1.4.3.3 Hematopoiesis regulation of innate immune memory

Trained immunity acts at the level of hematopoietic progenitor in the bone marrow to maintain the trained phenotype, which explains the long-term alteration in mature myeloid cells that are known to be short-lived (Netea et al., 2019). β-glucan stimulation was shown to promote hematopoiesis that flavors myelopoiesis, mediated by metabolic alterations in the progenitor cells (Mitroulis et al., 2018). This results in the establishment and long-term maintenance of novel open chromatin regions that are associated with genes involved in myeloid lineage commitment and innate immunity.

Cytokines play a crucial role in the induction of trained immunity through hematopoiesis regulation. Hematopoietic stem cells, which lack the β-glucan receptor dectin-1, act under the control of IL-1β induced in the bone marrow in response to β-glucan stimulation (Mitroulis et al., 2018). IL-1 priming was reported to enhance resistance against *Pseudomonas aeruginosa*, *Candida albicans*, *Listeria monocytogenes*, and *Staphylococcus aureus* in mice (Moorlag et al., 2018). Kaufmann et al. (2018) demonstrated that trained immunity induced by BCG vaccination diverts the bone marrow hematopoietic stem cells towards myelopoiesis in an IFNγ-dependent manner.

1.4.4 Contribution of innate immunity to vaccine immunogenicity

In many cases, antigen alone in a vaccine formula does not result in appropriate immune responses for development of immunity against infection (Mahla et al., 2013). Adjuvants are simple or complex compounds, which, when added to antigens used for vaccination, enhance their immunogenicity (Nawab et al., 2019). In mammalian studies, a number of PAMPs are considered as functional vaccine adjuvants, which mainly activate innate immune cells such as

macrophages and dendritic cells. β-glucan administration as an adjuvant with a candidosis vaccine was shown to enhance protection in mice against pathogenic *C. albicans* (Mahla et al., 2013). This adjuvant effect is associated with induction of innate cytokines such as IL-6, IL-12, TGF-β, and IL-1.

The insights in innate immune memory also open a new avenue in vaccinology to develop trained immunity-based vaccines (TIbV), targeting the memory of innate immune cells (Sánchez-Ramón et al., 2018). Induction of trained immunity can either increase the responsiveness of innate immune cells to pathogens or harness the activation state of dendritic cells to enhance T cell-mediated adaptive responses. TIbV is considered particularly useful when dealing with co-infections by multiple pathogens, or when conventional vaccines are not available. Conventional vaccines that trigger cross protection and vaccine adjuvants already fall in the TIbV concept.

As innate immune memory became a novel target to enhance vaccine immunogenicity in poultry, there is a need to understand the immunomodulation effect of trained immunity-inducers on disease resistance and growth performance. In chickens, several PAMPs have been classified as potential adjuvants for the use in vaccines to increase resistance against pathogenic infection. β-glucan, CpG Oligodeoxynucleotides and Poly I:C were shown to induce both specific and nonspecific immune responses to combat several bacterial, viral and parasitic diseases in avian species (Nawab et al., 2019). However, there is a lack of evidence whether trained immunity-inducers confer protective defense responses against the opportunistic infection of *C. perfringens*. My hypothesis is β-glucan redirect immune tolerance caused by early-life *C. perfringens* exposure, resulting in enhanced disease resistance against NE infection at later age.

Chapter 2: Materials and methods

2.1 Animals and establishment of a natural NE challenge

Ross 708 broiler chicks were obtained from a local hatchery (Sofina Foods) and housed in the Poultry Research Center at the University of Alberta, Edmonton, Canada. A total of 752 birds from three experiment flocks were involved in this project. Each of the three flocks were treated with a natural NE challenge model but with different levels of coccidiosis challenge intensity. The experimental flocks were reared in Specht pullet cages (21 × 23.5 × 17.5 inches, Specht Canada Inc.) or floor pens (0.9 × 1.4 m). Table 1 shows the flock size, housing type, and coccidial challenge intensity in each flock.

The natural NE infection model was developed using the three experimental flocks. Animals in flocks 1 and 3 were randomly assigned to two dietary treatments to evaluate the impact of antibiotic removal on NE development (flock 1: antibiotic treatment with 21 cages of 8 birds, and drug-free treatment with 22 cages of 8 birds; flock 3: each treatment with 8 pens of 18 birds). Animals in flock 2 were used for evaluating the immunomodulation effect of β-glucan, and were randomly assigned to three injection treatments (each with 5 cages of 8 birds).

Birds from all 3 flocks were fed a wheat-based diet formulated to meet or exceed the management guide recommendations for all nutrients (Table 2). The experimental diets were administered as a starter diet (day 1 to 13), grower diet (day 14 to 27), and finisher diet (day 28 to 40). Feed and water were provided *ad libitum*. Temperature and lighting were monitored daily and adjusted according to the Ross 708 guidelines (Aviagen, Inc., 2019). The wheat-based broiler chicken diets commonly used in Alberta include a xylanase to break down arabinoxylans

and decrease viscosity and increase nutrient digestibility (Lee et al., 2017). Elimination of the enzyme additive in our formulation in flocks 1 and 2 increased feed transit time and created conditions that promoted *C. perfringens* persistence (Choct et al., 2006). Another commonly used dietary predisposing factor, fishmeal, supplies abundant glycine and methionine that enhance *C. perfringens* proliferation and toxin production (Dahiya et al., 2007; Shojadoost et al., 2012; Wilkie et al., 2005). However, the usage of fishmeal is limited in broiler feed due to its high cost and low availability (Frempong et al., 2019). We therefore examined whether a fishmeal-free wheat-based diet with xylanase inclusion (flock 3) can be used for inducing experimental NE. On day 13, a concentrated dose of Coccivac-B52 vaccine (Merck Animal Health) containing live *Eimeria* oocysts was administered through oral gavage. Live coccidia vaccine challenges at 10× of regular concentration are commonly used to promote the development of experimental NE. This parasite-induced epithelium injury typically occurs before *C. perfringens* infection and no more than 7 days (Williams et al., 2003; Wu et al., 2011). In this study, two coccidial chanllenge concentration, 10× (flock 1) or 15× (flock 2 and 3), were tested for desired NE severity. Each bird received 1 ml of vaccine diluted in distilled water. On day 18, the feed was withdrawn for 24 h with animals being closely monitored for health over the subsequent 3 days. Figure 1 shows the predisposing factors application timeline in the natural NE challenge model.

2.2 Growth performance and sampling procedure

Growth performance was monitored for the starter phase, grower phase, and finisher phase (Table 2). Birds in each cage or pen were weighed as a group on day 1, 14, 28, and 40 (flock 1 and 2), or on day 1, 11, 26, and 38 (flock 3). Body weight gain, feed intake, and feed conversion ratio (FCR) were calculated on a cage or pen basis. For experimental flocks1 and 2, animals were

randomly selected and examined for NE disease status on day 17, 21, and 40 (flock 1: 8 birds per treatment, flock 2: 6 birds per treatment). Flock 3 was sampled on day 21 and day 40 (16 birds per treatment). For determining circulatory leukocyte profile, peripheral whole blood samples were collected into vacutainer tubes containing EDTA (BD Vacutainer) and maintained on ice until subsequent examination. Liver, Bursa of Fabricius, spleen, and empty small intestine were collected and weighed. Cecal contents were collected into 1.5 ml microtubes and kept at -20 °C for subsequent determination of *C. perfringens* abundance. The remaining small intestine was opened for examination of NE lesions. A 1.5 cm section of ileum was collected and fixed in 10% formaldehyde for morphologic analysis.

To determine carcass traits at slaughter, birds in flock 1 and flock 3 were processed at Alberta Chicken Producers Poultry Technology Centre. The live weight and carcass weight were determined from individual animals. The carcasses were subsequently cut up to evaluate the weight of pectoralis major muscle, pectoralis minor muscle, thighs, drumsticks, and wings.

2.3 Intestine lesion scoring

The NE-specific lesions in the small intestine were scored as described by Shojadoost et al. (2012) with some modification. Animals were scored from 0 to 3 based on the following criteria:

0: No gross lesion;

1: Thin or friable walls, or diffuse superficial fibrin;

2: Focal necrosis or ulceration, or non-removable fibrin deposit;

3: Multifocal necrosis or ulceration, or non-removable fibrin deposit.

Lesions more severe than score 3 were not observed.

2.4 Bacterial quantification

2.4.1 DNA extraction and purification

For samples collected from flock 1, 0.2 g of thawed cecal content was measured into a 2 ml tube with 0.3 g 0.1 mm diameter silica beads (Biospec). The cecal contents were washed with and resuspended in 1 ml of TN150 buffer (149 mM NaCl, 5.58 mM Tris-HCl, 4.38 mM Trometamol, pH 8.0) followed with a 3 min bead-beating at 5000 *g* (Mini BeadBeater, Fisher Scientific). After centrifugation at 14600 *g* for 5 min, the supernatant was transferred to a new 2 ml microtube. The DNA was purified by phenol and chloroform–isoamyl alcohol (24:1) method and precipitated with 100% ethanol at -20 °C overnight. The DNA pellet was washed twice with 500 µl 70% ethanol without disrupting the pellet and dissolved in 100 µl of Nuclease-free water. For flock 2, the extraction of *C. perfringens* DNA was performed as described by Feng et al. (2010). The QIAamp DNA Stool Mini Kit (QIAGEN) was used to lyse bacterial cells and extract DNA from cell lysates following the manufacturer's instructions for Gram-positive bacteria. The concentration and quality of DNA were measured using a ND-1000 spectrophotometer (NanoDrop Technologies) at 260 and 280 nm.

2.4.2 Quantitative real-time PCR (qRT-PCR)

The total *C. perfringens* population was quantified by qRT-PCR targeting the *16s rRNA* gene (Table 3). Commercial *C. perfringens* genomic DNA was serially diluted and was included on each plate to generate a standard curve for the absolute quantification of the bacteria population. The experiment was performed in QuantStudio™ 6 Flex System (Applied Biosystems) and data were analyzed with a QuantStudio rt-PCR Software v.1.3 (Applied Biosystems). Reactions of each sample were triplicated on a 96-well plate containing 20 µl reaction mixture in each well (1

µl 50 ng/µl DNA template, 1 µl 25 pmol/µl of forward and reverse primers, 10 µl Fast SYBR Green Master Mix, 7 µl Nuclease-free water). The amplification process started with an initial denaturation at 95 °C for 20 s followed by 40 cycles of annealing including 95 °C for 3 s and 62 °C for 30 s. As an indicator of amplification specificity, the melting curve of PCR products was generated by fluorescence collection during slow heating from 60 to 95 °C with a rate of 0.05 °C/s. The copy number of the target gene was calculated as described by Li et al. (2009) and expressed as copies/g digesta.

To detect the presence of the *netB* gene in cecal digesta, 20 µl reaction mixture was analyzed as described above with some modification (2 µl 50 ng/µl DNA template, 1 µl 25 pmol/µl of forward and reverse primers, 10 µl Fast SYBR Green Master Mix, 6 µl Nuclease-free water). The qPCR amplification cycle and calculation of the *netB* copy number were performed as described by Yang et al. (2018). Briefly, the amplification process started with 95 °C for 2 min, followed by 45 cycles of annealing including 95 °C for 5 s and 60 °C for 30 s. The specific *netB* amplicon was differentiated from non-specific products by the DNA melting curve. The average threshold cycle from the positive replicate samples was used for calculating the copy number of *netB*.

2.5 Intestine histology

Formaldehyde-fixed intestine tissue was dehydrated and embedded in paraffin wax. The tissue was sliced into 5 µm sections. The periodic acid-Schiff stain was used to determine villus height (VH), villus width, crypt depth (CD), and VH:CD ratio. The necrotic tissue sections were stained with hematoxylin & eosin staining procedure. Sections were examined under light microscopy. Measurement and image collection were performed in SeBaView software (Thermo Scientific).

2.6 Circulatory leukocyte profiles

Whole blood samples were lysed twice by ACK lysis buffer (ACK Gibco, USA) for 3 min to remove red blood cells. Samples were then centrifuged onto glass slides at 55 *g* for 6 min using a cytocentrifuge (Cytospin, Thermo Scientific) and stained using a HEMA 3 stain kit (Fisher Scientific) according to the manufacturer's specifications. Slides were rinsed with water and air-dried prior to observation using brightfield microscopy.

2.7 Immune priming

2.7.1 Preparation of heat-killed *C. perfringens*

Clostridium perfringens (ATCC® 13124™) were propagated according to manufacturer's protocols and maintained in cooked meat broth with 30% glycerol at -80 °C. 500 µl of frozen stock was inoculated into 10 ml of cooked meat broth, followed by overnight incubation at 37 °C under anaerobic conditions. The bacteria were washed twice with PBS and heat-killed by incubation at 80 °C for 30 min.

2.7.2 Intra-abdominal injection

The immune priming experiment was performed in flock 2. Upon placement, birds were randomly assigned to three priming treatments: PBS, *C. perfringens* (CP), and β-glucan+CP. Zymosan A (Sigma-Aldrich) was used as a source of β-glucan. The animal restraint method and intra-abdominal injection were performed as described by More Bayona (2019). The priming agonists were suspended in 300 µl of PBS-/- (no calcium/no magnesium). Accordingly, PBS birds served as the negative control and received an intra-abdominal injection of 300 µl of PBS; CP birds received 10^8 CFU of heat-killed *C. perfringens*; β-glucan+CP birds received 1 mg of

zymosan and 10^8 CFU of heat-killed *C. perfringens*. This bacterial load was determined by a preliminary screening trial which showed *C. perfringens* at 10^8 CFU recruited the highest number of infiltrating leukocytes into the abdominal cavity (figure 13). The animals were subsequently challenged with the natural NE model and sampled as described above.

2.8 Gene expression

Eight birds per treatment were sacrificed 4 h after immune priming to examine gene expression. The relative expression levels of selected genes were evaluated in leukocytes harvested from the abdominal cavity. Birds were dissected to expose the abdominal cavity, and infiltrating cells were lavaged with 3 ml of RPMI 1640 then resuspended in 1 ml Trizol reagent (InvitrogenLife Technologies) after centrifugation. RNA extraction, reverse transcription, and quantitative PCR were performed as previously described (Kamely et al., 2020). The transcriptional level of each target gene was corrected by the ribosomal RNA 28S gene (*R28S*) and calculated using the $2^{-\Delta\Delta CT}$ method (Livak et al., 2001). The data were normalized to chicken receiving PBS injection and results are expressed as fold changes. The primer sequences for each gene are shown in Table 4.

2.9 Leukocyte infiltration and respiratory burst

2.9.1 Cell collection and staining

The intra-abdominal leukocytes were collected as described above at 12 h after immune priming and counted using a hemocytometer chamber under light microscopy. The infiltrating leukocyte is confirmed by CD45 antibody staining (SouthernBiotech) and subset into heterophil, macrophage, and lymphocyte as described by More Bayona et al. (2017). ROS production was

determined by the CellROX® Oxidative Stress Reagent (Molecular Probes). NO production was determined by DAF-FM™ Nitric Oxide Indicator (Fisher Scientific). Both reagents were used according to the manufacturer's specifications. 10^6 intra-abdominal leukocytes were incubated with 0.2 µg of CD45 antibody, 5 µM of CellROX reagent and 1 µM of DAF-FM reagent for 30 min at 41 °C. Leukocytes were washed twice with PBS-/- and fixed in 1% formaldehyde for 20 min.

2.9.2 Imaging Flow cytometry

Abdominal leukocytes were analyzed by imaging flow cytometry following staining. Data was acquired on an ImageStream MKII multi-spectral flow cytometer (Amnis Corporation). A minimum of 10,000 cells were acquired and analyzed using IDEAS software (Amnis Corporation). Single dye control samples were prepared for compensation. Fluorescence-minus-one samples were used to minimize the possibility of false positive signals. Differentiation of leukocyte subsets was performed as described by More Bayona et al. (2019) using a dot plot of events scattered by size (x-axis) versus internal complexity (y-axis).

2.10 Statistical analysis

Statistical analyses were performed using the GraphPad Prism 8 software. Results were expressed as means ± SEM unless otherwise noted. Fisher's exact test was conducted to compare the lesion score between low- and high-coccidial challenged animals. To determine the effect of NE challenge on birds fed with or without antibiotics, average body weight on day 18 (before feed withdrawal) and 19 (after feed withdrawal) was analyzed by a two-way ANOVA model including dietary treatment and age (day) as main factors. T tests were conducted to compare growth performance, intestine weight, ileal pH, ileal morphology, and bacteria abundance.

Distribution of lesion status was analyzed by chi-square test. For evaluation of β-glucan as an early-life immune modulator, data were analyzed using one-way ANOVA followed by Tukey's multiple comparison test. Additionally, Dunnett's multiple comparison test was conducted to compare the relative organ weight from CP and β-glucan+CP treatments using PBS as control. $P < 0.05$ was defined as being statistically significant and $P<0.1$ was described as a tendency.

Table 1. Flock size, housing type, and coccidial challenge dosage in 3 experimental flocks. Coccidial pre-exposure was incorporated in the NE disease model through oral gavage of live Eimeria oocysts using Coccivac-B52 vaccine (Merck Animal Health).

	Flock 1	**Flock 2**	**Flock 3**
Flock size	348	120	288
Housing type	cage	cage	floor
Eimeria dosage[1]	10×	15×	15×

[1] A concentrated Coccivac-B52 vaccine was applied at 10× (flock 1) or 15× (flock 2 and 3) of regular dosage. Each bird received 1 ml of vaccine diluted in distilled water.

Table 2. Ingredient and calculated nutrient composition of experimental diets for birds during starter, grower, and finisher stage.

	Flocks 1 and 2			Flock 3		
	Starter (day 1-13)	Grower (day 14-27)	Finisher (day 28-40)	Starter (day 1-10)	Grower (day 11-25)	Finisher (day 26-38)
Ingredients (%)						
Canola meal	5	7.5	10	7.5	10	12
Fish meal	4	4	4	-	-	-
Soybean Meal	24.05	17.62	11.75	27.96	22.44	19.38
Wheat	62.25	65.44	67.18	59.18	61.46	61.40
Limestone	0.92	0.78	0.66	1.18	1.03	0.93
Monocalcium phosphate	0.43	0.20	-	1.00	0.75	0.57
NaCl	0.30	0.30	0.30	0.27	0.26	0.26
L-Lysine	0.06	0.06	0.92	0.10	0.07	0.02
DL-Methionine	0.26	0.22	0.2	0.30	0.25	0.23
L-Threonine	0.05	0.03	0.01	0.05	0.01	-
Hy-D® Premix[1]	0.05	0.05	0.05	0.05	0.05	0.05
Vitamin Mineral Premix[2]	0.5	0.5	0.5	0.5	0.5	0.5
Choline Chloride Premix[3]	0.05	0.05	0.05	0.05	0.05	0.05
Phytase[4]	0.01	0.01	0.01	0.01	0.01	0.01
Canola oil	1.62	2.79	3.92	1.86	3.12	4.63
Mycotoxin Binder	0.05	0.05	0.05	0.15	0.15	0.15
Xylanase[5]	-	-	-	0.05	0.05	0.05

[1]Provided 69 µg 25-hydroxycholecalciferol per kg diet
[2]Provided per kilogram of diet: vitamin A (retinyl acetate), 10,000 IU; cholecalciferol, 4,000 IU; vitamin E (DL-α-tocopheryl acetate), 50 IU; vitamin K, 4.0 mg; thiamine mononitrate (B_1), 4.0 mg; riboflavin (B_2), 10 mg; pyridoxine HCL (B_6), 5.0 mg; vitamin B_{12} (cobalamin), 0.02 mg; D-pantothenic acid, 15 mg; folic acid, 0.2 mg; niacin, 65 mg; biotin, 1.65 mg; iodine (ethylenediamine dihydroiodide), 1.65 mg; Mn (MnSO4H2O) , 120 mg; Cu, 20 mg; Zn, 100 mg, Se, 0.3 mg; Fe ($FeSO_4 \cdot 7H_2O$), 800 mg.
[3]Provided 100 mg choline per kg of diet.
[4]Provided 500 FTU phytase per kg of diet (Phyzyme XP, Danisco Animal Nutrition, Marlborough, UK).
[5]Biomin II (Biomin Canada Inc., Mont-St-Hilaire, Québec, Canada)

Table 3. Sequences of primers used in quantification of *Clostridium perfringens* by qPCR.

Target	Sense	Sequence	Reference
16s rRNA	Fw	GGGTTTCAACACCTCCGTG	AP017630.1
	Rv	GCAAGGGATGTCAAGTGTAGG	
netB	Fw	TGATACCGCTTCACATAAAGGTTGG	Yang et al., 2018
	Rv	ATAAGTTTCAGGCCATTTCATTTTTCCG	

Table 4. Primers used for the qPCR analysis of chicken mRNA.

Target	Sense	Sequence	Reference
R28S	Fw	GGCGAAGCCAGAGGAAACT	FM165415
	Rv	GACGACCGATTTGCACGTC	
IL-8	Fw	GGCTTGCTAGGGGAAATGA	AJ009800
	Rv	AGCTGACTCTGACTAGGAAACTGT	
IL-1β	Fw	AGGTCAACATCGCCACCTAC	NM_204524.1
	Rv	ACGAGATGGAAACCAGCAAC	
LITAF	Fw	GTTGACTTGGCTGTCGTGTG	AY765397.1
	Rv	TCAGAGCATCAACGCAAAAG	
TNF-a	Fw	CGCTCAGAACGACGTCAA	Rohde et al., 2018
	Rv	GTCGTCCACACCAACGAG	
IL6	Fw	AACAACCTCAACCTGCCCAA	HM179640.1
	Rv	GGAGAGCTTCGTCAGGCATT	
PKM	Fw	CACCCGCAATGACCAAACAG	NM_205469.1
	Rv	CCAGGTTCACACGGAGATCC	
PCK2	Fw	ATGGCTGCTCTCCTTTGTCC	NM_205470.1
	Rv	CGCCCAGAAGTCCCACTATC	
ADPGK	Fw	AGCTTGGAGGAGTTTCAGCC	NM_001293210
	Rv	CGTCTGTGTCGCATCTCCTT	
LDHA	Fw	GGGTGGATTGTTGGAGAGCA	NM_205284.1
	Rv	CCTCCTTCCAGTGCTCCTTG	
HIF1a	Fw	ACGTGTAAAGGCGTGCAAAA	Verwoolde et al., 2020
	Rv	CGTGAGTTGGGGTAGTCCAC	

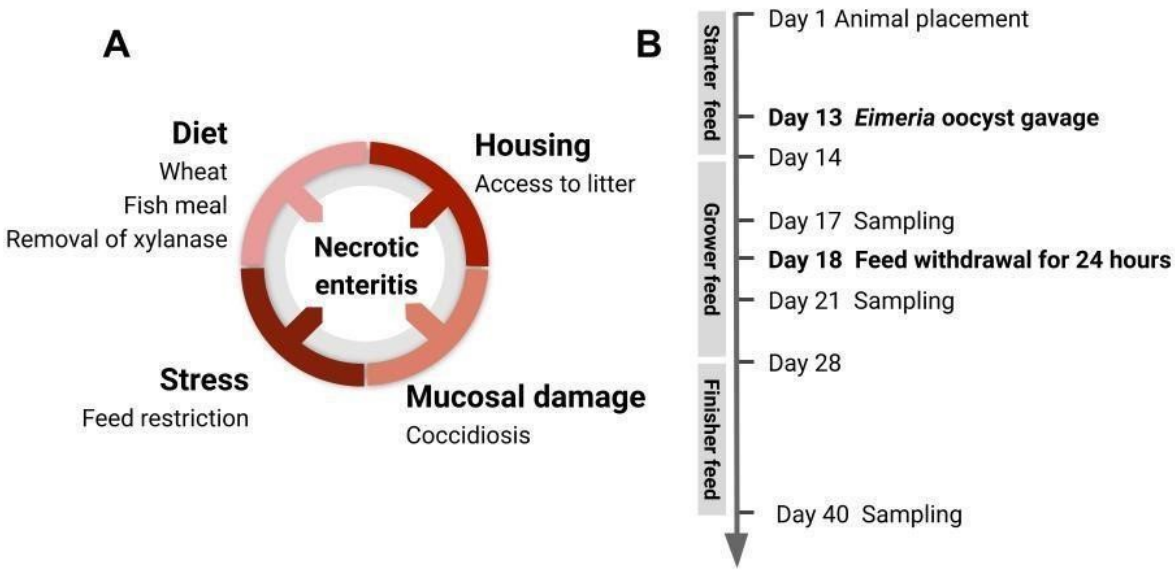

Figure 1. Induction of subclinical NE in broiler chicken by application of disease predisposing factors. (A) To promote natural development of the already-existing virulent *C. perfringens* in the gut, this disease model incorporated multiple predisposing factors: housing condition, diet components, coccidiosis-induced mucosal damage, and stressor leading to alteration of the gut environment. (B)Timeline of animal handling and sampling schedule.

Chapter 3: Optimization of a natural NE challenge model based on *C. perfringens* uptake from the farming environment

3.1 Introduction

Given the multifactorial nature of NE, reproduction of subclinical infections similar to field cases is known to be challenging. Ideally, experimental NE outbreaks should occur around week 3 post hatch, reflecting the timing when animals in the field are most at risk (Moore, 2016; Williams, 2005). Additionally, the model should yield a high incidence of necrotic lesions without severe mortality in the flock (Dierick et al., 2021). Efforts over the past decade suggest that concentrated live coccidial vaccines in combination with multiple dosages of *C. perfringens* culture result in NE infections that fulfill these criteria (Dierick et al., 2021; Gholamiandehkordi et al., 2007). Recent NE studies often adopt this dual-infection approach concurrently with application of dietary and /management risk factors (Dierick et al., 2019; Lee et al., 2013; M'Sadeq et al., 2015; Onrust et al., 2018; Wilson et al., 2018).

Induction of experimental NE using natural exposure to *C. perfringens* further mirrors conditions under which NE arises in commercial operations. This approach has gained prominence over the past decade (Abildgaard et al., 2010a; Calik et al., 2019; Emami et al., 2019 and 2021; Fernando et al., 2011; Løvland et al., 2003; Palliyeguru et al., 2010 and 2011). Compared to conventional models that drive infection via exogenous experimental application of *C. perfringens*, natural NE infection can A) simplify the disease challenge protocol; B) eliminate variation between models caused by different bacterial culture conditions, challenge route, dosage, timing and frequency; and C) develop infection most similar to the field condition. Importantly, *C. perfringens* undergoes a series of adaptations in response to fluctuation of gut environment, which modify

the disease-causing ability of this bacterium (Figure 2). Given the highly plastic phenotype that *C. perfringens* can display, natural infection also overcomes deviations commonly associated with *in vitro* manipulation, including changes to colonization efficacy and toxin production (Parreira et al., 2016). Thus, the natural infection approach can better recapitulate the microbial loads and other relevant physiological factors that contribute to NE pathogenesis.

Aiming to optimize current natural NE model, we incorporated a novel stressor, a 24-hour feed withdrawal at day 18 post hatch, apart from other commonly used risk factors for inducing experimental NE. This nutrient alteration in the gut lumen aims to disrupt the intestinal microbial community and promote development of pathogenic *C. perfringens*. This data set demonstrates the stepwise development of a natural NE infection model involving 3 experimental flocks. Our results suggested timely application of stress factors developed NE infection similar in the field situation, characterised by a highly prevalence of gut lesions in the flock with a low mortality rate.

3.2 Results

3.2.1 Mortality and clinical signs

The two cage-reared flocks yielded similar mortality during the experiment period. The overall mortality (day 1 to 40) was 1.15% in flock 1 and 1.39% in flock 2 (Table 5). All mortalities occurred within the first week, prior to coccidial vaccine dosing and feed withdrawal challenge. Birds did not show observable clinical signs, but bloody and mucous-containing feces were found in a few cages after feed withdrawal, indicating presence of diarrhea.

The floor-reared flock (flock 3) showed higher mortality at 3.82% compared to the cage-reared flocks (Table 5). Two mortality peaks were observed during the experiment period. The first peak occurred during week 1 and the second peak was found between weeks 3 to 5, after the 24-hour feed withdrawal was applied. The second mortality peak was directly triggered by feed withdrawal on day 18, which also caused depression and decreased mobility in a portion of birds.

3.2.2 Detection and quantification of *C. perfringens*

Total *C. perfringens* was quantified by qRT-PCR targeting the 16s gene. In flock 1, all sampled birds were found to be *C. perfringens* positive regardless of age (Figure 3A). The presence of *netB*, the hallmark of NE-causing strains, was detected in 12.5% of the samples on day 17, and increased to 31.5% on day 21. This is consistent with our expectation that the 24-hour feed withdrawal on day 18 further contributed to propagation of the virulent strains within the flock. The observed *C. perfringens* density was relatively high with *16s* abundance ranging from 10^7 to 10^9 copies per gram of cecal content (Figure 3B). A relatively lower *netB* abundance was observed at around 10^6 copies per gram of digesta. Quantification of *16s* and *netB* copies showed no significant difference between day 17 and day 21 ($P>0.05$). Flock 2 was reared in similar conditions as flock 1 but was challenged with higher dosage of *Eimeria* oocysts on day 13. However, the higher coccidial challenge level did not increase bacteria detection rate or bacteria abundance.

3.2.3 Gross examination of intestine lesion

In our study, mild lesions (score 1 and 2) were predominantly observed with a few birds scored with 3 (Figure 3E). Severe lesions (score greater than 3) were not observed in any of the experiment flocks. A total of 48 birds were sampled in experiment flock 1. Only 5 birds

(10.42%) had NE-specific lesions under 10× coccidial challenge (Table 5), and all the lesion-positive animals were observed on day 21. With increased intensity of coccidial challenge at 15×, flock 2 (n=54) and flock 3 (n=256) showed higher prevalence of birds carrying necrotic lesions (85.19% and 80.08%, respectively). Lesion prevalence in flocks reared in the wire-floored cage environment (flock 1 and 2) showed that coccidial challenge levels have profound impact on NE lesion development (Figure 3C). The 15× dosage of *Eimeria* vaccine gavage led to prevalent lesion development in the flock as early as day 17 and the lesions were also present at day 40. The high-coccidial challenge flock showed increased lesion score compared to the low-challenge flock on day 17 ($P<0.0001$), day 21 ($P=0.0045$) and day 40 ($P<0.0001$) (Figure 3D). The floor-rear flock (flock 3) with 15× coccidial challenge level yielded consistent high lesion prevalence on both sampling days as expected. The observed lesion prevalence was 75% (96/128) on day 21 and 93.75% (120/128) on day 40. Animals in flock 3 were not sampled on day 17.

3.2.4 Microscopic examination of intestine lesion

Representative histopathological images of the intestinal section are shown in Figure 3E. Intestine tissue scored at 0 with no gross lesion generally showed intact villus structure. However, examination under higher magnification revealed pathological changes including presence of *Eimeria* oocysts, mildly dilated capillaries, and capillary hemorrhage. Tissue lesions scored with 1 and 2 generally showed similar microscopic appearance though distinguished changes were observed in gross examination. Under microscopic examination, the necrotic region showed hyperemia, villus fusion, and separation of epithelium from the lamina propria. The necrotic tissue was usually covered by adherent fibrin and cellular debris. These pathological alterations were also observed in lesioned tissue with score 3. Noticeably, sloughed

mucosa leading to complete loss of villi was observed in certain areas within the lesioned tissue (Figure 3E).

3.3 Discussion

Bacteria quantification together with the characteristic pathology of NE, such as clinical signs and gut lesion, is indicative of successful induction of NE disease (McReynolds et al., 2004; Palliyeguru et al., 2010; Williams et al. 2003). *C. perfringens* can be found in high populations in NE-affected animals ranging from $10^6 \sim 10^9$ CFU (Abildgaard et al., 2010a; McDevitt et al., 2006; Williams, 2005). The *C. perfringens* population observed in our trial is consistent with those typically found in NE-affected animals. Interestingly, the *netB* gene was more prevalent on day 21 compared to day 17 (Figure 3A). This may be associated with the observed diarrhea on day 18 to 19, which could indicate that the experimental conditions promoted the spreading of the *netB*-carrying strains in the flock.

As noted by previous studies, there may be a poor correlation between the number of *C. perfringens* organisms in the digesta and the incidence or severity of NE, especially in subclinical form of the disease (Fernando et al., 2011). Subclinical NE is usually mild with no clinical signs or sudden increase in mortality (Fernando et al., 2011). Thus gut lesions are considered to be a sensitive disease indicator compared to clinical signs and mortality (Williams et al. 2003). In this study, gut lesions were found in all three experimental flocks and were confirmed with microscopic examination. Together, the observed bacterial load, clinical signs and pathological changes suggest NE occurrence in the flocks with disease severity peaked during weeks 3 to 4.

Many conventional NE disease models have shown lesion incidence peaks at a certain age and decline as the animal approaches slaughter (Gholamiandehkordi et al., 2007; Løvland et al., 2003). Natural NE infection induced by reused litter material from a previous flock (Palliyeguru et al., 2010), high stocking density and housing of birds on litter (Fernando et al., 2011; Lovland et al., 2003) have resulted in lesion prevalence ranging from 6.9% to 68.6%. However, to our knowledge, most studies conducted only one lesion examination during the rearing. In field conditions, subclinical NE-affected animals can be detected at slaughter with *C. perfringens*-associated lesions in liver and gut (Johansson et al., 2010; Løvland and Kaldhusdal, 1999). This suggests experimental NE models with persisting lesion occurrence better model NE cases in the field, where birds suffering from subclinical disease are kept without being treated. In this study, we thus performed multiple gut examinations throughout the rearing period. Our data shows coccidial challenge significantly promoted the development of gut NE lesions, affecting the occurrence and duration of necrotic lesion in the flocks. As noted by Stanley et al., (2014), *Eimeria spp.* caused significant changes in gut microbiota diversity and enabled *C. perfringens* to persist post challenge. *C. perfringens* inoculated in the absence of this predisposing factor fail to establish and maintain themselves in the gut flora.

Epidemiology studies revealed that birds kept on litter or raised in floor-type housing are more prone to NE occurrence compared to those reared in aviary and cage systems (Goossens et al., 2020; Kaldhusdal et al., 2016). Our data shows NE-related mortality was not observed in our cage-reared flocks even with 15× coccidial challenge dosage. A sudden but minor increase in mortality in the floor-reared flock was observed starting from day 18. In the *E. maxima*/*C. perfringens* dual infection model, NE lesions can be produced without mortality in animals reared in wire-cages (Williams et al., 2003), while similar models in floor-reared flock yielded

NE-induced mortality ranging from 8 to 12% (Hofacre et al., 2019; Wu et al., 2010). Natural NE infection induced by reused litter material from a previous poultry flock showed mortality during day 15 to 30 ranging from 1.5 to 4.9% across dietary treatments (Palliyeguru et al., 2010). Fernando et al. (2011), by housing birds on wood-shaving litter and removing antibiotic drugs, induced NE infection with mortality of 1.19%-1.66% during day 20-36. These findings and our observation are consistent with the mortality range reported in subclinical NE affected flocks (Løvland and Kaldhusdal, 2001). Recent work by Calik et al. (2019) and Emami et al. (2019) described a new natural NE disease model by spraying the same coccidiosis vaccine, as used in our study, on litter and feed upon bird placement. NE outbreak was observed on days 7 to 9 with overall mortality at around 12%. These findings showed higher mortality which peaked at an earlier in age compared to our study, but are consistent with our observation that the NE outbreak occurred 1 to 2 week after coccidial challenge by concentrated Coccivac®-B52 vaccine.

In conclusion, the NE infection model developed in this study is based on natural uptake of *C. perfringens* presented in the housing environment by the chicken. We incorporated multiple NE-associated risk factors to promote natural development of pathogens, and successfully reproduce the commonly observed sub-clinical NE. This will contribute to future research aiming at understanding and preventing this disease, by mimicking the natural development of NE in commercial poultry production.

Table 5. Mortality and overall lesion prevalence in 3 experimental flocks.

	Flock 1[1,3]	Flock 2[1,4]	Flock 3[2,4]
Overall mortality (%)	1.15%	1.39%	2.86%
Mortality (%), day 18-35	0	0	1.51%
Overall lesion prevalence	10.42%	85.19%	80.08%

[1]Animal were housed in pullet cages
[2]Animal were housed in floor pens
[3]Challenged with 10× coccidal vaccine on day 13
[4]Challenged with 15× coccidal vaccine on day 13

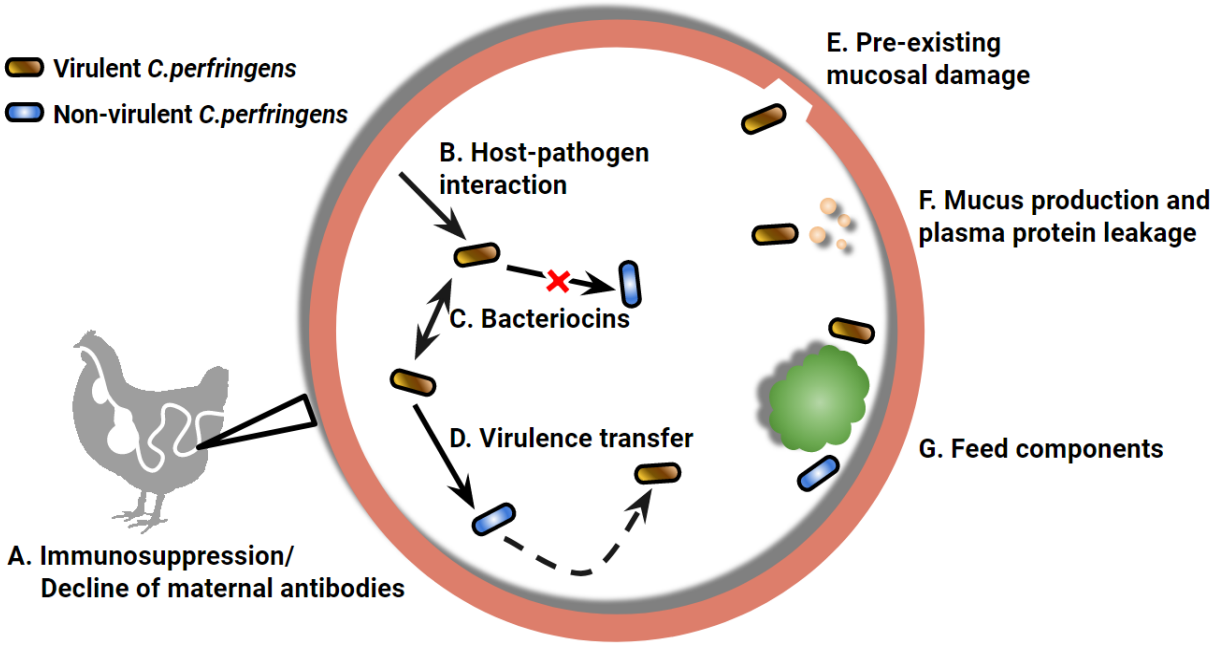

Figure 2. A wide range of environmental factors determine the manifestation of necrotic enteritis (NE) caused by *Clostridium perfringens*.

Animals are at higher risk to develop NE infection with impaired immune status (A), which can result from declining maternal antibodies and/or pre-exposure to immunosuppressive factors (Lee et al. 2011). The intestine environment also plays a role in virulence modulation of *C. perfringens*, the first example can be seen in the upregulated toxin expression when in close contact to epithelial cells (B) (Vidal et al., 2009). Complex microbiota environment, compared to culture medium, can differentially regulate virulence phenotype of the bacterium via inter and intra strain interactions through bacteriocin production (C) (Ohtani et al., 2015). Virulent strains are capable of producing bacteriocins that strongly inhibit non-virulent strains, as a mechanism to success in the pathogen-commensal competition (Barbara et al., 2008; Timbermont et al., 2009). Meanwhile, non-virulent strains can inherit the virulence genes horizontally (D), leading to the emergence of new strains that are capable of causing NE (Lacey et al., 2017). Pre-existing mucosal damage that exposes epithelial extracellular matrix (E), increased plasma protein leakage and mucus production (F) can provide extra advantage for pathogenic *C. perfringens*, which possess stronger binding ability and mucolytic activity (Collier et al., 2008; Martin and Smyth, 2010). Diet components comprising part of the gut environment are also key risk factors associated with NE development (G). For example, feeds rich in water-soluble non-starch polysaccharide, such as a wheat-based diet, can increase digesta viscosity, prolong transit time and promote pathogen retention (Annett et al., 2002; Shojadoost et al., 2012).

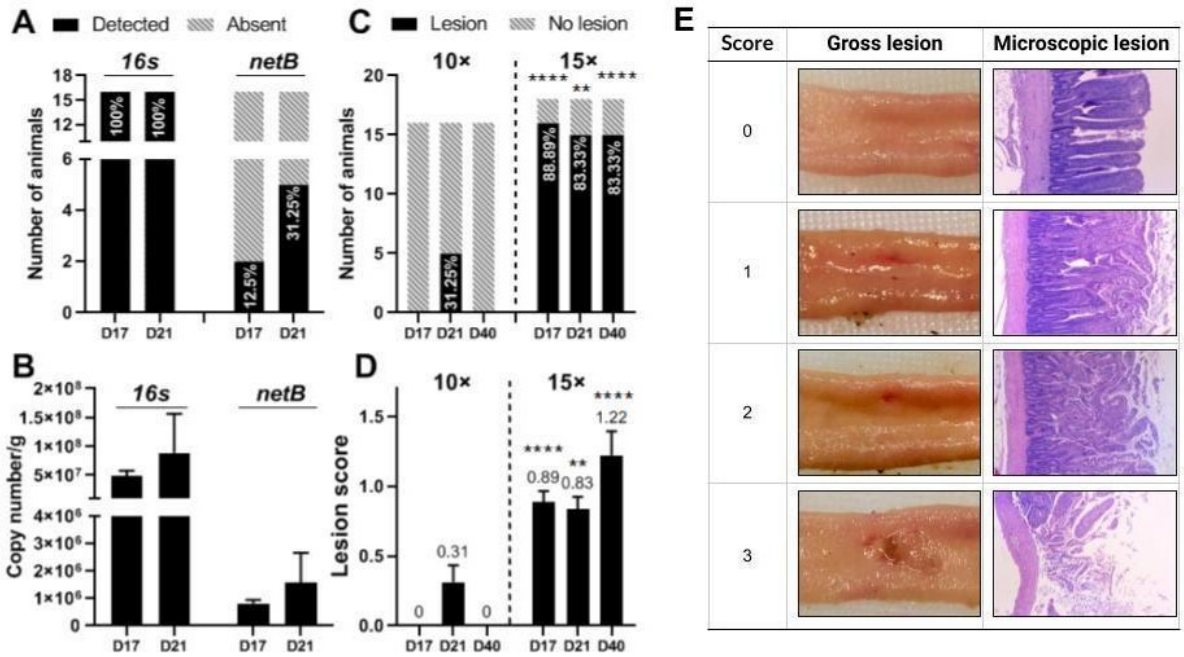

Figure 3. Quantification of *C. perfringens* and intestine lesion confirmed induction of subclinical necrotic enteritis using the natural infection model. (A) Detection of *C. perfringens 16s* and *netB* gene in cecal content by qRT-PCR. The percentages of animals detected with *16s* or *netB* are plotted within bars. Data were collected from flock 1 (n=16). (B) Abundance of *16s* and *netB* gene expressed as copy number/g of cecal content. Data were collected in flock 1. (C) Intestine gross lesion prevalence in flock 1 (challenged with 10× concentrated coccidial vaccine) and flock 2 (challenged with 15× concentrated coccidial vaccine). The percentages of animals detected with gross lesion are plotted within bars. Fisher's exact test was conducted to compare the difference between low and high coccidial challenged animals (D17: P<0.0001, D21: P=0.0045; D40: P<0.0001) (D) Lesion scoring results from experiment flock 1 and flock 2. A nonparametric Mann-Whitney test was used to compare the rank of lesion score between 2 coccidial challenge levels from the same age (D17: P<0.0001, D21: P=0.0045, D40: P<0.0001). (E)Severity of NE-specific lesions was scored from 0-3 based on intestine gross examination. Tissue at lesion site was processed for histology analysis. Original magnification of the images is 25×.

Chapter 4: The impact of NE infection on growth performance and disease severity under drug-free conditions

4.1 Introduction

Antibiotic administration at subtherapeutic levels have been used for decades in poultry production worldwide for growth promoting. This medication treatment is known to reshape microbiota diversity, suppress intestinal inflammatory response, thus increase efficiency of nutrient utilization (Diaz Carrasco et al., 2019). A 2003 publication summarized that in-feed antibiotic growth promotor can increase body weight gain up to 8% and decrease the feed conversion ratio up to 5% in chicken production (Butaye et al., 2003). Until the early 2000s, NE in broiler was effectively controlled by in-feed application of antimicrobial growth promoters, including avoparcin, avilamycin and bacitracin methylene disalicylate (BMD) (Prescott et al., 1978; Elwinger et al., 1998; Brennan et al., 2003; Løvland et al., 2003). However, recently, a growing number of studies have reported inconsistency in the effectiveness of antibiotic drugs, where in-feed antibiotic administration either mitigated or had no impact on NE disease severity (Calik et al., 2019; Fasina et al., 2016; M'Sadeq et al., 2015).

The inconsistency in the effectiveness of antibiotic growth promoters against NE in different studies may be attributed to the different antibiotic drugs, *C. perfringens* strains and challenge models used in different researches. Additionally, the emergence of antibiotic-resistant strains may cause decreased effectiveness of antibiotic growth promoters against NE. It was shown that *C. perfringens* isolates collected in Belgium showed dramatic increase in lincomycin resistance from 1980 to 2002 (49% vs 63%) (Martel et al., 2004). Swedish, Norwegian and Danish strains displayed various degrees of resistance to numerous common antibiotics, such as virginiamycin

and bacitracin (Johansson et al., 2004). In Canada, field isolates of *C. perfringens* were shown to possess high degree of bacitracin resistance (25% of the isolates) (Charlebois et al., 2012) or reduced susceptibility to bacitracin (64% of the isolates) (Slavić et al., 2011). Antibiotic resistance has aroused public attention concerning the transmission of antibiotic-resistant bacteria to humans through the food chain. The European Union banned the use of all antimicrobial growth promoters in poultry feed starting January 2006, and it is expected that reduction and removal of antibiotic use will be the future trend for global poultry production.

This research aimed to better understand the influence of antibiotic removal on naturally-occuring NE infections. This study involved data collected from flock 1 (10× coccidial challenge intensity, reared in cages) and flock 3 (15× coccidial challenge intensity, reared in floor pen). Upon arrival, chickens were randomly assigned to two dietary treatments: Antibiotic+coccidiostat (AC) and drug-free (DF). Diet for AC animals contain 0.05% antibiotic growth promoter (Bacitracin) and coccidiostat (Monensin) in the starter and grower diets, but not in the finisher diet. Animals were challenged by the natural NE infection, and their growth performance, health parameters and carcass traits were monitored.

4.2 Results

4.2.1 Medication reduced body weight decline in NE challenged animals

Feed was withdrawn from all chicken for 24h at day 18 to induce alteration of the intestine environment that promoted pathogenic *C. perfringens* development. Body weight was measured at the start and end of the feed withdrawal, significant body weight loss was observed in all animals ($P<0.0001$) (Figure 4A), while the average body weight did not differ between AC and

DF groups. However, the body weight decline in the DF animals was more severe compared to the AC animals (48.87 g versus 41.42 g, respectively) (P<0.0001) (Figure 4B).

4.2.2 Medicated animals showed decreased empty intestine weight and increased ileal pH

On days 17 and 21, eight animals from each treatment were sampled to determine empty small intestine weight, ileal pH, and microscopic morphology. AC and DF animals showed identical intestine to body weight ratio and ileal pH on day 17 (Figures 4C and D). On day 21, the AC animals had significantly lower intestine weight (P=0.0132) and higher ileal pH (P=0.0315).

4.2.3 Medication treatment caused higher abundance of cecal pathogenic *C. perfringens*

Total *C. perfringens* density was quantified by qRT-PCR targeting the 16s gene. Eight animals from each treatment were sampled on each of day 17, 21 and 40, and all birds were *C. perfringens*-positive. As shown in Figure 4F, the total *C. perfringens* population did not differ significantly between treatments on any of the sampling days. Presence of *netB*, the determining factor of NE-causing strains, was detected only in 2 animals, both from the medicated treatment group, on day 17. A higher number of samples were *netB* positive on day 21 (AC: 3/8; DF: 2/8). We then calculated the abundance of *netB* using only the positive samples (samples collected from day 17 and day 21 were combined in each treatment). Surprisingly, medication treatment significantly increased *netB* abundance in cecal digesta (P=0.0061) (Figure 4E). No significant differences were observed in ileal morphology in the aspect of villus height, villus width and crypt depth (Figures 4G and H).

4.2.4 Medication treatment did not improved production performance or gut lesion status

Flock 3 yielded higher incidence of gut lesion and higher lesion score. However, no significant differences were observed between the AC and DF groups in either flock 1 or flock 3 (Figures

5A and B). The medication treatment does not overall growth performance (feed conversion, body weight gain, feed intake (Figures 5C, D and E, respectively)) or carcass traits (Figure 5F). However, compromised starter FCR was observed in medicated chicken in both flocks. AC animals from flock 1 had significantly higher FCR compared to the DF group ($P<0.0001$). A similar tendency was observed in flock 3 ($P=0.0546$).

4.3 Discussion

We first determined the body weight loss during the feed withdrawal on day 18 to assess the acute responses of the animals. Our data shows that medication treatment reduced this short-term body weight decline associated with NE induction. Compared to mammals, chickens have a relatively short gastrointestinal tract, with an average digesta passage time less than 3.5 h (Hughes, 2008; Pan and Yu, 2014). Body weight loss during a day-long feed restriction is attributed to fat catabolism, glycolysis and proteolysis in the animals, besides the emptying of the digestive tract (Kim et al., 2006; Nieto et al. 1994; Warriss et al., 1988). The severe body weight loss in the DF group reflects increased metabolism rate in response to the NE induction. As noted in previous studies, *C. perfringens* infection affects the hepatic lipid metabolism, alters muscle lipid level and fatty acid composition, eventually leading to reduced growth (Qing et al., 2018).

In terms of overall growth performance, the protective effect of antibiotic drugs against NE in different studies are sometimes contradicting. It was shown that dietary antibiotics either increased (M'Sadeq et al., 2015), had no effect on growth performance (Fasina et al., 2016), or even hampered feed conversion during the first week (Calik et al., 2019). Meanwhile, the authors reported that antibiotic drugs were beneficial in terms of reducing *C. perfringens* load (Fasina et

al., 2016) and mortality rate (Calik et al., 2019). These findings are consistent with our observation that in-feed antibiotic application does not affect overall growth performance but showed protective effects against certain disease parameters.

The rise of antibiotic-resistant strains may be associated with the ineffectiveness of antibiotic drugs in some studies. NE pathogenicity loci in the genome of NE-causing *C. perfringens* harbour genes not only related to the virulence but antibiotic resistance, suggesting selective advantages of the pathogenic strains under antibiotic pressure (Bannam et al., 2011). This hypothesis is also supported by our data, which shows increased *netB* abundance in the medicated chickens. This observation expands the knowledge from previous studies, where antibiotic-fed chickens possessed a higher abundance of *Campylobacter* and *Salmonella* in the digesta (Kumar et al., 2018; Wei et al., 2013). As discussed in these studies, this can be attributed to bactericidal activities against sensitive bacteria that reduce the competition for nutrients.

Antibiotic supplementation reduces the intestinal thickness and weight, which is associated with reduced gastrointestinal microbiota diversity (Diaz Carrasco et al., 2019; Stutz et al., 1983). As expected, we observed reduced weight of the empty small intestine in the medicated group. We then measured digesta pH to examine whether fermentation activity of intestinal microbiota was affected, and found a higher ileal pH in antibiotic-fed birds. This is in agreement with previous findings that dietary antibiotic increased ileal pH compared to the drug-free diet (Vidanarachchi et. al, 2013). Increased pH indicates reduced abundance of SCFA-producing bacteria (Pan and Yu, 2014), which is associated with increased *C. perfringens* abundance due to less competition. It has been demonstrated that pH value and *C. perfringens* proliferation are positively correlated, as the bacteria growth is favoured under higher pH (Tsiouris et al., 2015). Our observation

therefore suggests the negative effect of antibiotics on the gut microbiota may not outweigh the beneficial bactericidal effect in the natural subclinical NE challenge model.

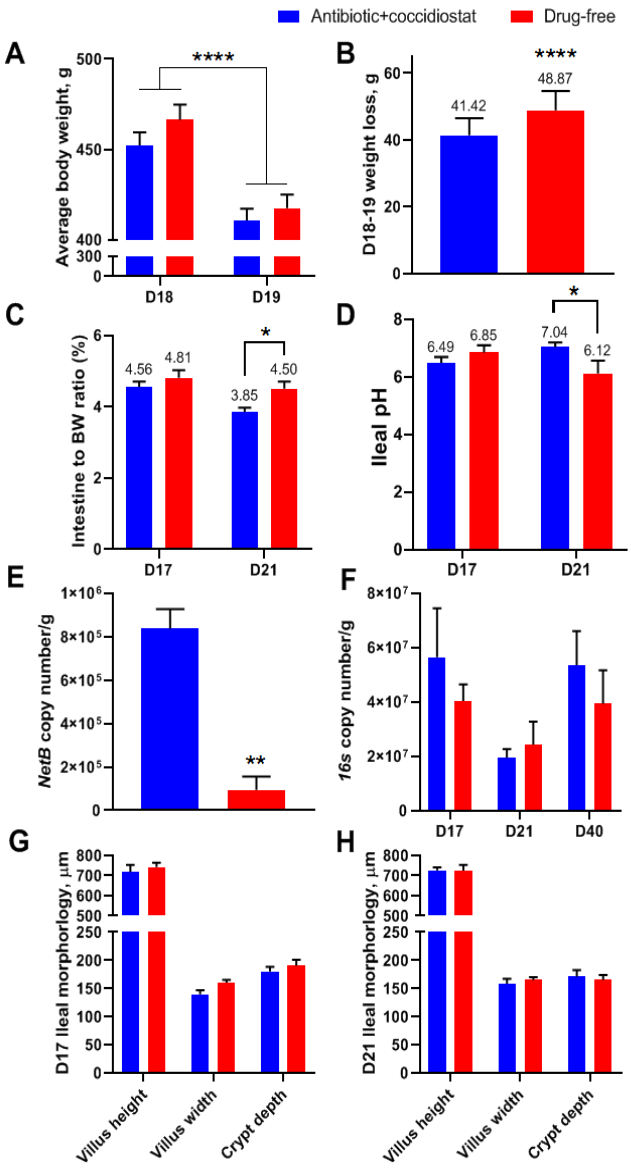

Figure 4. Effect of medication treatment on body weight decline, intestinal parameters and bacterial density in chicken. Birds from the antibiotic+coccidiostat treatment were fed with a diet contain 0.05% antibiotic growth promoter (Bacitracin) and coccidiostat (Monensin) in the starter and grower diets. Results were reported as mean ± SEM and analyzed by T tests unless otherwise noted. (A) Average body weight before (day 18) and after (day 19) feed restriction. Data were analyzed by a two-way ANOVA. (B) Average body weight decline (g per bird) during the 24-hour feed restriction. (C) Empty small intestine to body weight ratio on day 17 and 21. Intestinal digesta was removed before weighing. (D) pH value of ileal content on day 17 and 21. (E) Cecal *netB* copy number. *netB*-positive samples collected from day 17 and day 21 were combined in each treatment. (F) *C. perfringens 16s* copies in cecal content. (G) Ileal morphology on day 17. (H) Ileal morphology on day 21. *P < 0.05, **P < 0.01, and ****P < 0.0001 between treatments.

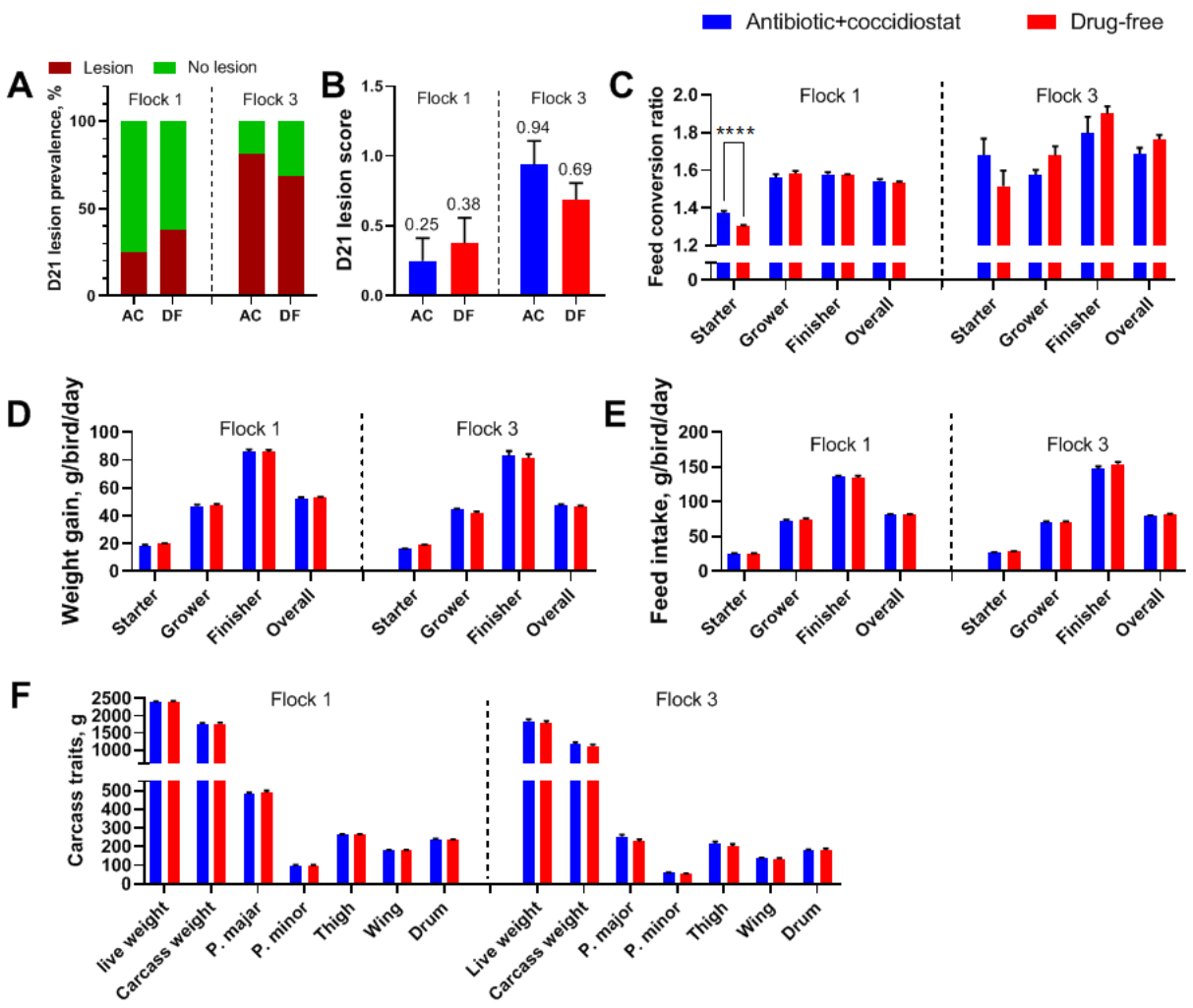

Figure 5. Effect of medication treatment on gut lesion profile and production performance. Birds from the antibiotic+coccidiostat treatment were fed with a diet contain 0.05% antibiotic growth promoter (Bacitracin) and coccidiostat (Monensin) in the starter and grower diets. Data were collected from flock 1 (10× coccidial challenge intensity, reared in cages) and flock 3 (15× coccidial challenge intensity, reared in floor pens). Results were reported as mean ± SEM and analyzed by T test unless otherwise noted. (A) Prevalence of gross lesion in sampled animals reported as percentage. Distribution of lesion status was analyzed by chi-square test. No significant difference was observed between medicated and drug-free animals in either flock. (B) Necrotic enteritis-specific lesion scores. (C) Feed conversion ratio, (D) Body weight gain and (E) Feed intake during starter phase, grower phase, finisher phase and throughout the production period. (F) Carcass traits determined at the age of day 40 after slaughtering and cut-up. ****P < 0.0001 between treatments. AC, antibiotic+coccidiostat. DF, drug-free.

Chapter 5: The effect of immunomodulation by β-glucan against NE infection

5.1 Introduction

Dietary antibiotic supplementation in poultry has been linked to issues of antibiotic resistance in medical cases (Mahmood et al., 2014). Removal of antibiotic growth promoters has been practiced in Europe since 2006, which was shown to subsequently increase occurrence of NE and other animal diseases (Kaldhusdal et al., 2016). This suggests an urgent need for effective alternatives to combat NE as antibiotics are phased out in poultry production. Vaccination is a well-developed strategy against many microbial infections, but the commensal nature of the causative agent, *C. perfringens*, has hindered the development of effective NE vaccines.

C. perfringens colonizes the chicken gut during hatch and forms part of its normal microbiome (Lee et al., 2011; Wigley, 2015). Repeated antigen exposure at the mucosal level is known to reduce or abrogate responsiveness towards systemic immunization with the same antigen (Mestecky et al., 2007). This creates a significant challenge for developing vaccines against *C. perfringens* since vaccination must overcome the immune tolerance towards gut-colonizing bacteria. As shown in mammals, the metabolites and structural components of *Clostridium spp.* trigger the development of tolerized phenotype in immune cells (Atarashi et al., 2011; Nagano et al., 2012).

Recent evidence suggests immune responsiveness against a recurring pathogen can be modified by the context during the initial exposure. β-glucan, a dectin-1 receptor agonist composing fungal cell walls, induces trained immunity and reverts tolerized immune status via epigenetic,

metabolic, and functional reprogramming of myeloid cells (Domínguez-Andrés et al., 2019b; Mitroulis et al., 2018; Novakovic et al., 2016). β-glucan applied as an adjuvant in a rabies vaccine was shown to initiate innate immune memory in dogs, enhancing immunogenicity of the vaccine (Paris et al., 2020). Verwoolde et al. (2020 and 2021) recently demonstrated that *in-vitro* β-glucan priming enhanced subsequent antimicrobial responses in chicken monocytes. These findings showcase the potential of β-glucan to enhance NE vaccine effectiveness by breaking commensal tolerance in neonatal birds. The goal of this chapter was to assess if the context of early-life exposure to *C. perfringens* may affect NE infection outcomes. The experiment was conducted in flock 2. β-glucan was co-administered with *C. perfringens* on day 1 of age, and the impact on disease severity and bird performance was subsequently determined using a natural NE infection model. Analysis of the cellular components, metabolic status and inflammatory profile was performed to further understand the environmental context in which *C. perfringens* was first encountered.

5.2 Results

5.2.1 β-glucan prevented reduction in feed efficiency caused by CP exposure

Chickens receiving CP injection at hatch showed significantly higher FCR during the grower phase compared to the β-glucan+CP group (P=0.0372) (Figure 6A). The overall FCR was also higher in the CP animals, compared to both PBS (P=0.0377) and β-glucan+CP (P=0.0137). This evidence indicated a reduced feed efficiency as a result of the early-life CP exposure. The overall FCR in β-glucan+CP treated animals was equal to those in the PBS control group (P=0.6714). Thus, β-glucan costimulation prevented the negative effect of early-life CP exposure in

subsequent NE challenge. No significant difference was observed in body weight gain and feed intake across treatments ($P > 0.05$) (Figure 6B and C).

5.2.2 β-glucan exposure improved ileal morphology on day 21

Field NE outbreaks typically occur between week 3 and 4 post-hatch (Moore, 2016). Thus, we assessed the effect of immune priming on ileal morphology on days 17 and 21. On day 17, prior to the feed withdrawal challenge on day 18, no significant differences among treatments were found in villus height, villus weight, crypt depth, or villus height-to-crypt depth ratio (VH:CD) (Figure 7A and B). On day 21, β-glucan+CP primed chicken showed significantly higher villus width compared to the PBS ($P=0.007$) and CP groups ($P=0.0076$) (Figure 7C). VH:CD was also higher in chickens exposed to β-glucan+CP compared to PBS ($P=0.0004$) and CP ($P=0.0007$) (Figure 7D).

5.2.3 Gut lesion score and *C. perfringens* abundance was not affected by early-life immune priming

To test the hypothesis that β-glucan induced a redirection of immune activity towards *C. perfringens* elimination, we quantified the NE lesion severity in the small intestine and *C. perfringens* abundance in the cecum. Figure 8 shows the number of lesion-positive animals found in each treatment. Animals primed with PBS, CP, and β-glucan+CP showed equivalent lesion scores on all sampling days ($P>0.05$) (Figure 9A).

We then quantified the abundance of total *C. perfringens* based on *16s* gene copies and NE-causing strains based on *netB* copies. There were no significant differences in the gene copies among the three treatments (Figures 9B and C). Thus, the protective effect of β-glucan is not associated with direct pathogen killing or prevention of bacterial colonization.

5.2.4 Spleen and liver weight decline was avoided by β-glucan co-exposure

Organ weights are commonly used in agricultural research as an indicator of health status. Thus, we measured the relative weight of the spleen, liver, empty small intestine, and bursa of Fabricius, reported as a percentage of live body weight (Figure 10). On day 17, the relative liver weight in the CP group was significantly lower compared to PBS animals (P=0.0425) (Figure 10A). A similar trend was observed on day 21 where the CP animals showed a decrease in the liver (P=0.0324) and spleen (P=0.0483) weight, compared to the PBS group (Figure 10B). Reduced organ weight was not observed when β-glucan was included at the time of CP injection.

5.2.5 β-glucan exposure increased circulatory heterophil percentage on day 21

To assess the robustness of host inflammatory responses against the NE outbreak, we examined blood leukocyte profiles at 21 days post-hatch. CP injection did not alter circulatory blood profile regarding percentages of heterophil, monocyte or lymphocyte subsets compared to PBS injection (Figure 11). β-glucan costimulation resulted in higher heterophil proportion (P=0.0286) and heterophil to lymphocyte (H:L) ratio (P=0.0365) in CP injected animals (Figures 11A and D). No significant difference was observed in monocyte or lymphocyte proportion (Figures 11B and C).

5.2.6 β-glucan enhanced glycolysis and cytokine expression in abdominal leukocytes

Enhanced HIF-1α-mediated glycolysis is well characterized in activated leukocytes (Cheng et al., 2014; Li et al., 2018). To examine whether β-glucan costimulation modified the context in which *C. perfringens* was first encountered, we measured the local cell metabolic and inflammatory status following the immune priming. We found enhanced HIF-1α expression in the β-glucan+CP birds compared to PBS (P=0.0007), while this was not observed when CP was

injected alone (Figure 12A). Similarly, CP injection did not increase the expression of glycolysis enzymes (P>0.05). While β-glucan co-exposure significantly enhanced the expression of the examined genes compared to PBS injection, including phosphoenolpyruvate carboxykinase 2 (PCK2) (P=0.0014), pyruvate kinase (PKM) (P= 0.0053), ADP-dependent glucokinase (ADPGK) (P=0.0039) and lactate dehydrogenase A (LDHA) (P=0.0092) (Figure 12B-E).

As for the transcript levels of pro-inflammatory cytokines, CP animals showed increased LiTAF (P=0.0117) compared to the PBS group but not in any other genes (P>0.05). Birds exposed to β-glucan+CP showed increased gene expression of IL-1β (P=0.0001), LiTAF (P=0.0406), TNF-α (P<0.0001), IL-6 (P=0.0070) and IL-8 (P=0.0032) compared to PBS animals (Figure 12F-J). β-glucan inclusion enhanced IL-1β (P=0.0012), TNF-α (P<0.0001), IL-6 (P=0.0107), and IL-8 (P=0.0118) compared to CP injection alone. The cytokine production profile revealed a robust immune activation when β-glucan was included.

5.2.7 β-glucan induced significant leukocyte infiltration at the priming site

To characterize the cellular components that mediate the pro-inflammatory responses against the microbial stimuli, we examined leukocyte infiltration following immune priming. Preliminary experiments indicated that β-glucan is a strong inducer of leukocyte infiltration into the abdominal cavity, while CP injection alone does not result in increased cell infiltration at the same level (Figure 13). In the formal trial, β-glucan+CP injection resulted in significant leukocyte recruitment into the abdominal cavity, compared to the PBS (P=0.0047) and CP (P=0.0085) (Figure 14A). Increases in the heterophil (P=0.0049), macrophage (P=0.0109), and lymphocyte (P=0.0064) population were found in the β-glucan+CP group, compared to PBS (figure 14B). Compared to CP injection alone, β-glucan inclusion significantly enhanced

heterophil (P=0.0088) and macrophage/monocyte (P=0.0098) recruitment, while the lymphocyte population remained unchanged (P>0.05).

5.2.8 β-glucan enhanced ROS production in abdominal macrophages

We next examined the functional changes of the infiltrating cell to further understand the environmental context during early *C. perfringens* exposure. ROS and NO production were measured 12 h post-injection to evaluate antimicrobial responses in activated leukocytes. Percentages of ROS- and NO-producing cells in total leukocytes did not vary among treatments (Figure 14C and E). CP injection alone did not increase ROS or NO production in either macrophage or heterophil subset, compared to PBS (Figure 14D), while β-glucan costimulation resulted in a higher proportion of ROS-producing macrophages (P=0.0181). Enhanced NO production was not observed in any cell subset (P>0.05) (Figure 14F).

5.3 Discussion

Repeated bacterial stimulations, such as LPS, can cause immune tolerance in chickens that reduces responsiveness against the same antigen (De Boever et al., 2008; Webel et al., 1998). β-glucan is well characterized as an immunomodulator that redirects the host immunity towards activation (Novakovic et al., 2016). Previous studies from our lab showed that β-glucan induces acute inflammation that is resolved 48 h following injection (More Bayona et al., 2017). Surprisingly, the current study reveals that intra-abdominal β-glucan injection conferred a long-term effect which encompassed the 40-day broiler chicken growth cycle. β-glucan costimulation during early-life *C. perfringens* exposure prevented FCR increase in NE-affected birds. To our knowledge, this is the first study to report the effect of β-glucan against NE via a single-dose administration at hatch.

NE infection typically leads to reduced mucosal surface area, shortened villi, and deeper crypts (Jayaraman et al., 2013; Tian et al., 2016). The crypt is a proliferating compartment that gives rise to cells on the villus. A high VH:CD indicates a well-differentiated intestinal structure with functional absorption capability (Fasina et al.,2013; Jeurissen et al., 2002). β-glucan regulates epithelial functions during NE infection, centered on enhancing cell survival, decreasing apoptosis, and generating compensatory signaling to reduce disease severity (Johnson et al., 2020). Previous studies have shown dietary β-glucan increased villus height, VH:CD ratio, and reduced crypt depth in NE chicken (M'Sadeq et al., 2015; Tian et al., 2016). Similarly, we observed a higher villus width and VH:CD ratio at day 21 in birds receiving β-glucan. The changes in intestinal morphology suggests a higher absorptive area and lower metabolic requirement in support of epithelium renewal (Jeurissen et al., 2002), thus favoring an increased growth efficiency.

Our data shows that intra-abdominal β-glucan injection failed to reduce *C. perfringens* abundance or lesion severity in the gut. This may indicate the protective effect of β-glucan was mediated by enhanced host disease resistance and can overshadow systemic tolerance responses elicited by gut *C. perfringens.* On the contrary, routine application of dietary β-glucan decreased *C. perfringens* density and reduced necrotic lesions (Ahiwe et al., 2019; Tian et al., 2016), which may be associated with direct growth inhibition of *C. perfringens* by β-glucan. Recent findings by Santovito et al. (2019) showed the antimicrobial activity of β-glucan against *C. perfringens in vitro*, which reduced growth rate of the pathogen.

Acute inflammation typically leads to increased liver and spleen weight, which are commonly measured in poultry and other agricultural species to evaluate immune competence against microbial challenge (Gore and Qureshi, 1997; Huang et al., 2019; Zhang et al., 2013).

Noticeably, the liver is characterized as a key mediator in acute phase responses during *C. perfringens* infection (Ruhnke et al., 2017; Saleem, 2012), and liver enlargement typically found in NE-affected animals is associated with robust systemic inflammation (Xue et al., 2018). Our data shows that CP animals had a lower liver and spleen weight compared to PBS, which may indicate a lesser extent of systemic response during NE challenge. Exposure to β-glucan at the time of early-life CP exposure resulted in higher liver and spleen weight during the NE outbreak. This is consistent with a previous study that dietary β-glucan increased spleen weight of NE-affected chickens and reduced NE severity (Ao et al., 2012). However, it was also reported that β-glucan either decreased or had no influence on spleen and liver weights (Ahiwe et al., 2019; Xue et al., 2017). The inconsistent findings may result from various NE challenge conditions, different sources, exposure routes, and doses of β-glucan. It is shown in human monocytes that exposure to the same antigen with different concentrations or exposure durations can develop distinct responses against subsequent infection (Ifrim et al., 2014).

Avian heterophils provide the first line of defence against bacterial insults facilitated by oxidative burst, microbicidal peptides and heterophil extracellular traps (Alkie et al., 2019). Dietary β-glucan increased chicken circulating heterophil numbers, percentages, oxidative burst activity, and H:L ratio during microbial infection (Cox et al., 2010a and 2010b; Huff et al., 2010; Lowry et al., 2005). We consistently found a higher circulatory heterophil percentage and H:L ratio in β-glucan-primed animals, reflecting the active antimicrobial response under the NE challenge. However, the exact mechanism by which β-glucan affects circulating heterophil number has not yet been identified, which indicates the need for further study. It may be associated with increased hematopoietic activity, as shown in mice, where intraperitoneal β-

glucan injection increases emergency myelopoiesis against a subsequent LPS challenge (Mitroulis et al., 2018).

Intra-abdominal administration of the immune priming agonist allowed us to evaluate the immune cell recruited to the challenge site. Our findings show β-glucan modifies leukocyte metabolic and functional status during the initial *C. perfringens* exposure. Coadministration of β-glucan induced stronger leukocyte infiltration, enhanced glycolysis, proinflammatory cytokine expression, and macrophage ROS production. The glycolytic genes analyzed in this dataset, namely ADPGK, PKM, LDHA, PCK2, contribute to immune activation via epigenetic, transcriptional, and post-translational modification of inflammatory genes (Cheng et al., 2014; Millet et al., 2016; Vachharajani et al., 2016). ADPGK is a glycolytic enzyme turning glucose to glucose-6-phosphate, which regulates mitochondrial ROS generation and IL-8 expression via NF-κB signaling (Kamiński et al., 2012). PKM catalyzes the dephosphorylation of phosphoenolpyruvate into pyruvate, and functions to phosphorylate transcription factor STAT3, leading to enhanced IL-1β and IL-6 production (Shirai et al., 2016; Weyand et al., 2017). LDHA is responsible for the conversion of pyruvate into lactate and NAD+, which are subsequently involved in modulating monocyte function via chromatin modification (Jang et al., 2012; Cheng et al., 2014). PCK2 converts oxaloacetate to phosphoenolpyruvate to fuel glycolysis, controlling the metabolic reprogramming during immune activation (Zhao et al., 2017). Here, the upregulated glycolytic genes in the β-glucan costimulation chickens suggest enhanced glycolysis supporting a robust inflammatory response. This change in environmental context during early-life *C. perfringens* exposure subsequently influences long-term disease resistance against the persisting enteropathogen. Remarkably, the strengthened antimicrobial resistance has

outweighed the metabolism cost during β-glucan-induced immune activation, which should be considered in application of immune modulation.

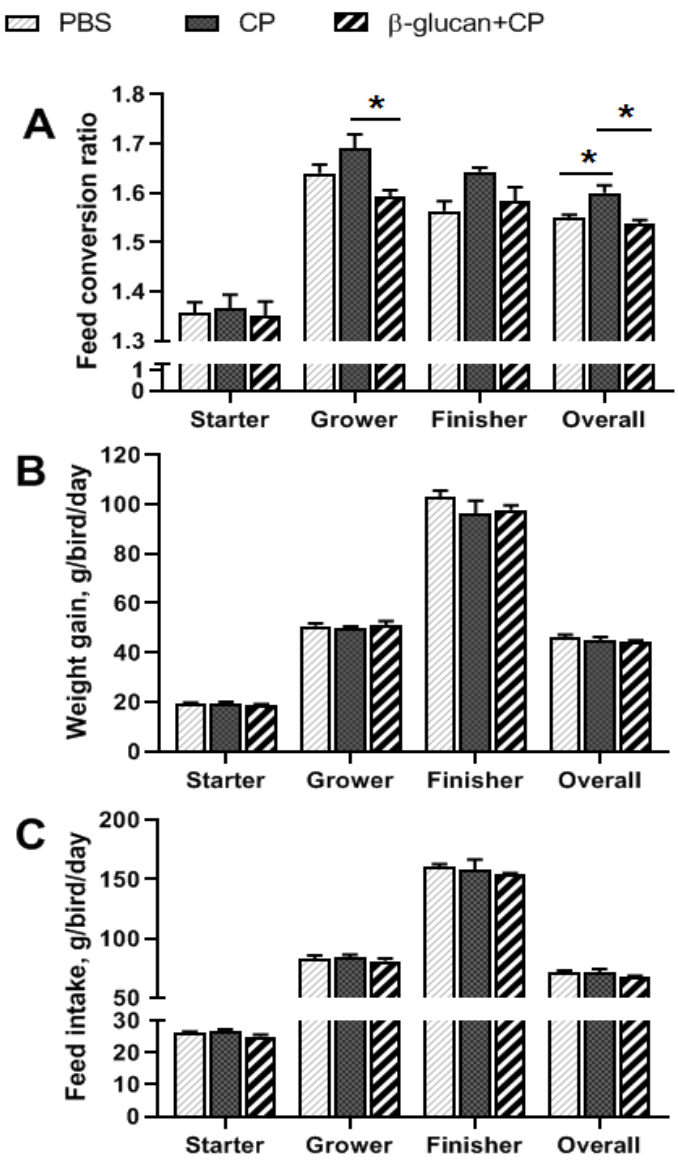

Figure 6. Impact of early-life immune priming by PBS, CP, and β-glucan+CP on (A) Feed conversion ratio, (B) Weight gain, and (C) Feed intake. PBS birds served as the negative control and received an intra-abdominal injection of 300 µl of PBS; CP birds received 10^8 CFU of heat-killed *C. perfringens*; β-glucan+CP birds received 1 mg of zymosan and 10^8 CFU of heat-killed *C. perfringens*. Animals received the immune priming treatment on day 1 of age and were subsequently challenged using a naturally-occurring necrotic enteritis challenge model, which included a 15x coccidiosis oral vaccination and a 24-hour feed withdrawal at 18 days of age. The performance parameters were monitored and reported for the starter (day 1-13), grower (day 14-27), finisher (day 28-40) phase and the overall growth period (day 1-40). One-way ANOVA and Tukey's multiple comparison test were conducted to compare the results between different priming treatments. *$P < 0.05$ between priming treatments. CP, *C. perfringens*.

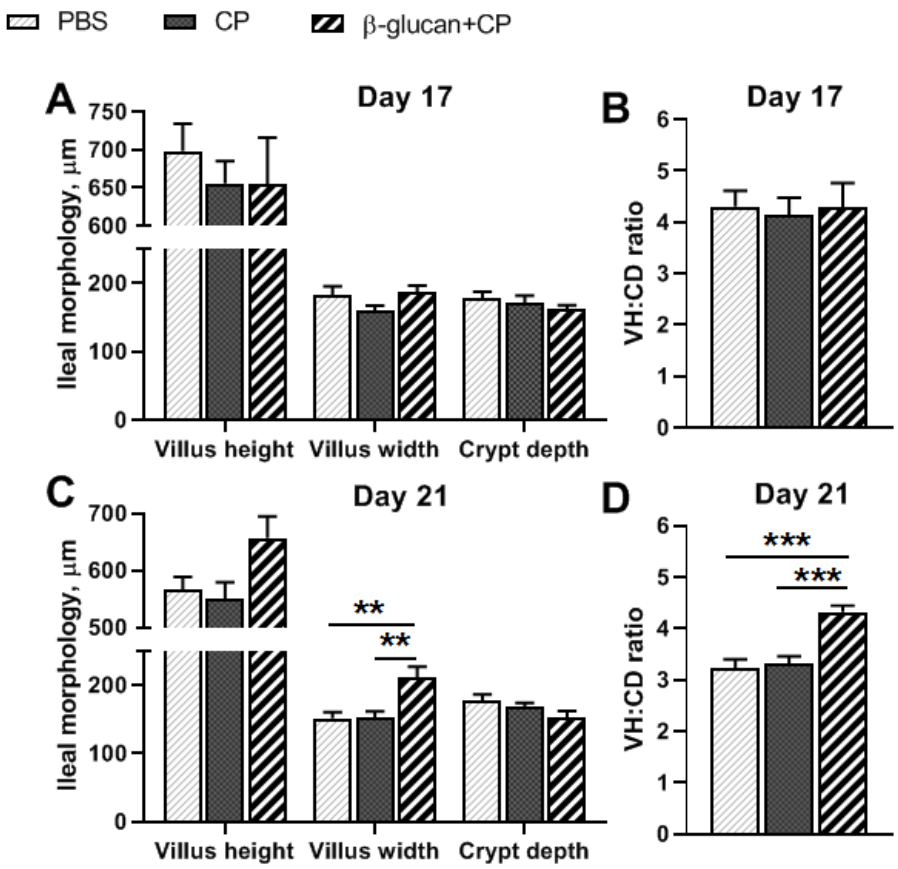

Figure 7. Impact of early-life immune priming by PBS, CP, and β-glucan+CP on ileal morphology following necrotic enteritis challenge. PBS birds served as the negative control and received an intra-abdominal injection of 300 µl of PBS; CP birds received 10^8 CFU of heat-killed *C. perfringens*; β-glucan+CP birds received 1 mg of zymosan and 10^8 CFU of heat-killed *C. perfringens*. Animals received the immune priming treatment on day 1 of age and were subsequently challenged using a naturally-occurring necrotic enteritis challenge model, which included a 15x coccidiosis oral vaccination and a 24-hour feed withdrawal at 18 days of age. Villus height, villus width, crypt depth, and villus height-to-crypt ratio were determined on day 17 (A and B) and on day 21 (C and D). Data represent mean ± SEM (n = 6). One-way ANOVA and Tukey's multiple comparison test were conducted to compare the mean between different priming treatments. **P < 0.01 and ***P < 0.001 between priming treatments. CP, *C. perfringens*. VH:CD ratio, villus height-to-crypt depth ratio.

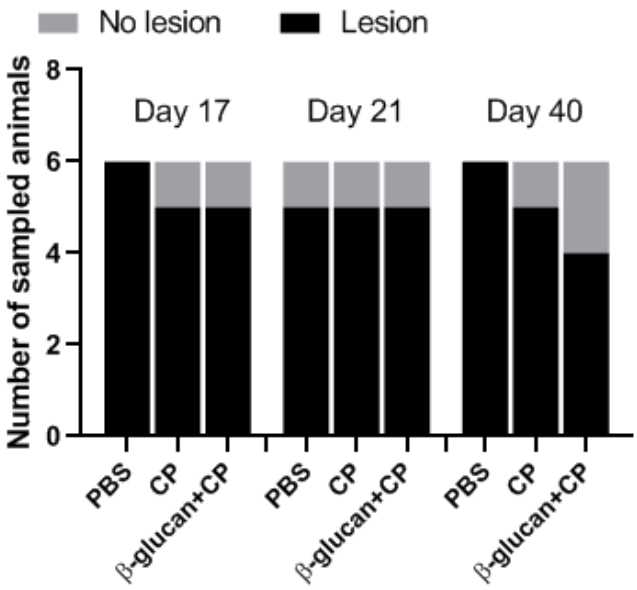

Figure 8. Impact of early-life immune priming by PBS, CP, and β-glucan+CP on the prevalence of animals with necrotic enteritis lesions. PBS birds served as the negative control and received an intra-abdominal injection of 300 µl of PBS; CP birds received 10^8 CFU of heat-killed *C. perfringens*; β-glucan+CP birds received 1 mg of zymosan and 10^8 CFU of heat-killed *C. perfringens*. Animals received the immune priming treatment on day 1 of age and were subsequently challenged using a naturally-occurring necrotic enteritis challenge model, which included a 15x coccidiosis oral vaccination and a 24-hour feed withdrawal at 18 days of age. Animals were examined for necrotic enteritis lesions on day 17, 21 and 40. The distribution of lesion status was analyzed by the chi-square test. No significant differences were found between treatments. CP, *C. perfringens.*

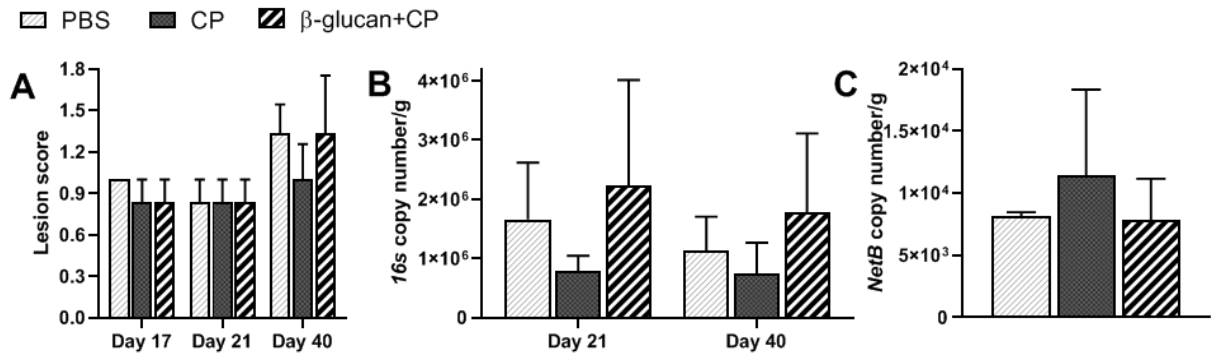

Figure 9. Impact of early-life immune priming by PBS, CP, and β-glucan+CP on (A) Lesion score, (B) Total *C. perfringens* density, and (C) *NetB* abundance. PBS birds served as the negative control and received an intra-abdominal injection of 300 µl of PBS; CP birds received 10^8 CFU of heat-killed *C. perfringens*; β-glucan+CP birds received 1 mg of zymosan and 10^8 CFU of heat-killed *C. perfringens*. Animals received the immune priming treatment on day 1 of age and were subsequently challenged using a naturally-occurring necrotic enteritis challenge model, which included a 15x coccidiosis oral vaccination and a 24-hour feed withdrawal at 18 days of age. The intestinal necrotic lesion scores (A) were determined on day 17, 21 and 40. Total *C. perfringens* density (B) was quantified on day 21 and 40 based on *16s* gene copies. *NetB* gene copies (C) were used to evaluate the abundance of pathogenic *C. perfringens* (Samples collected from day 17 and day 21 were combined in each treatment). Data represent mean ± SEM (n = 6). Results were analyzed by one-way ANOVA and Tukey's multiple comparison test. No significant differences were found between treatments. CP, *C. perfringens.*

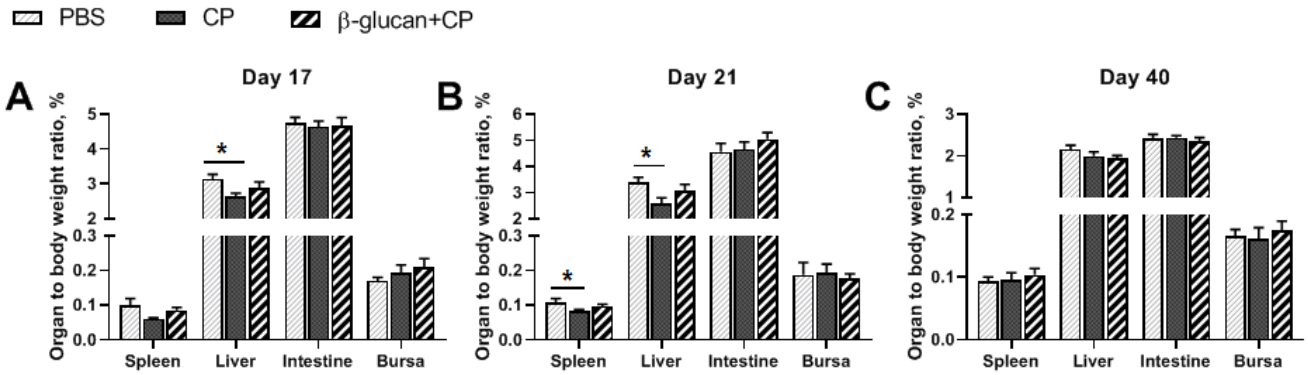

Figure 10. Impact of early-life immune priming by PBS, CP, and β-glucan+CP on relative organ weight following necrotic enteritis challenge. PBS birds served as the negative control and received an intra-abdominal injection of 300 µl of PBS; CP birds received 10^8 CFU of heat-killed *C. perfringens*; β-glucan+CP birds received 1 mg of zymosan and 10^8 CFU of heat-killed *C. perfringens*. Animals received the immune priming treatment on day 1 of age and were subsequently challenged using a naturally-occurring necrotic enteritis challenge model, which included a 15x coccidiosis oral vaccination and a 24-hour feed withdrawal at 18 days of age. Relative weights of the spleen, liver, empty small intestine, and bursa of Fabricius were determined on day 17 (A), day 21 (B), and day 40 (C). Data represent mean ± SEM (n = 6). One-way ANOVA and Dunnett's multiple comparison test were conducted to compare the mean between different priming treatments using PBS as control. *P < 0.05 between priming treatments. CP, *C. perfringens*.

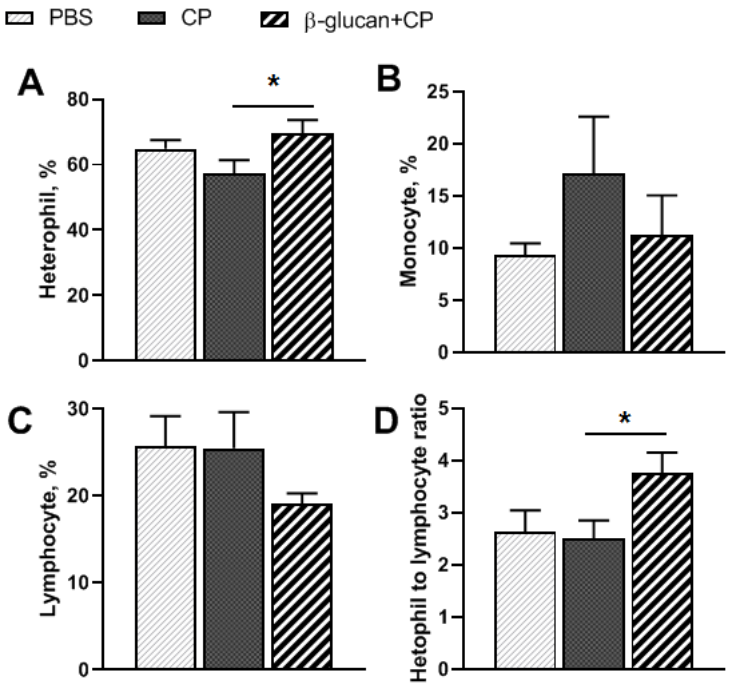

Figure 11. Impact of early-life immune priming by PBS, CP, and β-glucan+CP on circulatory leukocyte profile following necrotic enteritis challenge. PBS birds served as the negative control and received an intra-abdominal injection of 300 µl of PBS; CP birds received 10^8 CFU of heat-killed *C. perfringens*; β-glucan+CP birds received 1 mg of zymosan and 10^8 CFU of heat-killed *C. perfringens*. Animals received the immune priming treatment on day 1 of age and were subsequently challenged using a naturally-occurring necrotic enteritis challenge model, which included a 15x coccidiosis oral vaccination and a 24-hour feed withdrawal at 18 days of age. The percentages of heterophil (A), monocyte (B), lymphocyte (C), and heterophil to lymphocyte ratio (D) were determined on day 21. Data represent mean ± SEM (n = 6). Results were analyzed by one-way ANOVA followed by Sidak's multiple comparison test. *P < 0.05 between priming treatments. CP, *C. perfringens*.

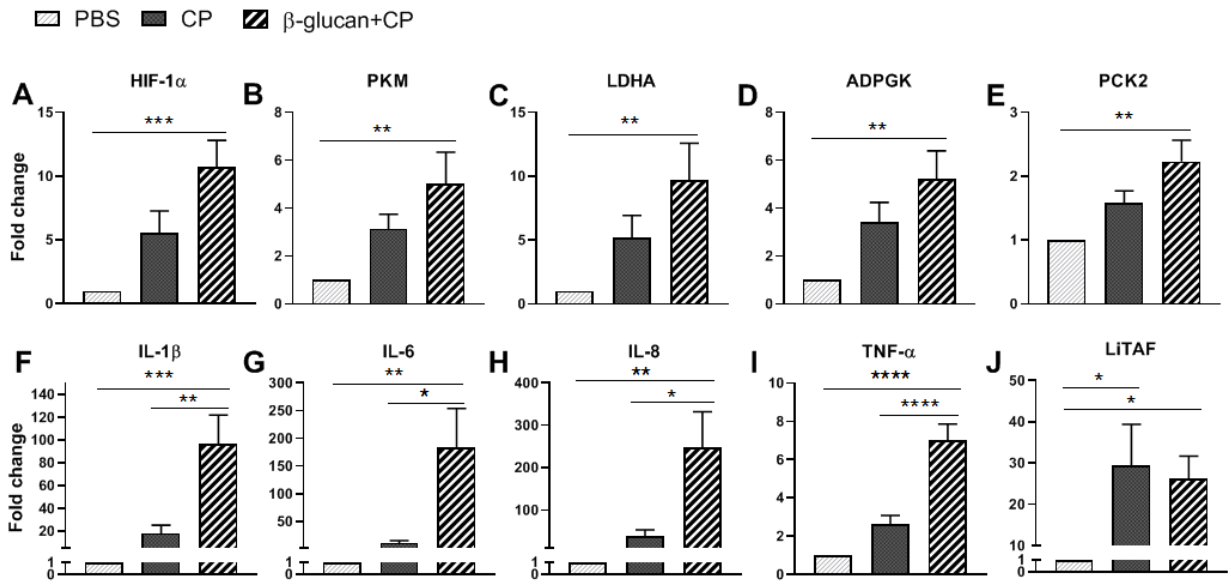

Figure 12. Changes in metabolic gene and inflammatory cytokine expression following initial *C. perfringens* exposure with and without β-glucan costimulation. PBS birds served as the negative control and received an intra-abdominal injection of 300 µl of PBS; CP birds received 10^8 CFU of heat-killed *C. perfringens*; β-glucan+CP birds received 1 mg of zymosan and 10^8 CFU of heat-killed *C. perfringens*. mRNA abundance of the examined genes was determined 4h after the injection treatment on day 1 of age. The result was expressed as fold changes and analyzed by one-way ANOVA followed by Tukey's multiple comparison test. Data represent mean ± SEM (n = 8). *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001 between priming treatments. CP, *C. perfringens*.

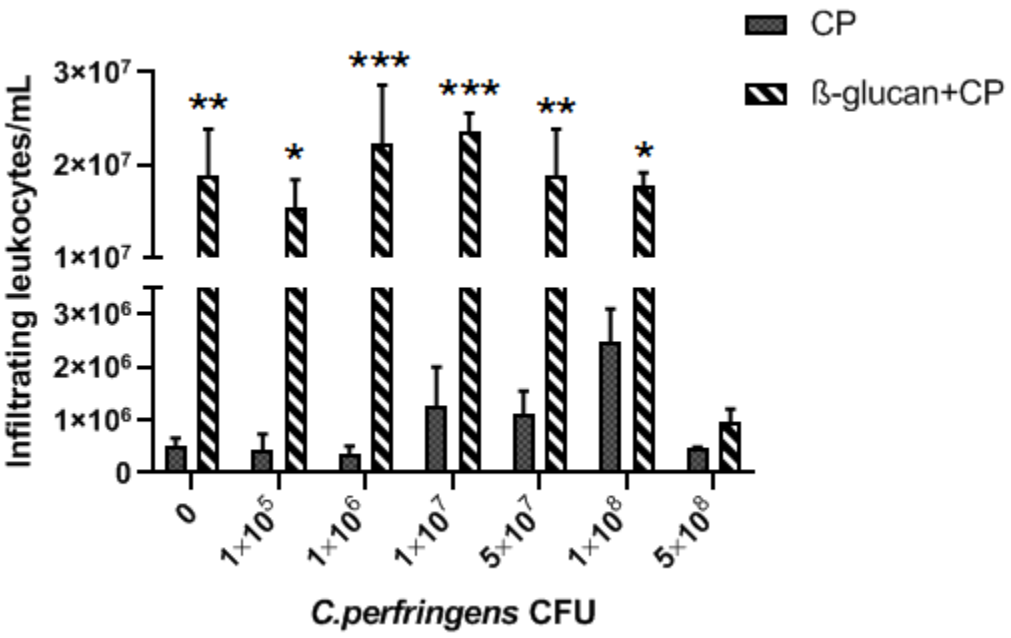

Figure 13. Impact of *C. perfringens* concentration on leukocyte infiltration with and without β-glucan inclusion. At 12 h post injection (at 1 day of age), the number of total infiltrating leukocytes were determined and analyzed by two-way ANOVA. Both *C. perfringens* concentration ($P<0.0001$) and β-glucan inclusion ($P=0.0167$) affected leukocyte infiltration significantly. Comparison of cell number between CP and β-glucan+CP was made using Sidak's multiple comparisons test and significant differences are labeled above the bar. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$ between priming treatments. CP, *C. perfringens.*

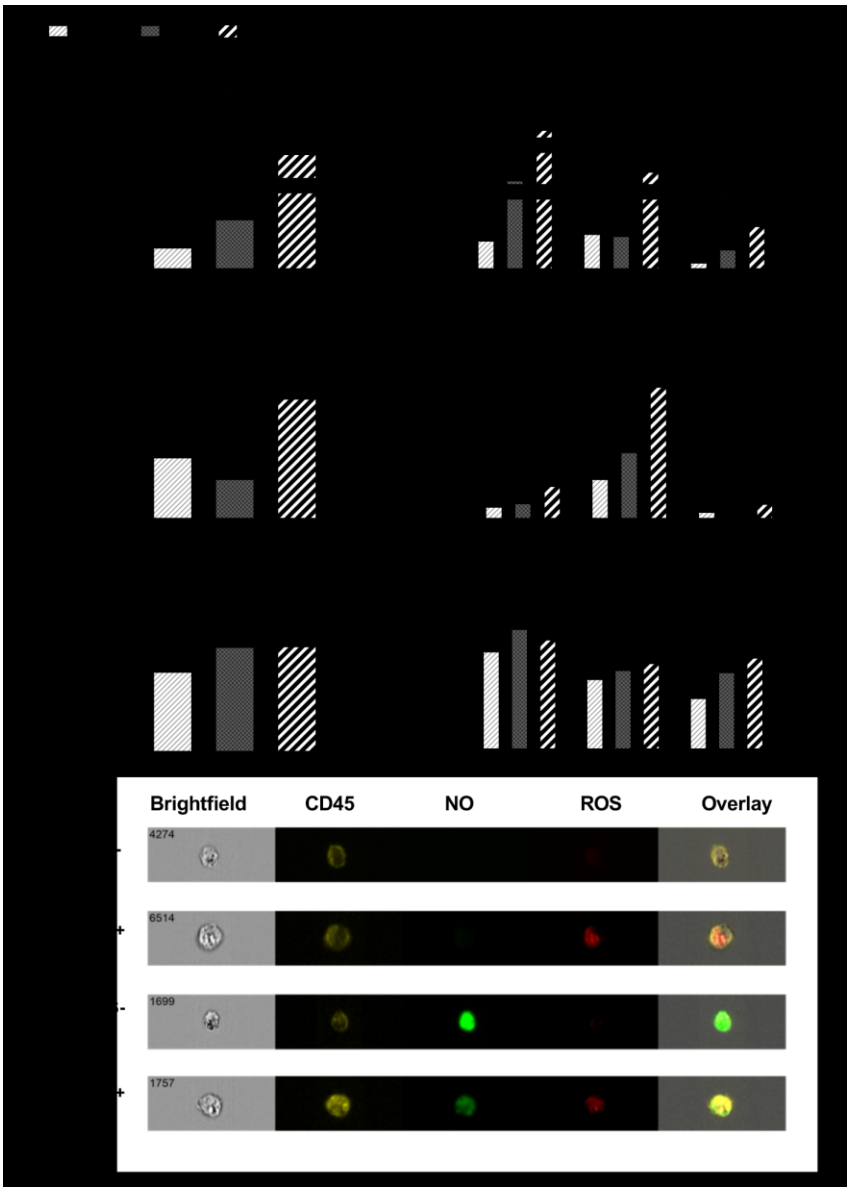

Figure 14. Changes in infiltrating leukocyte composition and function following initial *C. perfringens* exposure with and without β-glucan costimulation. PBS birds served as the negative control and received an intra-abdominal injection of 300 µl of PBS; CP birds received 10^8 CFU of heat-killed *C. perfringens*; β-glucan+CP birds received 1 mg of zymosan and 10^8 CFU of heat-killed *C. perfringens*. At 12 h post injection on day 1 of age, the total infiltrating leukocytes were counted (A) and were subsequently examined for capacity of producing ROS (C) and NO (E). The cells were subset into heterophils, macrophages, and lymphocytes to reveal the composition changes (B). ROS (D) and NO (F) production among distinct phagocyte subsets were determined. Data were collected in imaging flow cytometry and representative images are shown in panel G. Data represent mean ± SEM (n = 4). Results were analyzed by one-way ANOVA followed by Tukey's multiple comparison test. *P < 0.05 and **P < 0.01 between priming treatments. CP, *C. perfringens*.

Chapter 6: General discussion

6.1 Introduction

As the poultry industry moves away from antibiotic use, maintenance of a healthy flock requires a multi-faceted strategy that includes good husbandry, strong biosecurity practices, proper feed formulation, and veterinary care (Wilde et al., 2019). However, the lack of reliable disease models for experimental NE induction have held back NE research progress. My first goal was to develop a challenge model that accurately mimics the NE infection in a commercial setting (Chapter 3). This part of my work produced a novel challenge model that will take NE research to the next level. It considers a number of critical factors in reproducing NE disease: predisposing environment, natural uptake of the pathogens, timing and severity of disease outbreaks, and long-standing bacterial tolerance. With this simple yet effective challenge model available, I next examined the impact of antibiotic removal on production performance and disease severity of NE-affected chickens (Chapter 4). My data shows that antibiotic treatment failed to improve overall production performance, and negatively affected intestinal health parameters. Use of in-feed antibiotic was associated with increased *netB* density in the cecum. This findings support the opinion that the emergence of antibiotic-resistant pathogens are hampering the nutritional and economic potential of poultry (Andrew Selaledi et al., 2020). In answer to the urgent need for antibiotic alternatives to combat NE, my next dataset demonstrates the protection longevity of β-glucan via a single intra-abdominal administration in day-old chicks (Chapter 5). The timing of β-glucan administration is relevant to routine vaccine programs in practical poultry production. This work provides further understanding on the immunomodulatory effect of β-glucan, an adjuvant candidate to enhance immunogenicity of NE

vaccines. Future studies should evaluate the effect of β-glucan as a vaccine adjuvant for NE prevention.

6.2 Summary of findings

6.2.1 Appropriate combination of risk factors induced NE mimicking the field cases

This challenge model incorporated access to litter, coccidial exposure, feed composition, and feeding stress to reproduce NE infections typically encountered in the field. The stepwise optimization process eventually achieved the commonly observed subclinical infection 3 weeks post hatch. NE development is confirmed by gut lesion pathology, clinical signs, and mortality rate. Under cage-reared conditions, birds challenged with a 15× coccidial vaccine showed overall NE lesion prevalence 8-fold higher than those with 10× coccidial vaccine challenge. NE-associated mortality was observed only in a floor-reared flock.

Conventional NE models usually involve repeated oral inoculations of coccidial oocysts and *C. perfringens* for consecutive days (Gholamiandehkordi et al., 2007; Jayaraman et al., 2013; McReynolds et al., 2004; Park et al., 2008; Williams et al., 2003; Wu et al., 2010). One of the remarkable advantages of our natural NE model is the simplicity in the challenge schedule. Coccidial inoculation typically takes 30 minutes for 2 experienced technicians to gavage 100 birds, while feed withdrawal can be done within an hour even in large flocks. Experience from our research facility showed consistent induction of NE across studies using this protocol. Reduced complexity in the challenge schedule can limit animal stress and treatment inconsistency between different personnel, thus contributing to persistent induction of NE disease.

Feed withdrawal introduced on day 18 in this natural NE challenge is important to trigger timely development of disease outbreak. At the same time, this approach is believed to cause limited stress to the animals and considered humane when used properly. Pathogenic *C. perfringens* has stronger ability in binding extracellular matrices and utilizing nutrients released from host intestine tissue, thus showing selective survival advantage over non-pathogenic strains during feed withdrawal (Timbermont et al., 2009 and 2014; Wade et al., 2015). Feed restriction is often used in conventional NE challenges, not as a designated stressor but as a measure to ensure uniformity of inoculation treatments (Shojadoost et al., 2012). In those situations, the *C. perfringens* inoculation is administered mixed with feed, and feed is removed overnight so that the birds will eagerly eat the inoculated feed.

The NE-causing *C. perfringens* are of high diversity in terms of genomic content, with varied ability of causing intestinal damage. The growing understanding in the differences between strains isolated from animals of different health status and geographical regions highlights the need to carefully select appropriate strains to use in experimental NE models. It was reported that two *C. perfringens* strains, both isolated from NE-affected chickens and carrying *NetB*, showed varied virulence and produced different levels of disease severity in experimentally-induced NE (Gharib-Naseri et al., 2019). The natural infection approach in NE reproduction would allow propagation of pathogenic strains that are locally prevalent. Knowledge gained using this challenge model can likely be applied across wide geographic regions. This would contribute to a better understanding of NE development thus more targeted control strategies against the regionally prevalent pathogenic strains.

6.2.2 Antibiotic inclusion prevented short-term body weight decline but negatively impacted gut health

This research aimed to understand the impacts of antibiotic removal from poultry feed on NE severity and growth performance. My data shows that supplementation of antibiotics was partially protective against body weight decline, but it negatively affected fermentation activity and pathogen load in the gut. In NE infection driven by inoculation of *C. perfringens* culture, supplementation of antibiotics had resulted in increased growth performance and reduced disease severity (Prescott et al., 1978; Elwinger et al., 1998; Brennan et al., 2003; M'Sadeq et al., 2015). In contrast, under naturally-occuring NE challenges, antibiotic treatment was shown to hampered early-age feed conversion, though had no effect on overall growth performance (Calik et al., 2019). This is consistent with our findings, where medicated birds showed impaired feed conversion during the starter phase but equivalent growth performance compared to drug-free animals. Since we observed n increased *netB* abundance in cecal digesta of antibiotic-fed birds, one may speculate that experimentally-inoculated *C. perfringens* is less resistant compared to the naturally-occurring strains. Antibiotics are often administered in small amounts for the purpose of promoting growth (Diaz Carrasco et al., 2018). This long-term, low-dose exposure may promote the establishment of various antibiotic resistance mechanisms within gut microbial communities (Andrew Selaledi et al., 2020).

6.2.3 β-glucan increased NE resistance by influencing the initial responses against *C. perfringens*

β-glucan was recently characterized as the primary driver of trained immunity that revert tolerized phenotype of innate immune cells based on mammalian studies. In chickens, routine

supplementation of dietary β-glucan was shown to modulate immune responses and improve performance parameters under NE infection (Ahiwe et al., 2019; Johnson et al., 2020; M'Sadeq et al., 2015; Tian et al., 2016). However, a gap remained whether β-glucan confers immune redirection against the immune tolerance caused by *C. perfringens*. Answers to this question will contribute to NE vaccine design by effectively breaking the systemic tolerance against the gut-colonized pathogen. My work demonstrates that β-glucan co-administered with *C. perfringens* at hatch conferred long-term benefit on intestine morphology that improved feed conversion. This is associated with metabolic and functional changes in leukocytes following the initial stimulation. β-glucan co-stimulation enhanced glycolysis, proinflammatory cytokine expression, and ROS production. These results suggest an immune redirection from unresponsiveness toward activation during the early pathogen encounter, which prepared the animals for stronger disease resistance at later age.

Compared to the traditionally used antibiotic approach, immune modulation by β-glucan is more effective against the naturally-occurring NE challenge (Chapter 4 and 5). This suggests immune activation by β-glucan could be the solution to enhance NE resistance during the post-antibiotic era. Indeed, the capacity of β-glucan to enhance vaccine immunogenicity is being actively experimented, with positive effects against Newcastle disease and avian influenza (Le et al., 2011; Wang et al., 2014). In the biomedicine field, vaccinology research aimed at taking advantage of trained immunity is quickly evolving. In 2017, CpG was approved by the FDA as an adjuvant in a hepatitis B vaccine, whose ability to trigger trained immunity has been recognized (Owen et al., 2020). Chapter 5 of this thesis demonstrates early-life metabolic and functional changes in leukocytes was translated into a stronger NE resistance throughout the

rearing period. This contributes to understanding the gap between development of trained immunity in chicken monocytes *in vitro* and the protective effect via routine application *in vivo*.

6.3 Future directions

Much progress has been made in the past decade in understanding NE pathogenesis due to the maturation of high-throughput sequencing techniques. There is abundant data generated in the host transcriptome response, microRNA regulation profile, gut microbiota shift and bacteria transcriptome responses during NE infection (Dinh et al., 2014; Parreira et al., 2016; Rengaraj et al., 2016; Wu et al. 2014). However, the data is predominantly collected based on disease triggered by exogenous *C. perfringens* inoculation. It is necessary to identify the alternatively regulated virulence factors of *C. perfringens* and immune response of the host during a natural infection, which is important to understand the complex NE disease. For research aimed at prophylaxis of NE, it is critical to conduct the evaluation under conditions accurately mimicking the disease development under practical production conditions. The infection model presented in this study was able to reproduce the commonly observed subclinical NE, regarding the severity of symptoms, timing of lesion development, and rate of mortality. By allowing the pathogen to develop *in vivo*, NE researchers will be able to evaluate a prophylactic strategy at an early stage of disease development, when the damage to the animal is most reversible thus ideal to be targeted.

Mammalian studies have revealed that β-glucan mediated trained immunity is driven by metabolic reprogramming, hematopoiesis regulation, and epigenetic modifications. Although trained immunity in chicken monocytes are also known to be regulated by metabolic shift toward enhanced glycolysis (Verwoolde et al., 2020), there is much that remains to be elucidated. It is

still unclear whether intestinal epithelial differentiation and immune defense can be modulated by β-glucan via induction of trained immunity. It is critical to further understand the cell types and intra/inter-cellular mechanisms responsible for conferring the stronger NE resistance via trained immunity. One direction is to reveal the impact of trained immunity on intestinal epithelium differentiation and morphology. Previous studies suggest gut residence macrophages are critical for regulating intestinal stem cells activities (Sehgal et al., 2018). Remarkably, the differentiation and function of gut epithelium are under the control of HIF-1 (Fachi et al., 2019) and glycolysis metabolite lactate (Allaire et al., 2018), the key players in induction of trained immunity.

References

Abdolmaleki M, Yeap SK, Tan SW, Satharasinghe DA, Bello MB, Jahromi MZ, Bejo MH, Omar AR, Ideris A. Effects of Newcastle disease virus infection on chicken intestinal intraepithelial natural killer cells. Frontiers in Immunology. 2018 Jun 20;9:1386.

Abildgaard L, Hojberg O, Schramm A, Balle KM, Engberg RM. The effect of feeding a commercial essential oil product on *Clostridium perfringens* numbers in the intestine of broiler chickens measured by real-time PCR targeting the α-toxin-encoding gene (*plc*). Animal Feed Science and Technology. 2010a May 11;157(3-4):181-9.

Abildgaard L, Sondergaard TE, Engberg RM, Schramm A, Højberg O. In vitro production of necrotic enteritis toxin B, NetB, by netB-positive and netB-negative *Clostridium perfringens* originating from healthy and diseased broiler chickens. Veterinary Microbiology. 2010b Jul 29;144(1-2):231-5.

Ahiwe EU, Chang'a EP, Abdallh ME, Al-Qahtani M, Kheravii SK, Wu S, Graham H, Iji PA. Dietary hydrolysed yeast cell wall extract is comparable to antibiotics in the control of subclinical necrotic enteritis in broiler chickens. British Poultry Science. 2019 Nov 2;60(6):757-65.

Al-Khalaifah H, Al-Nasser A. Cytokines as effective elements of the avian immune system. Journal of Microbiology and Genetics: JMGE-119. DOI. 2018;10:2574-7371.

Allaire JM, Crowley SM, Law HT, Chang SY, Ko HJ, Vallance BA. The intestinal epithelium: central coordinator of mucosal immunity. Trends in Immunology. 2018 Sep 1;39(9):677-96.

Alkie TN, Yitbarek A, Hodgins DC, Kulkarni RR, Taha-Abdelaziz K, Sharif S. Development of innate immunity in chicken embryos and newly hatched chicks: A disease control perspective. Avian Pathology. 2019 Jul 4;48(4):288-310.

Andrew Selaledi L, Mohammed Hassan Z, Manyelo TG, Mabelebele M. The current status of the alternative use to antibiotics in poultry production: An African perspective. Antibiotics. 2020 Sep;9(9):594.

Annett CB, Viste JR, Chirino-Trejo M, Classen HL, Middleton DM, Simko E. Necrotic enteritis: effect of barley, wheat and corn diets on proliferation of *Clostridium perfringens* type A. Avian Pathology. 2002 Dec 1;31(6):598-601.

Ao Z, Kocher A, Choct M. Effects of dietary additives and early feeding on performance, gut development and immune status of broiler chickens challenged with *Clostridium perfringens*. Asian-Australasian Journal of Animal Sciences. 2012 Apr;25(4):541.

Arts RJ, Novakovic B, ter Horst R, Carvalho A, Bekkering S, Lachmandas E, Rodrigues F, Silvestre R, Cheng SC, Wang SY, Habibi E. Glutaminolysis and fumarate accumulation integrate immunometabolic and epigenetic programs in trained immunity. Cell Metabolism. 2016 Dec 13;24(6):807-19.

Atarashi K, Tanoue T, Shima T, Imaoka A, Kuwahara T, Momose Y, Cheng G, Yamasaki S, Saito T, Ohba Y, Taniguchi T. Induction of colonic regulatory T cells by indigenous *Clostridium* species. Science. 2011 Jan 21;331(6015):337-41.

Aviagen, Inc. 2019. Ross Broiler Nutrition Specifications. Aviagen, Inc. Huntsville, AL. USA.

Aychek T, Jung S. The axis of tolerance. Science. 2014 Mar 28;343(6178):1439-40.

Bannam TL, Yan XX, Harrison PF, Seemann T, Keyburn AL, Stubenrauch C, Weeramantri LH, Cheung JK, McClane BA, Boyce JD, Moore RJ. Necrotic enteritis-derived *Clostridium perfringens* strain with three closely related independently conjugative toxin and antibiotic resistance plasmids. MBio. 2011 Nov 1;2(5).

Barbara AJ, Trinh HT, Glock RD, Songer JG. Necrotic enteritis-producing strains of *Clostridium perfringens* displace non-necrotic enteritis strains from the gut of chicks. Veterinary Microbiology. 2008 Jan 25;126(4):377-82.

Bekkering S, Blok BA, Joosten LA, Riksen NP, van Crevel R, Netea MG. In vitro experimental model of trained innate immunity in human primary monocytes. Clinical and Vaccine Immunology. 2016 Dec 1;23(12):926-33

Bekkering S, Arts RJ, Novakovic B, Kourtzelis I, van der Heijden CD, Li Y, Popa CD, Ter Horst RO, van Tuijl J, Netea-Maier RT, van de Veerdonk FL. Metabolic induction of trained immunity through the mevalonate pathway. Cell. 2018 Jan 11;172(1-2):135-46.

Bernier G, Phaneuf JB, Filion R. Necrotic enteritis in broilers. I. Clinoco-pathological aspect. Canadian Journal of Comparative Medicine: Revue Canadienne de Médecine Comparée. 1974 Jul 1;38(3):280-5.

Bortoluzzi C, Vieira BS, Hofacre C, Applegate TJ. Effect of different challenge models to induce necrotic enteritis on the growth performance and intestinal microbiota of broiler chickens. Poultry Science. 2019 Jul 1;98(7):2800-12.

Bouwmeester T, Bauch A, Ruffner H, Angrand PO, Bergamini G, Croughton K, Cruciat C, Eberhard D, Gagneur J, Ghidelli S, Hopf C. A physical and functional map of the human TNF-α/NF-κB signal transduction pathway. Nature Cell Biology. 2004 Feb;6(2):97-105.

Bradley JR. TNF-mediated inflammatory disease. The Journal of Pathology: A Journal of the Pathological Society of Great Britain and Ireland. 2008 Jan;214(2):149-60.

Brennan J, Skinner J, Barnum DA, Wilson J. The efficacy of bacitracin methylene disalicylate when fed in combination with narasin in the management of necrotic enteritis in broiler chickens. Poultry Science. 2003 Mar 1;82(3):360-3.

Breous E, Somanathan S, Vandenberghe LH, Wilson JM. Hepatic regulatory T cells and Kupffer cells are crucial mediators of systemic T cell tolerance to antigens targeting murine liver. Hepatology. 2009 Aug;50(2):612-21.

Brisbin JT, Gong J, Sharif S. Interactions between commensal bacteria and the gut-associated immune system of the chicken. Animal Health Research Reviews. 2008 Jun 1;9(1):101.

Broom LJ. Host–microbe interactions and gut health in poultry—focus on innate responses. Microorganisms. 2019 May;7(5):139.

Butaye P, Devriese LA, Haesebrouck F. Antimicrobial growth promoters used in animal feed: effects of less well known antibiotics on gram-positive bacteria. Clinical Microbiology Reviews. 2003 Apr 1;16(2):175-88.

Byrne KA, Loving CL, McGill JL. Innate immunomodulation in food animals: evidence for trained immunity? Frontiers in Immunology. 2020 Jun 5;11:1099.

Calik A, Omara II, White MB, Evans NP, Karnezos TP, Dalloul RA. Dietary non-drug feed additive as an alternative for antibiotic growth promoters for broilers during a necrotic enteritis challenge. Microorganisms. 2019 Aug;7(8):257.

Cao L, Yang XJ, Li ZJ, Sun FF, Wu XH, Yao JH. Reduced lesions in chickens with *Clostridium perfringens*-induced necrotic enteritis by *Lactobacillus fermentum* 1.2029. Poultry Science. 2012 Dec 1;91(12):3065-71.

Claver JA, Quaglia AI. Comparative morphology, development, and function of blood cells in nonmammalian vertebrates. Journal of Exotic Pet Medicine. 2009 Apr 1;18(2):87-97.

CDC. Surveillance for foodborne disease outbreaks. Atlanta, GA United States, 2012, Annual Report. US Department of Health and Human Services, CDC, 2014

Chang CH, Curtis JD, Maggi Jr LB, Faubert B, Villarino AV, O'Sullivan D, Huang SC, Van Der Windt GJ, Blagih J, Qiu J, Weber JD. Posttranscriptional control of T cell effector function by aerobic glycolysis. Cell. 2013 Jun 6;153(6):1239-51.

Charlebois A, Jalbert LA, Harel J, Masson L, Archambault M. Characterization of genes encoding for acquired bacitracin resistance in *Clostridium perfringens*. PLoS One. 2012 Sep 6;7(9):e44449.

Cheng SC, Quintin J, Cramer RA, Shepardson KM, Saeed S, Kumar V, Giamarellos-Bourboulis EJ, Martens JH, Rao NA, Aghajanirefah A, Manjeri GR. mTOR-and HIF-1α–mediated aerobic glycolysis as metabolic basis for trained immunity. Science. 2014 Sep 26;345(6204):1250684.

Chistiakov DA, Bobryshev YV, Kozarov E, Sobenin IA, Orekhov AN. Intestinal mucosal tolerance and impact of gut microbiota to mucosal tolerance. Frontiers in Microbiology. 2015 Jan 13;5:781.

Choct M, Sinlae M, Al-Jassim RA, Pettersson D. Effects of xylanase supplementation on between-bird variation in energy metabolism and the number of *Clostridium perfringens* in broilers fed a wheat-based diet. Australian Journal of Agricultural Research. 2006 Aug 30;57(9):1017-21.

Chomarat P, Banchereau J, Davoust J, Palucka AK. IL-6 switches the differentiation of monocytes from dendritic cells to macrophages. Nature Immunology. 2000 Dec;1(6):510-4.

Collier CT, Hofacre CL, Payne AM, Anderson DB, Kaiser P, Mackie RI, Gaskins HR. Coccidia-induced mucogenesis promotes the onset of necrotic enteritis by supporting *Clostridium perfringens* growth. Veterinary Immunology and Immunopathology. 2008 Mar 15;122(1-2):104-15.

Cox CM, Stuard LH, Kim S, McElroy AP, Bedford MR, Dalloul RA. Performance and immune responses to dietary β-glucan in broiler chicks. Poultry Science. 2010a Sep 1;89(9):1924-33.

Cox CM, Sumners LH, Kim S, McElroy AP, Bedford MR, Dalloul RA. Immune responses to dietary β-glucan in broiler chicks during an *Eimeria* challenge. Poultry Science. 2010b Dec 1;89(12):2597-607.

Crhanova M, Hradecka H, Faldynova M, Matulova M, Havlickova H, Sisak F, Rychlik I. Immune response of chicken gut to natural colonization by gut microflora and to *Salmonella enterica serovar enteritidis* infection. Infection and Immunity. 2011 Jul 1;79(7):2755-63.

Dahiya JP, Hoehler D, Van Kessel AG, Drew MD. Dietary encapsulated glycine influences *Clostridium perfringens* and *Lactobacilli* growth in the gastrointestinal tract of broiler chickens. The Journal of Nutrition. 2007 Jun 1;137(6):1408-14.

De Boever S, Beyaert R, Vandemaele F, Baert K, Duchateau L, Goddeeris B, De Backer P, Croubels S. The influence of age and repeated lipopolysaccharide administration on body temperature and the concentration of interleukin-6 and IgM antibodies against lipopolysaccharide in broiler chickens. Avian Pathology. 2008 Feb 1;37(1):39-44.

Derache C, Esnault E, Bonsergent C, Le Vern Y, Quéré P, Lalmanach AC. Differential modulation of β-defensin gene expression by *Salmonella Enteritidis* in intestinal epithelial cells from resistant and susceptible chicken inbred lines. Developmental & Comparative Immunology. 2009 Sep 1;33(9):959-66.

Diaz Carrasco JM, Redondo EA, Pin Viso ND, Redondo LM, Farber MD, Fernandez Miyakawa ME. Tannins and bacitracin differentially modulate gut microbiota of broiler chickens. BioMed Research International. 2018 Feb 21;2018.

Diaz Carrasco JM, Casanova NA, Fernández Miyakawa ME. Microbiota, gut health and chicken productivity: what is the connection? Microorganisms. 2019 Oct;7(10):374.

Dierick E, Hirvonen OP, Haesebrouck F, Ducatelle R, Van Immerseel F, Goossens E. Rapid growth predisposes broilers to necrotic enteritis. Avian Pathology. 2019 Sep 3;48(5):416-22.

Dierick E, Ducatelle R, Van Immerseel F, Goossens E. Research Note: The administration schedule of coccidia is a major determinant in broiler necrotic enteritis models. Poultry Science. 2021 Mar 1;100(3):100806.

Dinh H, Hong YH, Lillehoj HS. Modulation of microRNAs in two genetically disparate chicken lines showing different necrotic enteritis disease susceptibility. Veterinary Immunology and Immunopathology. 2014 May 15;159(1-2):74-82.

Domínguez-Andrés J, Joosten LA, Netea MG. Induction of innate immune memory: the role of cellular metabolism. Current Opinion in Immunology. 2019a Feb 1;56:10-6.

Domínguez-Andrés J, Novakovic B, Li Y, Scicluna BP, Gresnigt MS, Arts RJ, Oosting M, Moorlag SJ, Groh LA, Zwaag J, Koch RM. The itaconate pathway is a central regulatory node linking innate immune tolerance and trained immunity. Cell Metabolism. 2019b Jan 8;29(1):211-20.

Dupré-Crochet S, Erard M, Nüβe O. ROS production in phagocytes: why, when, and where?. Journal of Leukocyte Biology. 2013 Oct;94(4):657-70.

Eckmann L. Innate immunity and mucosal bacterial interactions in the intestine. Current Opinion in Gastroenterology. 2004 Mar 1;20(2):82-8.

Elleder D, Kaspers B. After TNF-alpha, still playing hide-and-seek with chicken genes. Poultry Science. 2019 Oct 1.

Elwinger K, Berndtson E, Engström B, Fossum O, Waldenstedt L. Effect of antibiotic growth promoters and anticoccidials on growth of *Clostridium perfringens* in the caeca and on performance of broiler chickens. Acta Veterinaria Scandinavica. 1998 Dec;39(4):433-41.

Engström BE, Fermer C, Lindberg A, Saarinen E, Båverud V, Gunnarsson A. Molecular typing of isolates of *Clostridium perfringens* from healthy and diseased poultry. Veterinary Microbiology. 2003 Jul 17;94(3):225-35.

Engström BE, Johansson A, Aspan A, Kaldhusdal M. Genetic relatedness and netB prevalence among environmental *Clostridium perfringens* strains associated with a broiler flock affected by mild necrotic enteritis. Veterinary Microbiology. 2012 Sep 14;159(1-2):260-4.

Emami NK, Calik A, White MB, Young M, Dalloul RA. Necrotic enteritis in broiler chickens: the role of tight junctions and mucosal immune responses in alleviating the effect of the disease. Microorganisms. 2019 Aug;7(8):231.

Emami NK, White MB, Calik A, Kimminau EA, Dalloul RA. Managing broilers gut health with antibiotic-free diets during subclinical necrotic enteritis. Poultry Science. 2021 Feb 16:101055.

Fachi JL, de Souza Felipe J, Pral LP, da Silva BK, Corrêa RO, de Andrade MC, da Fonseca DM, Basso PJ, Câmara NO, e Souza ÉL, dos Santos Martins F. Butyrate protects mice from *Clostridium difficile*-induced colitis through an HIF-1-dependent mechanism. Cell Reports. 2019 Apr 16;27(3):750-61.

Fan H, Cook JA. Molecular mechanisms of endotoxin tolerance. Journal of Endotoxin Research. 2004 Apr;10(2):71-84.

Fang FC. Antimicrobial reactive oxygen and nitrogen species: concepts and controversies. Nature Reviews Microbiology. 2004 Oct;2(10):820-32.

Fasina YO, Newman MM, Stough JM, Liles MR. Effect of *Clostridium perfringens* infection and antibiotic administration on microbiota in the small intestine of broiler chickens. Poultry Science. 2016 Feb 1;95(2):247-60.

Fasina YO, Olowo YL. Effect of a commercial yeast-based product (Maxigen®) on intestinal villi morphology and growth performance of broiler chickens. International Journal of Poultry Science. 2013 Jan 1;12(1):09-14.

Feng Y, Gong J, Yu H, Jin Y, Zhu J, Han Y. Identification of changes in the composition of ileal bacterial microbiota of broiler chickens infected with *Clostridium perfringens*. Veterinary Microbiology. 2010 Jan 6;140(1-2):116-21.

Fernando PS, Rose SP, Mackenzie AM, Silva SS. Effect of diets containing potato protein or soya bean meal on the incidence of spontaneously-occurring subclinical necrotic enteritis and the physiological response in broiler chickens. British Poultry Science. 2011 Feb 1;52(1):106-14.

Fialkow L, Wang Y, Downey GP. Reactive oxygen and nitrogen species as signaling molecules regulating neutrophil function. Free Radical Biology and Medicine. 2007 Jan 15;42(2):153-64.

Freedman JC, Theoret JR, Wisniewski JA, Uzal FA, Rood JI, McClane BA. *Clostridium perfringens* type A–E toxin plasmids. Research in Microbiology. 2015 May 1;166(4):264-79.

Freitas-Lopes MA, Mafra K, David BA, Carvalho-Gontijo R, Menezes GB. Differential location and distribution of hepatic immune cells. Cells. 2017 Dec;6(4):48.

Fu Y, Glaros T, Zhu M, Wang P, Wu Z, Tyson JJ, Li L, Xing J. Network topologies and dynamics leading to endotoxin tolerance and priming in innate immune cells. PLoS Computational Biology. 2012 May 17;8(5):e1002526.

Gao B, Jeong WI, Tian Z. Liver: an organ with predominant innate immunity. Hepatology. 2008 Feb;47(2):729-36.

Gasteiger G, Rudensky AY. Interactions between innate and adaptive lymphocytes. Nature Reviews Immunology. 2014 Sep;14(9):631-9.

Gaucher ML, Perron GG, Arsenault J, Letellier A, Boulianne M, Quessy S. Recurring necrotic enteritis outbreaks in commercial broiler chicken flocks strongly influence toxin gene carriage and species richness in the resident *Clostridium perfringens* population. Frontiers in Microbiology. 2017 May 17;8:881.

Genovese KJ, He H, Swaggerty CL, Kogut MH. The avian heterophil. Developmental & Comparative Immunology. 2013 Nov 1;41(3):334-40.

Gharib-Naseri K, Kheravii SK, Keerqin C, Morgan N, Swick RA, Choct M, Wu SB. Two different *Clostridium perfringens* strains produce different levels of necrotic enteritis in broiler chickens. Poultry Science. 2019 Dec 1;98(12):6422-32.

Gholamiandekhordi AR, Ducatelle R, Heyndrickx M, Haesebrouck F, Van Immerseel F. Molecular and phenotypical characterization of *Clostridium perfringens* isolates from poultry flocks with different disease status. Veterinary Microbiology. 2006 Mar 10;113(1-2):143-52.

Gholamiandehkordi AR, Timbermont L, Lanckriet A, van den Broeck W, Pedersen K, Dewulf J, Pasmans F, Haesebrouck F, Ducatelle R, van Immerseel F. Quantification of gut lesions in a subclinical necrotic enteritis model. Avian Pathology. 2007 Oct 1;36(5):375-82.

Gibson MS, Kaiser P, Fife M. The chicken IL-1 family: evolution in the context of the studied vertebrate lineage. Immunogenetics. 2014 Aug;66(7):427-38.

Göbel TW, Schneider K, Schaerer B, Mejri I, Puehler F, Weigend S, Staeheli P, Kaspers B. IL-18 stimulates the proliferation and IFN-γ release of CD4+ T cells in the chicken: conservation of a Th1-like system in a nonmammalian species. The Journal of Immunology. 2003 Aug 15;171(4):1809-15.

Gooz M. ADAM-17: the enzyme that does it all. Critical Reviews in Biochemistry and Molecular Biology. 2010 Apr 1;45(2):146-69.

Goossens E, Van Erum J, De Gussem M, Van Limbergen T, Callens C, De Zutter L, Ducatelle R, Van Immerseel F. Incidence and associated risk factors of necrotic enteritis in Belgian layer pullet flocks. Avian Pathology. 2020 Sep 2;49(5):476-85.

Gore AB, Qureshi MA. Enhancement of humoral and cellular immunity by vitamin E after embryonic exposure. Poultry Science. 1997 Jul 1;76(7):984-91.

Gourbal B, Pinaud S, Beckers GJ, Van Der Meer JW, Conrath U, Netea MG. Innate immune memory: An evolutionary perspective. Immunological Reviews. 2018 May;283(1):21-40.

Guo S, Li C, Liu D, Guo Y. Inflammatory responses to a *Clostridium perfringens* type a strain and α-toxin in primary intestinal epithelial cells of chicken embryos. Avian Pathology. 2015 Mar 4;44(2):81-91

Han Z, Zhang Y, Wang C, Liu X, Jiang A, Liu Z, Wang J, Yang Z, Wei Z. Ochratoxin A-triggered chicken heterophil extracellular traps release through reactive oxygen species production dependent on activation of NADPH oxidase, ERK, and p38 MAPK signaling pathways. Journal of Agricultural and Food Chemistry. 2019 Sep 15;67(40):11230-5.

Hedl M, Li J, Cho JH, Abraham C. Chronic stimulation of Nod2 mediates tolerance to bacterial products. Proceedings of the National Academy of Sciences. 2007 Dec 4;104(49):19440-5.

He H, Genovese KJ, Kogut MH. Modulation of chicken macrophage effector function by TH1/TH2 cytokines. Cytokine. 2011 Mar 1;53(3):363-9.

Herb M, Schramm M. Functions of ROS in macrophages and antimicrobial immunity. Antioxidants. 2021 Feb;10(2):313.

Hermans PG, Morgan KL. Prevalence and associated risk factors of necrotic enteritis on broiler farms in the United Kingdom; a cross-sectional survey. Avian Pathology. 2007 Feb 1;36(1):43-51.

Hoerr FJ. Pathogenesis of enteric diseases. Poultry Science. 1998 Aug 1;77(8):1150-5.

Hofacre CL. Necrotic enteritis, currently a billion dollar disease: is there anything new on the horizon. In Proceedings of Alltech's 17th Annual Symposium Science and Technology in the Feed Industry 2001 (pp. 79-86). Nottingham: Nottingham University Press.

Hofacre CL, Smith JA, Mathis GF. An optimist's view on limiting necrotic enteritis and maintaining broiler gut health and performance in today's marketing, food safety, and regulatory climate. Poultry Science. 2018 Jun 1;97(6):1929-33.

Hofacre C, Reynolds D, Mathis G, Lumpkins B, Ollis N, Smith J, Demey V. Effect of a competitive exclusion culture in a necrotic enteritis challenge model in broilers. Journal of Applied Poultry Research. 2019 Jun 1;28(2):350-5.

Hong YH, Dinh H, Lillehoj HS, Song KD, Oh JD. Differential regulation of microRNA transcriptome in chicken lines resistant and susceptible to necrotic enteritis disease. Poultry Science. 2014 Jun 1;93(6):1383-95.

Hong YH, Lillehoj HS, Lee SH, Park DW, Lillehoj EP. Molecular cloning and characterization of chicken lipopolysaccharide-induced TNF-α factor (LITAF). Developmental & Comparative Immunology. 2006 Jan 1;30(10):919-29.

Horiuchi T, Mitoma H, Harashima SI, Tsukamoto H, Shimoda T. Transmembrane TNF-α: structure, function and interaction with anti-TNF agents. Rheumatology. 2010 Jul 1;49(7):1215-28.

Huang Y, Chen Z. Inflammatory bowel disease related innate immunity and adaptive immunity. American Journal of Translational Research. 2016;8(6):2490.

Huang XY, Sun WY, Yan ZQ, Shi HR, Yang QL, Wang PF, Li SG, Liu LX, Zhao SG, Gun SB. Novel insights reveal anti-microbial gene regulation of piglet intestine immune in response to *Clostridium perfringens* infection. Scientific Reports. 2019 Feb 13;9(1):1-2.

Huff GR, Huff WE, Farnell MB, Rath NC, De Los Santos FS, Donoghue AM. Bacterial clearance, heterophil function, and hematological parameters of transport-stressed turkey poults supplemented with dietary yeast extract. Poultry Science. 2010 Mar 1;89(3):447-56.

Hughes RJ. Relationship between digesta transit time and apparent metabolisable energy value of wheat in chickens. British Poultry Science. 2008 Nov 1;49(6):716-20.

Ifrim DC, Quintin J, Joosten LA, Jacobs C, Jansen T, Jacobs L, Gow NA, Williams DL, van der Meer JW, Netea MG. Trained immunity or tolerance: opposing functional programs induced in human monocytes after engagement of various pattern recognition receptors. Clinical and Vaccine Immunology. 2014 Apr;21(4):534-45.

Iles KE, Forman HJ. Macrophage signaling and respiratory burst. Immunologic Research. 2002 Aug;26(1):95-105.

Immerseel FV, Buck JD, Pasmans F, Huyghebaert G, Haesebrouck F, Ducatelle R. *Clostridium perfringens* in poultry: an emerging threat for animal and public health. Avian Pathology. 2004 Dec 1;33(6):537-49.

Janeway Jr CA, Medzhitov R. Innate immune recognition. Annual Review of Immunology. 2002 Apr;20(1):197-216.

Jang SY, Kang HT, Hwang ES. Nicotinamide-induced mitophagy event mediated by high NAD+/NADH ratio and SIRT1 protein activation. Journal of Biological Chemistry. 2012 Jun 1;287(23):19304-14.

Jayaraman S, Thangavel G, Kurian H, Mani R, Mukkalil R, Chirakkal H. *Bacillus subtilis* PB6 improves intestinal health of broiler chickens challenged with *Clostridium perfringens*-induced necrotic enteritis. Poultry Science. 2013 Feb 1;92(2):370-4.

Jeurissen SH, Lewis F, Mroz Z, Rebel JM. Parameters and techniques to determine intestinal health of poultry as constituted by immunity, integrity, and functionality. Current Issues in Intestinal Microbiology. 2002 Mar;3(1):1-4.

Jiao Y, Wu L, Huntington ND, Zhang X. Crosstalk between gut microbiota and innate immunity and its implication in autoimmune diseases. Frontiers in Immunology. 2020 Feb 21;11:282.

Johansson A. *Clostridium perfringens* the causal agent of necrotic enteritis in poultry. 2006.

Johansson A, Aspán A, Kaldhusdal M, Engström BE. Genetic diversity and prevalence of *netB* in *Clostridium perfringens* isolated from a broiler flock affected by mild necrotic enteritis. Veterinary Microbiology. 2010 Jul 29;144(1-2):87-92.

Johansson A, Greko C, Engström BE, Karlsson M. Antimicrobial susceptibility of Swedish, Norwegian and Danish isolates of *Clostridium perfringens* from poultry, and distribution of tetracycline resistance genes. Veterinary Microbiology. 2004 Apr 19;99(3-4):251-7.

Johnson CN, Hashim MM, Bailey CA, Byrd JA, Kogut MH, Arsenault RJ. Feeding of yeast cell wall extracts during a necrotic enteritis challenge enhances cell growth, survival and immune signaling in the jejunum of broiler chickens. Poultry Science. 2020 Jun 1;99(6):2955-66.

Juul-Madsen HR, Viertlböeck B, Härtle S, Smith AL, Göbel TW. Innate immune responses. In Avian Immunology 2014 Jan 1 (pp. 121-147). Academic Press.

Kaiser P. Advances in avian immunology—prospects for disease control: a review. Avian Pathology. 2010 Oct 1;39(5):309-24.

Kaiser P, Stäheli P. Avian cytokines and chemokines. In Avian Immunology 2014 Jan 1 (pp. 189-204). Academic Press.

Kaldhusdal M, Benestad SL, Løvland A. Epidemiologic aspects of necrotic enteritis in broiler chickens–disease occurrence and production performance. Avian Pathology. 2016 May 3;45(3):271-4.

Kaldhusdal M, Skjerve E. Association between cereal contents in the diet and incidence of necrotic enteritis in broiler chickens in Norway. Preventive Veterinary Medicine. 1996 Aug 1;28(1):1-6.

Kamely M, He W, Wakaruk J, Whelan R, Naranjo V, Barreda D. Impact of reduced dietary crude protein in the starter phase on immune development and response of broilers throughout the growth period. Frontiers in Veterinary Science. 2020;7:436.

Kamiński MM, Sauer SW, Kamiński M, Opp S, Ruppert T, Grigaravičius P, Grudnik P, Gröne HJ, Krammer PH, Gülow K. T cell activation is driven by an ADP-dependent glucokinase linking enhanced glycolysis with mitochondrial reactive oxygen species generation. Cell Reports. 2012 Nov 29;2(5):1300-15.

Karnati HK, Pasupuleti SR, Kandi R, Undi RB, Sahu I, Kannaki TR, Subbiah M, Gutti RK. TLR-4 signalling pathway: MyD88 independent pathway up-regulation in chicken breeds upon LPS treatment. Veterinary Research Communications. 2015 Mar 1;39(1):73-8.

Kaspers B, Kaiser P. Avian antigen-presenting cells. In Avian Immunology 2014 Jan 1 (pp. 169-188). Academic Press.

Keyburn AL, Boyce JD, Vaz P, Bannam TL, Ford ME, Parker D, Di Rubbo A, Rood JI, Moore RJ. NetB, a new toxin that is associated with avian necrotic enteritis caused by *Clostridium perfringens*. PLoS Pathogen. 2008 Feb 8;4(2):e26.

Khalique A, Zeng D, Shoaib M, Wang H, Qing X, Rajput DS, Pan K, Ni X. Probiotics mitigating subclinical necrotic enteritis (SNE) as potential alternatives to antibiotics in poultry. AMB Express. 2020 Dec;10(1):1-0.

Kim DK, Lillehoj HS, Jang SI, Lee SH, Hong YH, Cheng HH. Transcriptional profiles of host-pathogen responses to necrotic enteritis and differential regulation of immune genes in two inbreed chicken lines showing disparate disease susceptibility. Plos One. 2014 Dec 11;9(12):e114960.

Kim WH, Lillehoj HS, Lim Y, Min W, Sullivan YB, Kakach L, LaBresh JW. Development and characterization of mouse monoclonal antibodies reactive with chicken CXCLi2. Developmental & Comparative Immunology. 2017 Jul 1;72:30-6.

Kim WH, Chaudhari AA, Lillehoj HS. Involvement of T cell immunity in avian coccidiosis. Frontiers in Immunology. 2019 Nov 22;10:2732.

Kim DH, Yoo YM, Kim SH, Jang BG, Park BY, Cho SH, Seong PN, Hah KH, Lee JM, Kim YK, Hwang IH. Effect of the length of feed withdrawal on weight loss, yield and meat color of broiler. Asian-Australasian Journal of Animal Sciences. 2006 Nov 27;20(1):106-11.

Koyasu S, Moro K. Role of innate lymphocytes in infection and inflammation. Frontiers in Immunology. 2012 May 7;3:101.

Kaufmann E, Sanz J, Dunn JL, Khan N, Mendonça LE, Pacis A, Tzelepis F, Pernet E, Dumaine A, Grenier JC, Mailhot-Léonard F. BCG educates hematopoietic stem cells to generate protective innate immunity against tuberculosis. Cell. 2018 Jan 11;172(1-2):176-90.

Kleessen B, Hartmann L, Blaut M. Fructans in the diet cause alterations of intestinal mucosal architecture, released mucins and mucosa-associated bifidobacteria in gnotobiotic rats. British Journal of Nutrition. 2003 May;89(5):597-606.

Kumar S, Chen C, Indugu N, Werlang GO, Singh M, Kim WK, Thippareddi H. Effect of antibiotic withdrawal in feed on chicken gut microbial dynamics, immunity, growth performance and prevalence of foodborne pathogens. PloS One. 2018 Feb 14;13(2):e0192450.

Lacey JA, Allnutt TR, Vezina B, Van TT, Stent T, Han X, Rood JI, Wade B, Keyburn AL, Seemann T, Chen H. Whole genome analysis reveals the diversity and evolutionary relationships between necrotic enteritis-causing strains of *Clostridium perfringens*. BMC Genomics. 2018 Dec 1;19(1):379.

Lacey JA, Johanesen PA, Lyras D, Moore RJ. Genomic diversity of necrotic enteritis-associated strains of *Clostridium perfringens*: a review. Avian Pathology. 2016 May 3;45(3):302-7.

Lacey JA, Keyburn AL, Ford ME, Portela RW, Johanesen PA, Lyras D, Moore RJ. Conjugation-mediated horizontal gene transfer of *Clostridium perfringens* plasmids in the chicken gastrointestinal tract results in the formation of new virulent strains. Applied and Environmental Microbiology. 2017 Dec 15;83(24).

Latham T, Mackay L, Sproul D, Karim M, Culley J, Harrison DJ, Hayward L, Langridge-Smith P, Gilbert N, Ramsahoye BH. Lactate, a product of glycolytic metabolism, inhibits histone deacetylase activity and promotes changes in gene expression. Nucleic Acids Research. 2012 Jun 1;40(11):4794-803.

Latorre JD, Adhikari B, Park SH, Teague KD, Graham LE, Mahaffey BD, Baxter MF, Hernandez-Velasco X, Kwon YM, Ricke SC, Bielke LR. Evaluation of the epithelial barrier function and ileal microbiome in an established necrotic enteritis challenge model in broiler chickens. Frontiers in Veterinary Science. 2018 Aug 21;5:199.

Le TH, Le TB, Doan TH, Quyen DV, Le KX, Pham VC, Nagataki M, Nomura H, Ikeue Y, Watanabe Y, Agatsuma T. The adjuvant effect of sophy beta-glucan to the antibody response in poultry immunized by the avian influenza A H5N1 and H5N2 vaccines. Journal of Microbiology and Biotechnology. 2011;21(4):405-11.

Lee SA, Apajalahti J, Vienola K, González-Ortiz G, Fontes CM, Bedford MR. Age and dietary xylanase supplementation affects ileal sugar residues and short chain fatty acid concentration in the ileum and caecum of broiler chickens. Animal Feed Science and Technology. 2017 Dec 1;234:29-42.

Lee SH, Lillehoj HS, Jang SI, Lillehoj EP, Min W, Bravo DM. Dietary supplementation of young broiler chickens with Capsicum and turmeric oleoresins increases resistance to necrotic enteritis. British Journal of Nutrition. 2013 Sep;110(5):840-7.

Lee KW, Lillehoj HS, Jeong W, Jeoung HY, An DJ. Avian necrotic enteritis: experimental models, host immunity, pathogenesis, risk factors, and vaccine development. Poultry Science. 2011 Jul 1;90(7):1381-90.

Lepp D, Roxas B, Parreira VR, Marri PR, Rosey EL, Gong J, Songer JG, Vedantam G, Prescott JF. Identification of novel pathogenicity loci in *Clostridium perfringens* strains that cause avian necrotic enteritis. PloS One. 2010 May 24;5(5):e10795.

Lepp D, Gong J, Songer JG, Boerlin P, Parreira VR, Prescott JF. Identification of accessory genome regions in poultry *Clostridium perfringens* isolates carrying the netB plasmid. Journal of Bacteriology. 2013 Mar 15;195(6):1152-66.

Li GH, Mine Y, Hincke MT, Nys Y. Isolation and characterization of antimicrobial proteins and peptide from chicken liver. Journal of Peptide Science: an official publication of the European Peptide Society. 2007 Jun;13(6):368-78.

Li M, Penner GB, Hernandez-Sanabria E, Oba M, Guan LL. Effects of sampling location and time, and host animal on assessment of bacterial diversity and fermentation parameters in the bovine rumen. Journal of Applied Microbiology. 2009 Dec;107(6):1924-34.

Li C, Wang Y, Li Y, Yu Q, Jin X, Wang X, Jia A, Hu Y, Han L, Wang J, Yang H. HIF1α-dependent glycolysis promotes macrophage functional activities in protecting against bacterial and fungal infection. Scientific Reports. 2018 Feb 26;8(1):1-1.

Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. Methods. 2001 Dec 1;25(4):402-8.

Long JR. Necrotic enteritis in broiler chickens I. A review of the literature and the prevalence of the disease in Ontario. Canadian Journal of Comparative Medicine. 1973 Jul;37(3):302.

Lopetuso LR, Scaldaferri F, Petito V, Gasbarrini A. Commensal Clostridia: leading players in the maintenance of gut homeostasis. Gut Pathogens. 2013 Dec 1;5(1):23.

Løvland A, Kaldhusdal M. Liver lesions seen at slaughter as an indicator of necrotic enteritis in broiler flocks. FEMS Immunology & Medical Microbiology. 1999 Jul 1;24(3):345-51.

Løvland A, Kaldhusdal M. Severely impaired production performance in broiler flocks with high incidence of *Clostridium perfringens*-associated hepatitis. Avian Pathology. 2001 Feb 1;30(1):73-81.

Løvland A, Kaldhusdal M, Reitan LJ. Diagnosing *Clostridium perfringens*-associated necrotic enteritis in broiler flocks by an immunoglobulin G anti-alpha-toxin enzyme-linked immunosorbent assay. Avian Pathology. 2003 Oct 1;32(5):527-34.

Løvland A, Kaldhusdal M, Redhead K, Skjerve E, Lillehaug A. Maternal vaccination against subclinical necrotic enteritis in broilers. Avian Pathology. 2004 Feb 1;33(1):81-90.

Lowry VK, Farnell MB, Ferro PJ, Swaggerty CL, Bahl A, Kogut MH. Purified β-glucan as an abiotic feed additive up-regulates the innate immune response in immature chickens against

Salmonella enterica serovar Enteritidis. International Journal of Food Microbiology. 2005 Feb 15;98(3):309-18.

Luu M, Visekruna A. Short-chain fatty acids: Bacterial messengers modulating the immunometabolism of T cells. European Journal of Immunology. 2019 Jun;49(6):842-8.

Mackinnon KM, He H, Swaggerty CL, McReynolds JL, Genovese KJ, Duke SE, Nerren JR, Kogut MH. In ovo treatment with CpG oligodeoxynucleotides decreases colonization of Salmonella enteriditis in broiler chickens. Veterinary Immunology and Immunopathology. 2009 Feb 15;127(3-4):371-5.

Maes M, Anderson G, Kubera M, Berk M. Targeting classical IL-6 signalling or IL-6 trans-signalling in depression? Expert Opinion on Therapeutic Targets. 2014 May 1;18(5):495-512.

Mahla RS, Reddy CM, Prasad D, Kumar H. Sweeten PAMPs: role of sugar complexed PAMPs in innate immunity and vaccine biology. Frontiers in Immunology. 2013 Sep 2;4:248.

Mahmood K, Rahman SU, Hussain I, Abbas RZ, Khaliq T, Arif J, Mahmood F. Non-antibiotic strategies for the control of necrotic enteritis in poultry. World's Poultry Science Journal. 2014 Dec;70(4):865-79.

Martel A, A. Devriese L, Cauwerts K, De Gussem K, Decostere A, Haesebrouck F. Susceptibility of *Clostridium perfringens* strains from broiler chickens to antibiotics and anticoccidials. Avian Pathology. 2004 Feb 1;33(1):3-7.

Martin TG, Smyth JA. The ability of disease and non-disease producing strains of *Clostridium perfringens* from chickens to adhere to extracellular matrix molecules and Caco-2 cells. Anaerobe. 2010 Oct 1;16(5):533-9.

Martin TG, Smyth JA. Prevalence of netB among some clinical isolates of *Clostridium perfringens* from animals in the United States. Veterinary Microbiology. 2009 Apr 14;136(1-2):202-5.

McDevitt, R. M., Brooker, J. D., Acamovic, T., & Sparks, N. H. C. (2006). Necrotic enteritis: a continuing challenge for the poultry industry. World's Poultry Science Journal, 62(2), 221-247.

McLoughlin RM, Jenkins BJ, Grail D, Williams AS, Fielding CA, Parker CR, Ernst M, Topley N, Jones SA. IL-6 trans-signaling via STAT3 directs T cell infiltration in acute inflammation. Proceedings of the National Academy of Sciences. 2005 Jul 5;102(27):9589-94.

McReynolds JL, Byrd JA, Anderson RC, Moore RW, Edrington TS, Genovese KJ, Poole TL, Kubena LF, Nisbet DJ. Evaluation of immunosuppressants and dietary mechanisms in an experimental disease model for necrotic enteritis. Poultry Science. 2004 Dec 1;83(12):1948-52.

Mebius RE, Kraal G. Structure and function of the spleen. Nature Reviews Immunology. 2005 Aug;5(8):606-16.

Meijerink N, van Haarlem DA, Velkers FC, Stegeman AJ, Rutten VP, Jansen CA. Analysis of chicken intestinal natural killer cells, a major IEL subset during embryonic and early life. Developmental & Comparative Immunology. 2021 Jan 1;114:103857.

estecky J, Russell MW, Elson CO. Perspectives on mucosal vaccines: is mucosal tolerance a barrier?. The Journal of Immunology. 2007 Nov 1;179(9):5633-8.

Miki Y, Miyamoto K, Kaneko-Hirano I, Fujiuchi K, Akimoto S. Prevalence and characterization of enterotoxin gene-carrying *Clostridium perfringens* isolates from retail meat products in Japan. Applied and Environmental Microbiology. 2008 Sep 1;74(17):5366-72.

Millet P, Vachharajani V, McPhail L, Yoza B, McCall CE. GAPDH binding to TNF-α mRNA contributes to posttranscriptional repression in monocytes: a novel mechanism of communication between inflammation and metabolism. The Journal of Immunology. 2016 Mar 15;196(6):2541-51.

Mills KH, Dunne A. Immune modulation: IL-1, master mediator or initiator of inflammation. Nature Medicine. 2009 Dec;15(12):1363-4.

Menk AV, Scharping NE, Moreci RS, Zeng X, Guy C, Salvatore S, Bae H, Xie J, Young HA, Wendell SG, Delgoffe GM. Early TCR signaling induces rapid aerobic glycolysis enabling distinct acute T cell effector functions. Cell Reports. 2018 Feb 6;22(6):1509-21.

Mitroulis I, Ruppova K, Wang B, Chen LS, Grzybek M, Grinenko T, Eugster A, Troullinaki M, Palladini A, Kourtzelis I, Chatzigeorgiou A. Modulation of myelopoiesis progenitors is an integral component of trained immunity. Cell. 2018 Jan 11;172(1-2):147-61.

Moore RJ. Necrotic enteritis predisposing factors in broiler chickens. Avian Pathology. 2016 May 3;45(3):275-81.

Moorlag SJ, Röring RJ, Joosten LA, Netea MG. The role of the interleukin-1 family in trained immunity. Immunological Reviews. 2018 Jan;281(1):28-39.

Mora ZV, Macías-Rodríguez ME, Arratia-Quijada J, Gonzalez-Torres YS, Nuño K, Villarruel-López A. *Clostridium perfringens* as foodborne pathogen in broiler production: Pathophysiology and potential strategies for controlling necrotic enteritis. Animals. 2020 Sep;10(9):1718.

More Bayona J. Applications of acute inflammation in livestock immunity. 2019. Doctoral dissertation. University of Alberta.

More Bayona J, Karuppannan AK, Barreda DR. Contribution of leukocytes to the induction and resolution of the acute inflammatory response in chickens. Developmental & Comparative Immunology. 2017 Sep 1;74:167-77.

Mot D, Timbermont L, Delezie E, Haesebrouck F, Ducatelle R, Van Immerseel F. Day-of-hatch vaccination is not protective against necrotic enteritis in broiler chickens. Avian Pathology. 2013 Apr 1;42(2):179-84.

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

M'Sadeq SA, Wu SB, Choct M, Forder R, Swick RA. Use of yeast cell wall extract as a tool to reduce the impact of necrotic enteritis in broilers. Poultry Science. 2015 May 1;94(5):898-905.

Munro DA, Hughes J. The origins and functions of tissue-resident macrophages in kidney development. Frontiers in Physiology. 2017 Oct 25;8:837.

Nagano Y, Itoh K, Honda K. The induction of Treg cells by gut-indigenous *Clostridium*. Current Opinion in Immunology. 2012 Aug 1;24(4):392-7.

Nagy N, Busalt F, Halasy V, Kohn M, Schmieder S, Fejszak N, Kaspers B, Härtle S. In and out of the bursa—the role of CXCR4 in chicken B cell development. Frontiers in Immunology. 2020 Jul 14;11:1468.

Nathan C, Shiloh MU. Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens. Proceedings of the National Academy of Sciences. 2000 Aug 1;97(16):8841-8.

Nauerby B, Pedersen K, Madsen M. Analysis by pulsed-field gel electrophoresis of the genetic diversity among *Clostridium perfringens* isolates from chickens. Veterinary Microbiology. 2003 Jul 17;94(3):257-66.

Nawab A, An L, Wu J, Li G, Liu W, Zhao Y, Wu Q, Xiao M. Chicken toll-like receptors and their significance in immune response and disease resistance. International Reviews of Immunology. 2019 Nov 2;38(6):284-306.

Nerren JR, Kogut MH. The selective Dectin-1 agonist, curdlan, induces an oxidative burst response in chicken heterophils and peripheral blood mononuclear cells. Veterinary Immunology and Immunopathology. 2009 Jan 15;127(1-2):162-6.

Netea MG, Joosten LA, Latz E, Mills KH, Natoli G, Stunnenberg HG, O'Neill LA, Xavier RJ. Trained immunity: a program of innate immune memory in health and disease. Science. 2016 Apr 22;352(6284).

Netea MG, Schlitzer A, Placek K, Joosten LA, Schultze JL. Innate and adaptive immune memory: an evolutionary continuum in the host's response to pathogens. Cell Host & Microbe. 2019 Jan 9;25(1):13-26.

Nieto R, Palmer RM, Fernandez-Figares I, Perez L, Prieto C. Effect of dietary protein quality, feed restriction and short-term fasting on protein synthesis and turnover in tissues of the growing chicken. British Journal of Nutrition. 1994 Oct;72(4):499-507.

Novakovic B, Habibi E, Wang SY, Arts RJ, Davar R, Megchelenbrink W, Kim B, Kuznetsova T, Kox M, Zwaag J, Matarese F. β-Glucan reverses the epigenetic state of LPS-induced immunological tolerance. Cell. 2016 Nov 17;167(5):1354-68.

Nowell VJ, Poppe C, Parreira VR, Jiang YF, Reid-Smith R, Prescott JF. *Clostridium perfringens* in retail chicken. Anaerobe. 2010 Jun 1;16(3):314-5.

Okeke EB, Uzonna JE. The pivotal role of regulatory T cells in the regulation of innate immune cells. Frontiers in Immunology. 2019 Apr 9;10:680.

Oláh I, Nagy N, Vervelde L. Structure of the avian lymphoid system. In Avian Immunology 2014 Jan 1 (pp. 11-44). Academic Press.

Olkowski AA, Wojnarowicz C, Chirino-Trejo M, Laarveld B, Sawicki G. Sub-clinical necrotic enteritis in broiler chickens: novel etiological consideration based on ultra-structural and molecular changes in the intestinal tissue. Research in Veterinary Science. 2008 Dec 1;85(3):543-53.

Onrust L, Van Driessche K, Ducatelle R, Schwarzer K, Haesebrouck F, Van Immerseel F. Valeric acid glyceride esters in feed promote broiler performance and reduce the incidence of necrotic enteritis. Poultry Science. 2018 Jul 1;97(7):2303-11.

Owen AM, Fults JB, Patil NK, Hernandez A, Bohannon JK. TLR agonists as mediators of trained immunity: mechanistic insight and immunotherapeutic potential to combat infection. Frontiers in Immunology. 2020;11:3866.

Paiva D, McElroy A. Necrotic enteritis: applications for the poultry industry. Journal of Applied Poultry Research. 2014 Sep 1;23(3):557-66.

Palliyeguru MW, Rose SP, Mackenzie AM. Effect of dietary protein concentrates on the incidence of subclinical necrotic enteritis and growth performance of broiler chickens. Poultry Science. 2010 Jan 1;89(1):34-43.

Palliyeguru MC, Rose SP, Mackenzie AM. Effect of trypsin inhibitor activity in soya bean on growth performance, protein digestibility and incidence of sub-clinical necrotic enteritis in broiler chicken flocks. British Poultry Science. 2011 Jun 1;52(3):359-67.

Pan D, Yu Z. Intestinal microbiome of poultry and its interaction with host and diet. Gut Microbes. 2014 Jan 1;5(1):108-19.

Paris S, Chapat L, Martin-Cagnon N, Durand PY, Piney L, Cariou C, Bergamo P, Bonnet JM, Poulet H, Freyburger L, De Luca K. β-glucan as trained immunity-based adjuvants for rabies vaccines in dogs. Frontiers in Immunology. 2020;11.

Parish WE. Necrotic enteritis in the fowl (*Gallus Gallus Domesticus*): I. Histopathology of the disease and isolation of a strain of *Clostridium welchii*. Journal of Comparative Pathology and Therapeutics. 1961 Jan 1;71:377-IN33.

Pan D, Yu Z. Intestinal microbiome of poultry and its interaction with host and diet. Gut Microbes. 2014 Jan 1;5(1):108-19.

Park SS, Lillehoj HS, Allen PC, Park DW, FitzCoy S, Bautista DA, Lillehoj EP. Immunopathology and cytokine responses in broiler chickens coinfected with *Eimeria maxima* and *Clostridium perfringens* with the use of an animal model of necrotic enteritis. Avian Diseases. 2008 Mar;52(1):14-22.

Parreira VR, Costa M, Eikmeyer F, Blom J, Prescott JF. Sequence of two plasmids from *Clostridium perfringens* chicken necrotic enteritis isolates and comparison with *C. perfringens* conjugative plasmids. PLoS One. 2012 Nov 26;7(11):e49753.

Parreira VR, Russell K, Athanasiadou S, Prescott JF. Comparative transcriptome analysis by RNAseq of necrotic enteritis *Clostridium perfringens* during in vivo colonization and in vitro conditions. BMC Microbiology. 2016 Dec 1;16(1):186.

Pétrilli V, Dostert C, Muruve DA, Tschopp J. The inflammasome: a danger sensing complex triggering innate immunity. Current Opinion in Immunology. 2007 Dec 1;19(6):615-22.

Poh TY, Pease J, Young JR, Bumstead N, Kaiser P. Re-evaluation of chicken CXCR1 determines the true gene structure: CXCLi1 (K60) and CXCLi2 (CAF/interleukin-8) are ligands for this receptor. Journal of Biological Chemistry. 2008 Jun 13;283(24):16408-15.

Predonzani A, Calì B, Agnellini AH, Molon B. Spotlights on immunological effects of reactive nitrogen species: when inflammation says nitric oxide. World Journal of Experimental Medicine. 2015 May 20;5(2):64.

Prescott JF, Sivendra R, Barnum DA. The use of bacitracin in the prevention and treatment of experimentally-induced necrotic enteritis in the chicken. The Canadian Veterinary Journal. 1978 Jul;19(7):181.

Qi X, Liu C, Li R, Zhang H, Xu X, Wang J. Modulation of the innate immune-related genes expression in H9N2 avian influenza virus-infected chicken macrophage-like cells (HD11) in

response to *Escherichia coli* LPS stimulation. Research in Veterinary Science. 2017 Apr 1;111:36-42.

Qing X, Zeng D, Wang H, Ni X, Lai J, Liu L, Khalique A, Pan K, Jing B. Analysis of hepatic transcriptome demonstrates altered lipid metabolism following *Lactobacillus johnsonii* BS15 prevention in chickens with subclinical necrotic enteritis. Lipids in Health and Disease. 2018 Dec;17(1):1-0.

Quintin J, Saeed S, Martens JH, Giamarellos-Bourboulis EJ, Ifrim DC, Logie C, Jacobs L, Jansen T, Kullberg BJ, Wijmenga C, Joosten LA. *Candida albicans* infection affords protection against reinfection via functional reprogramming of monocytes. Cell Host & Microbe. 2012 Aug 16;12(2):223-32.

Qureshi MA. Avian macrophage and immune response: an overview. Poultry Science. 2003 May 1;82(5):691-8.

Qureshi MA, Heggen CL, Hussain I. Avian macrophage: effector functions in health and disease. Developmental & Comparative Immunology. 2000 Mar 1;24(2-3):103-19.

Ramirez-Carrozzi VR, Nazarian AA, Li CC, Gore SL, Sridharan R, Imbalzano AN, Smale ST. Selective and antagonistic functions of SWI/SNF and Mi-2β nucleosome remodeling complexes during an inflammatory response. Genes & Development. 2006 Feb 1;20(3):282-96.

Rasid O, Chevalier C, Camarasa TM, Fitting C, Cavaillon JM, Hamon MA. H3K4me1 supports memory-like NK cells induced by systemic inflammation. Cell Reports. 2019 Dec 17;29(12):3933-45.

Remus A, Hauschild L, Andretta I, Kipper M, Lehnen CR, Sakomura NK. A meta-analysis of the feed intake and growth performance of broiler chickens challenged by bacteria. Poultry Science. 2014 May 1;93(5):1149-58.

Rengaraj D, Truong AD, Lee SH, Lillehoj HS, Hong YH. Expression analysis of cytosolic DNA-sensing pathway genes in the intestinal mucosal layer of necrotic enteritis-induced chicken. Veterinary Immunology and Immunopathology. 2016 Feb 1;170:1-2.

Riera Romo M, Pérez-Martínez D, Castillo Ferrer C. Innate immunity in vertebrates: an overview. Immunology. 2016 Jun;148(2):125-39.

Rincón M, Anguita J, Nakamura T, Fikrig E, Flavell RA. Interleukin (IL)-6 directs the differentiation of IL-4–producing CD4+ T cells. The Journal of Experimental Medicine. 1997 Feb 3;185(3):461-70.

Robinson MW, Harmon C, O'Farrelly C. Liver immunology and its role in inflammation and homeostasis. Cellular & Molecular Immunology. 2016 May;13(3):267-76.

Rohde F, Schusser B, Hron T, Farkašová H, Plachý J, Härtle S, Hejnar J, Elleder D, Kaspers B. Characterization of chicken tumor necrosis factor-α, a long missed cytokine in birds. Frontiers in Immunology. 2018 Apr 17;9:605.

Rood JI, Keyburn AL, Moore RJ. NetB and necrotic enteritis: the hole movable story. Avian Pathology. 2016 May 3;45(3):295-301.

Rose-John S. IL-6 trans-signaling via the soluble IL-6 receptor: importance for the pro-inflammatory activities of IL-6. International Journal of Biological Sciences. 2012;8(9):1237.

Ruhnke I, Andronicos NM, Swick RA, Hine B, Sharma N, Kheravii SK, Wu SB, Hunt P. Immune responses following experimental infection with *Ascaridia galli* and necrotic enteritis in broiler chickens. Avian Pathology. 2017 Nov 2;46(6):602-9.

Russo RC, Garcia CC, Teixeira MM, Amaral FA. The CXCL8/IL-8 chemokine family and its receptors in inflammatory diseases. Expert Review of Clinical Immunology. 2014 May 1;10(5):593-619.

Saeed S, Quintin J, Kerstens HH, Rao NA, Aghajanirefah A, Matarese F, Cheng SC, Ratter J, Berentsen K, van der Ent MA, Sharifi N. Epigenetic programming of monocyte-to-macrophage differentiation and trained innate immunity. Science. 2014 Sep 26;345(6204):1251086.

Saleem G. Necrotic enteritis, disease induction, predisposing factors and novel biochemical markers in broilers chickens. Doctoral dissertation. University of Glasgow. 2012

Sánchez-Ramón S, Conejero L, Netea MG, Sancho D, Palomares Ó, Subiza JL. Trained immunity-based vaccines: a new paradigm for the development of broad-spectrum anti-infectious formulations. Frontiers in Immunology. 2018 Dec 17;9:2936.

Santaolalla R, Abreu MT. Innate immunity in the small intestine. Current Opinion in Gastroenterology. 2012 Mar;28(2):124.

Santovito E, Greco D, Marquis V, Raspoet R, D'Ascanio V, Logrieco AF, Avantaggiato G. Antimicrobial activity of yeast cell wall products against *Clostridium perfringens*. Foodborne Pathogens and Disease. 2019 Sep 1;16(9):638-47.

Sarson AJ, Wang Y, Kang Z, Dowd SE, Lu Y, Yu H, Han Y, Zhou H, Gong J. Gene expression profiling within the spleen of *Clostridium perfringens*-challenged broilers fed antibiotic-medicated and non-medicated diets. BMC Genomics. 2009 Dec;10(1):1-3.

Schmidt-Arras D, Rose-John S. IL-6 pathway in the liver: From physiopathology to therapy. Journal of Hepatology. 2016 Jun 1;64(6):1403-15.

Schneider K, Klaas R, Kaspers B, Staeheli P. Chicken interleukin-6: cDNA structure and biological properties. European Journal of Biochemistry. 2001 Aug 1;268(15):4200-6.

Sehgal A, Donaldson DS, Pridans C, Sauter KA, Hume DA, Mabbott NA. The role of CSF1R-dependent macrophages in control of the intestinal stem-cell niche. Nature Communications. 2018 Mar 28;9(1):1-7.

Shao Y, Wang Z, Tian X, Guo Y, Zhang H. Yeast β-d-glucans induced antimicrobial peptide expressions against *Salmonella* infection in broiler chickens. International Journal of Biological Macromolecules. 2016 Apr 1;85:573-84.

Sharrock J, Sun JC. Innate immunological memory: from plants to animals. Current Opinion in Immunology. 2020 Feb 1;62:69-78.

Shirai T, Nazarewicz RR, Wallis BB, Yanes RE, Watanabe R, Hilhorst M, Tian L, Harrison DG, Giacomini JC, Assimes TL, Goronzy JJ. The glycolytic enzyme PKM2 bridges metabolic and inflammatory dysfunction in coronary artery disease. Journal of Experimental Medicine. 2016 Mar 7;213(3):337-54.

Shojadoost B, Vince AR, Prescott JF. The successful experimental induction of necrotic enteritis in chickens by *Clostridium perfringens*: a critical review. Veterinary Research. 2012 Dec 1;43(1):74.

Skinner JT, Bauer S, Young V, Pauling G, Wilson J. An economic analysis of the impact of subclinical (mild) necrotic enteritis in broiler chickens. Avian Diseases. 2010 Dec;54(4):1237-40.

Slavić Đ, Boerlin P, Fabri M, Klotins KC, Zoethout JK, Weir PE, Bateman D. Antimicrobial susceptibility of *Clostridium perfringens* isolates of bovine, chicken, porcine, and turkey origin from Ontario. Canadian Journal of Veterinary Research. 2011 Apr 1;75(2):89-97.

Smale ST, Tarakhovsky A, Natoli G. Chromatin contributions to the regulation of innate immunity. Annual Review of Immunology. 2014 Mar 21;32:489-511.

Smith CK, AbuOun M, Cawthraw SA, Humphrey TJ, Rothwell L, Kaiser P, Barrow PA, Jones MA. Campylobacter colonization of the chicken induces a proinflammatory response in mucosal tissues. FEMS Immunology & Medical Microbiology. 2008 Oct 1;54(1):114-21.

Stanley D, Keyburn AL, Denman SE, Moore RJ. Changes in the caecal microflora of chickens following *Clostridium perfringens* challenge to induce necrotic enteritis. Veterinary Microbiology. 2012 Sep 14;159(1-2):155-62.

Stanley D, Wu SB, Rodgers N, Swick RA, Moore RJ. Differential responses of cecal microbiota to fishmeal, *Eimeria* and *Clostridium perfringens* in a necrotic enteritis challenge model in chickens. PloS One. 2014 Aug 28;9(8):e104739.

Stutz MW, Johnson SL, Judith FR. Effects of diet, bacitracin, and body weight restrictions on the intestine of broiler chicks. Poultry Science. 1983 Aug 1;62(8):1626-32.

Sun H, Liu P, Nolan LK, Lamont SJ. Novel pathways revealed in bursa of Fabricius transcriptome in response to extraintestinal pathogenic *Escherichia coli* (ExPEC) infection. PloS One. 2015 Nov 10;10(11):e0142570.

Swaggerty CL, McReynolds JL, Byrd JA, Pevzner IY, Duke SE, Genovese KJ, He H, Kogut MH. Selection for pro-inflammatory mediators produces chickens more resistant to *Clostridium perfringens*-induced necrotic enteritis. Poultry Science. 2016 Feb 1;95(2):370-4.

Taylor Jr RL, McCorkle Jr FM. A landmark contribution to poultry science—Immunological function of the bursa of Fabricius. Poultry Science. 2009 Apr 1;88(4):816-23.

Tang Y, Foster N, Jones MA, Barrow PA. Model of persistent Salmonella infection: *Salmonella enterica serovar Pullorum* modulates the immune response of the chicken from a Th17-type response towards a Th2-type response. Infection and Immunity. 2018 Aug;86(8).

Tian X, Shao Y, Wang Z, Guo Y. Effects of dietary yeast β-glucans supplementation on growth performance, gut morphology, intestinal *Clostridium perfringens* population and immune response of broiler chickens challenged with necrotic enteritis. Animal Feed Science and Technology. 2016 May 1;215:144-55.

Tiegs G, Lohse AW. Immune tolerance: what is unique about the liver. Journal of Autoimmunity. 2010 Feb 1;34(1):1-6.

Timbermont L, De Smet L, Van Nieuwerburgh F, Parreira VR, Van Driessche G, Haesebrouck F, Ducatelle R, Prescott J, Deforce D, Devreese B, Van Immerseel F. Perfrin, a novel bacteriocin associated with netB positive *Clostridium perfringens* strains from broilers with necrotic enteritis. Veterinary Research. 2014 Dec 1;45(1):40.

Timbermont, L., Haesebrouck, F., Ducatelle, R., & Van Immerseel, F. (2011). Necrotic enteritis in broilers: an updated review on the pathogenesis. Avian Pathology, 40(4), 341-347.

Timbermont L, Lanckriet A, Pasmans F, Haesebrouck F, Ducatelle R, Van Immerseel F. Intra-species growth-inhibition by *Clostridium perfringens* is a possible virulence trait in necrotic enteritis in broilers. Veterinary Microbiology. 2009 Jun 12;137(3-4):388-91.

Truong AD, Hong YH, Lillehoj HS. RNA-seq profiles of immune related genes in the spleen of necrotic enteritis-afflicted chicken lines. Asian-Australasian Journal of Animal Sciences. 2015 Oct;28(10):1496.

Tsiouris V, Georgopoulou I, Batzios C, Pappaioannou N, Ducatelle R, Fortomaris P. The effect of cold stress on the pathogenesis of necrotic enteritis in broiler chicks. Avian Pathology. 2015 Nov 2;44(6):430-5.

Vachharajani VT, Liu T, Wang X, Hoth JJ, Yoza BK, McCall CE. Sirtuins link inflammation and metabolism. Journal of Immunology Research. 2016 Oct;2016.

Van Immerseel F, Rood JI, Moore RJ, Titball RW. Rethinking our understanding of the pathogenesis of necrotic enteritis in chickens. Trends in Microbiology. 2009 Jan 1;17(1):32-6.

Verwoolde MB, van den Biggelaar RH, van Baal J, Jansen CA, Lammers A. Training of primary chicken monocytes results in enhanced pro-inflammatory responses. Veterinary Sciences. 2020 Sep;7(3):115.

Verwoolde MB, van den Biggelaar RH, de Vries Reilingh G, Arts JA, van Baal J, Lammers A, Jansen CA. Innate immune training and metabolic reprogramming in primary monocytes of broiler and laying hens. Developmental & Comparative Immunology. 2021 Jan 1;114:103811.

Vidanarachchi JK, Mikkelsen LL, Constantinoiu CC, Choct M, Iji PA. Natural plant extracts and prebiotic compounds as alternatives to antibiotics in broiler chicken diets in a necrotic enteritis challenge model. Animal Production Science. 2013 Oct 30;53(12):1247-59.

Vignais PV. The superoxide-generating NADPH oxidase: structural aspects and activation mechanism. Cellular and Molecular Life Sciences CMLS. 2002 Oct;59(9):1428-59.

Villagrán-de la Mora Z, Vázquez-Paulino O, Avalos H, Ascencio F, Nuño K, Villarruel-López A. Effect of a synbiotic mix on lymphoid organs of broilers infected with *Salmonella typhimurium* and *Clostridium perfringens*. Animals. 2020 May;10(5):886.

Volf J, Polansky O, Varmuzova K, Gerzova L, Sekelova Z, Faldynova M, Babak V, Medvecky M, Smith AL, Kaspers B, Velge P. Transient and prolonged response of chicken cecum mucosa to colonization with different gut microbiota. PLoS One. 2016 Sep 29;11(9):e0163932.

Wade B, Keyburn A. The true cost of necrotic enteritis. Poultry World. 2015;31(7):16-7.

Wade B, Keyburn AL, Haring V, Ford M, Rood JI, Moore RJ. The adherent abilities of *Clostridium perfringens* strains are critical for the pathogenesis of avian necrotic enteritis. Veterinary Microbiology. 2016 Dec 25;197:53-61.

Wade B, Keyburn AL, Seemann T, Rood JI, Moore RJ. Binding of *Clostridium perfringens* to collagen correlates with the ability to cause necrotic enteritis in chickens. Veterinary Microbiology. 2015 Nov 18;180(3-4):299-303.

Wang B, Hussain A, Zhou Y, Zeng Z, Wang Q, Zou P, Gong L, Zhao P, Li W. *Saccharomyces boulardii* attenuates inflammatory response induced by *Clostridium perfringens* via TLR4/TLR15-MyD8 pathway in HD11 avian macrophages. Poultry Science. 2020 Nov 1;99(11):5356-65.

Wang N, Liang H, Zen K. Molecular mechanisms that influence the macrophage M1–M2 polarization balance. Frontiers in Immunology. 2014 Nov 28;5:614.

Wang T, Liu H, Lian G, Zhang SY, Wang X, Jiang C. HIF1α-induced glycolysis metabolism is essential to the activation of inflammatory macrophages. Mediators of Inflammation. 2017 Oct;2017.

Wang M, Yang R, Zhang L, Meng X, Fei C, Zhang K, Wang X, Zheng W, Xiao S, Zhang S, Xue F. Sulfated glucan can improve the immune efficacy of Newcastle disease vaccine in chicken. International Journal of Biological Macromolecules. 2014 Sep 1;70:193-8.

Warriss PD, Kestin SC, Brown SN, Bevis EA. Depletion of glycogen reserves in fasting broiler chickens. British Poultry Science. 1988 Mar 1;29(1):149-54.

Webel DM, Johnson RW, Baker DH. Lipopolysaccharide-induced reductions in food intake do not decrease the efficiency of lysine and threonine utilization for protein accretion in chickens. The Journal of Nutrition. 1998 Oct 1;128(10):1760-6.

Wei S, Gutek A, Lilburn M, Yu Z. Abundance of pathogens in the gut and litter of broiler chickens as affected by bacitracin and litter management. Veterinary Microbiology. 2013 Oct 25;166(3-4):595-601.

Weyand CM, Zeisbrich M, Goronzy JJ. Metabolic signatures of T-cells and macrophages in rheumatoid arthritis. Current Opinion in Immunology. 2017 Jun 1;46:112-20.

Wigley P. Blurred lines: pathogens, commensals, and the healthy gut. Frontiers in Veterinary Science. 2015 Oct 1;2:40.

Wilde S, Jiang Y, Tafoya AM, Horsman J, Yousif M, Vazquez LA, Roland KL. Salmonella-vectored vaccine delivering three *Clostridium perfringens* antigens protects poultry against necrotic enteritis. PloS One. 2019 Feb 12;14(2):e0197721.

Wilkie DC, Van Kessel AG, White LJ, Laarveld B, Drew MD. Dietary amino acids affect intestinal *Clostridium perfringens* populations in broiler chickens. Canadian Journal of Animal Science. 2005 Jun 1;85(2):185-93.

Williams RB. Intercurrent coccidiosis and necrotic enteritis of chickens: rational, integrated disease management by maintenance of gut integrity. Avian Pathology. 2005 Jun 1;34(3):159-80.

Williams RB, Marshall RN, La Ragione RM, Catchpole J. A new method for the experimental production of necrotic enteritis and its use for studies on the relationships between necrotic enteritis, coccidiosis and anticoccidial vaccination of chickens. Parasitology Research. 2003 May;90(1):19-26.

Wilson KM, Chasser KM, Duff AF, Briggs WN, Latorre JD, Barta JR, Bielke LR. Comparison of multiple methods for induction of necrotic enteritis in broilers. I. Journal of Applied Poultry Research. 2018 Dec 1;27(4):577-89.

Wu SB, Rodgers N, Choct M. Optimized necrotic enteritis model producing clinical and subclinical infection of *Clostridium perfringens* in broiler chickens. Avian Diseases. 2010 Sep;54(3):1058-65.

Wu SB, Stanley D, Rodgers N, Swick RA, Moore RJ. Two necrotic enteritis predisposing factors, dietary fishmeal and *Eimeria* infection, induce large changes in the caecal microbiota of broiler chickens. Veterinary Microbiology. 2014 Mar 14;169(3-4):188-97.

Xue GD, Wu SB, Choct M, Swick RA. Effects of yeast cell wall on growth performance, immune responses and intestinal short chain fatty acid concentrations of broilers in an experimental necrotic enteritis model. Animal Nutrition. 2017 Dec 1;3(4):399-405.

Xue GD, Barekatain R, Wu SB, Choct M, Swick RA. Dietary L-glutamine supplementation improves growth performance, gut morphology, and serum biochemical indices of broiler chickens during necrotic enteritis challenge. Poultry Science. 2018 Apr 1;97(4):1334-41.

Yang WY, Chou CH, Wang C. Characterization of toxin genes and quantitative analysis of netB in necrotic enteritis (NE)-producing and non-NE-producing *Clostridium perfringens* isolated from chickens. Anaerobe. 2018 Dec 1;54:115-20.

Zahoor I, Ghayas A, Basheer A. Genetics and genomics of susceptibility and immune response to necrotic enteritis in chicken: a review. Molecular Biology Reports. 2018 Feb;45(1):31-7.

Zhang ZF, Cho JH, Kim IH. Effects of Bacillus subtilis UBT-MO2 on growth performance, relative immune organ weight, gas concentration in excreta, and intestinal microbial shedding in broiler chickens. Livestock Science. 2013 Aug 1;155(2-3):343-7.

Zhang B, Lv Z, Li H, Guo S, Liu D, Guo Y. Dietary l-arginine inhibits intestinal *Clostridium perfringens* colonisation and attenuates intestinal mucosal injury in broiler chickens. British Journal of Nutrition. 2017 Sep;118(5):321-32.

Zhao J, Li J, Fan TW, Hou SX. Glycolytic reprogramming through PCK2 regulates tumor initiation of prostate cancer cells. Oncotarget. 2017 Oct 13;8(48):83602.

Zhou H, Lepp D, Pei Y, Liu M, Yin X, Ma R, Prescott JF, Gong J. Influence of pCP1NetB ancillary genes on the virulence of *Clostridium perfringens* poultry necrotic enteritis strain CP1. Gut Pathogens. 2017 Dec;9(1):1-7.